Investigation of Mechanisms of Multiple Drug Resistance using Polymerase Chain Reaction

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph. D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Lorraine O'Driscoll Date: 24/11/94

ABSTRACT

Investigation of Mechanisms of Multiple Drug Resistance using Polymerase Chain Reaction.

Control of transcription plays a critical role in the multi-step process that regulates gene expression. Understanding the regulation of gene expression therefore depends, in part, on the ability to accurately measure defined mRNA species in cell populations and tissues.

In this study, the presence of a number of mRNAs putatively involved in the determination of multiple drug resistance (MDR) was investigated, using reverse transcriptase-polymerase chain reaction (RT-PCR). This technique was evaluated at a semi-quantitative and a quantitative level, giving due consideration to the choice of primers for the RT and PCR reactions, controls, reproducibility of the technique, *etc.* mRNA levels were initially studied in sensitive cell lines and their corresponding MDR variants. Methods were then developed which enabled analysis of intact mRNA from paired normal and tumour biopsies and archival tissues (which were embedded in paraffin).

Levels of two proteins (p-glycoprotein and Topoisomerase IIα) known to have a role in MDR were studied using Western blotting techniques to establish if the mRNA and corresponding protein levels correlated.

MDR 1 expression was induced in cultured cells by short-term exposure to a chemotherapeutic drug. This induction corresponded to drug concentration and was associated with physiological signs of stress.

A hammer-head ribozyme to MDR 1 was transfected into both a resistant cell line and a clonal subpopulation of its sensitive parental cell line (as a control). PCR (DNA) and RT-PCR (mRNA) experiments were conducted to verify the presence and successful transcription of the ribozyme in the cells. Toxicity assays were performed to establish if the presence of the ribozyme affected the MDR profile of the cell lines.

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TABLE OF CONTENTS

Chapter 1

Introduction

INTRODUCTION

1.1 Multiple Drug Resistance

Failure of chemotherapy is a significant problem in the treatment of neoplastic disease. Despite initial sensitivity to a particular chemotherapeutic drug, some tumours become progressively unresponsive to the same or different anti-tumour drugs *i.e.* they acquire resistance. Combination chemotherapy was introduced to alleviate this problem. However, variants can arise which are resistant not only to the drugs that have been administered, but are cross-resistant to a broad range of other compounds. Some tumours appear to be inherently resistant to many forms of chemotherapy. The phenomenon described is termed multiple drug resistance (MDR) and may be observed in tumour cells *in vivo* and cultured cells *in vitro*. The emergence of multiple drug resistance is the major cause of death in patients with many types of cancer, including small cell lung cancer, breast cancer, ovarian cancer and acute leukemia (Kaye, 1988). Some tumour types *e.g.* colon carcinoma are inherently resistant, rarely responding to chemotherapy. In general, multiple drug resistance is characterised by resistance to a defined range of structurally and mechanistically unrelated natural product anti-cancer drugs, including the anthracyclines, vinca alkaloids, epipodophyllotoxins, colchicine, actinomycin D and Taxol. Sensitivity to alkylating agents (*e.g.* chlorambucil, cyclophosphamide, cisplatin), antimetabolites (*e.g.* cytosine arabinoside, methotrexate, 5-fluorouracil) or platinum compounds appears unaltered (Beck and Danks, 1991; Nielsen and Skovsgaard, 1992; Germann *et al.*, 1993; Clynes *et al.*, 1993; Hill, 1993).

The MDR phenotype was first described in 1970 by Biedler and Riehm, in studies of Chinese hamster lung cells and P388 leukemia cells. The causes of this phenomenon *in vivo* have since been realised as complex, possibly determined by a combination of the tumour characteristics. These include properties of resting phase cells, adequacy of blood supply, *etc.* and specific

cellular mechanisms such as increased drug efflux from cells, increased levels of drug detoxification, increased DNA repair, failure of activation of anti-tumour agents and alterations in the intracellular target of the drug (Judson, 1992).

1.1.1 MDR 1

Multiple drug resistance is associated with a reduction in intracellular drug accumulation and increased cellular drug efflux. The decreased intracellular drug accumulation in multiple drug resistance was first described in 1976 by Juliano and Ling) and has been found to result, at least in part, from an increased rate of drug efflux occuring by an energy-dependent mechanism (Dano, 1973; Skovsgaard, 1978; Inaba *et al.*, 1979; Fojo *et al.*, 1985; Willingham *et al.*, 1986). This has been associated with the presence of a 170 kDa plasma membrane glycoprotein (p-glycoprotein) (Kartner *et al.*, 1983) and correlated with specific genes encoding p-glycoprotein (Riordan *et al.*, 1985). The MDR gene family consists of two members in humans, namely MDR 1 and MDR 3 (sometimes termed MDR 2) (van der Bliek *et al.*, 1986; Roninson *et al.*, 1986; Chen *et al.*, 1986; 1990; Schinkel *et al.*, 1991; Chin *et al.*, 1989) and three in rodents (Endicott *et al.*, 1987; Ng *et al.*, 1989; Gros *et al.*, 1986a, 1988; Hsu *et al.*, 1989; Croop *et al.*, 1989; Raymond et al., 1990). The human MDR 1 gene encodes a 4.5 - 5.0 Kb mRNA (Roninson *et al.*, 1986; Gros *et al.*, 1986 (a; b).

Increased expression of a specific human gene encoding p-glycoprotein (the MDR gene) is a common, but not universal, phenomenon in multidrug resistant cells (Clynes, 1993). This over-expression of the transmembrane efflux pump may be as a result of gene amplification (Riordan *et al.*, 1985; van der Bliek *et al.*, 1986, 1988; Scotto *et al.* 1986; Clynes *et al.*, 1990; Raymond *et al.*, 1990). Gene amplification, however, is not necessary for multiple drug resistance *in vitro*, suggesting that the MDR 1 gene can be transcriptionally or translationally regulated (Benard *et al.*, 1989; Bradley *et al.*, 1989; Biedler,

1992). Fojo *et al.* (1987) reported a correlation between the level of MDR 1 mRNA and the degree of multiple drug resistance in several cell lines and in gene transfer experiments. Increased MDR 1 gene expression has been shown to occur prior to gene amplification in the development of drug resistance in tissue culture (Fojo *et al.*, 1987; Bradley *et al.*, 1989). Conflicting ideas exist as to the significance of this. Fojo *et al.* (1987) suggested that analysis of MDR 1 mRNA may prove to be useful from a clinical point of view for guiding chemotherapy. Harris and Hochhauser (1992), however, warned that measurement of MDR 1 mRNA alone may give a misleading impression of the amount of functional p-glycoprotein present. This is due to the fact that at intermediate drug concentrations, amplification was found to be significantly increased; while at high drug levels p-glycoprotein levels increased without further changes in mRNA or gene copy number, suggesting translational modifications or mutations.

Direct proof for the role of MDR 1 in multiple drug resistance has been obtained by transfection experiments, where expression of a full-length cDNA copy of the human MDR 1 gene in a drug sensitive cell, conferred a complete multiple drug resistant phenotype (Ueda *et al.*, 1987). However, over-expression of MDR 1, although generally, is not always associated with multiple drug resistance. Harris and Hochhauser (1992) reported studies of a cell line, derived from a lymph node metastasis of a colon adenocarcinoma, where increased expression was not accompanied by increased adriamycin or vincristine resistance. Similarly, the multiple drug resistance phenomenon is not always associated with MDR 1 over-expression (Gervasoni *et al.*, 1991; Coley *et al.*, 1993). While some drug-resistant tumours over-express p-glycoprotein *e.g.* multiple myeloma and lymphoma, other tumours do not *e.g.* small cell and non-small cell lung cancers (Futscher *et al.*, 1994).

The significance of MDR 1 expression in clinical cancer must be considered. It has been suggested that p-glycoprotein expression may play different roles in multiple drug resistant

cells selected *in vitro* and in clinically resistant human tumours (Roninson, 1992). Whereas MDR 1 expression may be the major, and possibly the only, determinant of the multiple drug resistant phenotype in some cultured cells (as they represent genetic mutations arising at low frequencies), it may be just one of many such mechanisms in a tumour. This may be due to the fact that tumours growing *in vivo* represent a heterogenous population of cells in which pleiotropic changes affecting various parameters of cell growth are constantly occuring (Nicolson, 1984). Consistent high levels of MDR 1 mRNA or p-glycoprotein expression have been found mainly in tumours derived from normal tissues, which themselves have elevated levels of p-glycoprotein *i.e.* adrenal cancer, renal cancer and colon cancer (Kaye *et al.*, 1988).

MDR 1 expression, in at least some types of tumours, may be just one of the traits of a sub-population of tumour cells possessing a complex of features, associated with resistance to different types of treatment and more aggressive behaviour (Roninson, 1992). This is supported by the fact that MDR 1 expression in human solid tumours is often heterogenous and in some tumour types it is preferentially associated with areas of apparent invasive growth (Roninson *et al.*, 1989; Weinstein *et al.*, 1991). MDR 1 expression has been associated with tumours that show drug resistance either clinically (Epstein and Barlogie, 1989; Ro *et al.*, 1990; Robey-Cafferty *et al.*, 1990; Pirker *et al.*, 1991) or in *in vitro* clonigenic assays (Salmon *et al.*, 1989; Kanamaru *et al.*, 1989). However, this expression has also been shown to correlate with resistance to combination chemotherapy, including drugs that should not affect p-glycoprotein (Epstein and Barlogie, 1989; Chan *et al.*, 1990). This is consistent with the suggestion that p-glycoprotein expression in some tumour types occurs in a population of cells that possess multiple changes making them resistant to various cytotoxic drugs.

1.1.2 MDR 3

MDR 3, a second gene in the MDR gene family has been identified in humans (termed MDR 2 by some authors) (Lincke *et al.*, 1991; Nooter and Herweijer, 1991, Roninson, 1992). Both the human MDR 1 and MDR 3 genes are located on the long arm of chromosome 7, linked within 230 Kb (Callen *et al.*, 1987). Although the MDR 1 and MDR 3 genes encode highly homologous proteins, suggesting that the MDR 3 gene product also functions as an efflux pump (Herweijer *et al.*, 1990), MDR 3 does not seem to be involved in drug resistance. This is shown by the fact that insertion of human MDR 3 cDNA into mammalian expression vectors does not result in resistance to drugs associated with multiple drug resistance. No function of the MDR 3 gene has been identified, as yet (van der Bliek *et al.*, 1988; Gros *et al.*, 1988; Schinkel *et al.*, 1991).

MDR 3 has been reported to be amplified to the same extent as MDR 1 in only some cell lines studied. In other cell lines it was amplified either to a lesser extent than MDR 1, or not at all (undetectable) (Hsu *et al.*, 1989; Roninson *et al.*, 1991; Borst and van der Bliek, 1991). This difference was even more obvious at the RNA and protein levels. All the multiple drug resistant cell lines were found to express MDR 1, which correlated with the level of drug resistance in studies documented by Roninson (1992). However, the MDR 3 mRNA levels were either very low or undectable, even in those cell lines where MDR 1 and MDR 3 genes were amplified to the same extent (Chin *et al.*, 1989; Raymond *et al.*, 1990; Borst and van der Bliek, 1991; Roninson *et al.*, 1991). Roninson (1992) suggested that as MDR 1 and MDR 3 are linked in genomic DNA amplification of MDR 3 may result as a chance co-amplification of sequences flanking the essential MDR 1 gene. Herweijer *et al.* (1990), however, based on results of their studies suggested that MDR 3 may contribute to the primary resistance of (B-cell) leukemias.

1.1.3 MRP

P-glycoprotein, the product of the MDR 1 gene, although strongly associated with multiple drug resistance, is not solely responsible, or indeed involved, in all forms of multiple drug resistance. In addition to p-glycoprotein mediated multiple drug resistance ("typical"), at least two other types of multiple drug resistance have been described *i.e.* an "atypical" form due to alterations in the level of drug sensitivity of Topoisomerase IIα and a "true" form of non-p-glycoprotein (non-p-gp) mediated multiple drug resistance (Zaman *et al.*, 1993).

Cole *et al.* (1992a) isolated a gene from a non-p-gp multiple drug resistant cell line, termed MRP, which has been suggested to be a multiple drug resistant gene. This gene, mapped to chromosome 16p13.1, encodes a 190-kDa membrane bound protein (Krishnamachary and Center, 1993) which has minor sequence homology to p-glycoprotein. The resulting mRNA has been estimated to be approximately 7.8 - 8.2 kb (Cole *et al.*, 1992a).

Criteria supporting the involvement of MRP in multiple drug resistance include circumstantial evidence *i.e.* like p-glycoprotein, it is a member of the adenosine triphosphate (ATP)- binding cassette (ABC) superfamily of transport genes (Hyde *et al.*, 1990; Cole *et al.*, 1992a). Furthermore, MRP has been found to be over-expressed in a number of different non-p-gp multiple drug resistant human tumour cell lines selected with adriamycin (Cole *et al.*, 1992a; Slovak *et al.*, 1993; Zaman *et al.*, 1993; Krishnamachary *et al.*, 1994) with the mRNA transcript level correlating well with that of drug resistance (Marquardt *et al.*, 1990). More direct evidence has recently been reported including the fact that transfection of full-length MRP cDNA contained in an expression vector into sensitive cells has been shown to result in multiple drug resistant phenotype (Grant *et al.*, 1994).

Over-expression of the MRP gene has been shown to occur mainly as a result of gene amplification. This is consistent

with the fact that reversion to drug sensitivity has been associated with loss of gene amplification and a significant decrease in mRNA expression (Cole *et al.*, 1992a). MRP over-expression, however, has not been associated multiple drug resistance resulting from mitoxantrone selection (Futscher *et al.*, 1994) and cannot account for all forms of non-p-gp multiple drug resistance (Zaman *et al.*, 1993).

Recent studies of anaplastic carcinoma of the thyroid showed high MRP mRNA expression levels in both carcinomas and cell lines (Sugawara *et al.*, 1994). Further analysis of MRP transcript levels should be performed in human tumour specimens to establish the potential clinical relevance of MRP (Futscher *et al.* (1994).

1.1.4 Phase I and Phase II Metabolising Enzymes

Multiple drug resistance may result due to alterations in phase I or II metabolising enzymes, resulting in failure of activation of anti-tumour agents and / or increased levels of drug detoxification. Some of the drugs involved in chemotherapy, including mitomycin C, VP-16, cyclophosphamide and a number of the anthracyclines, require metabolic activation before they can exert their toxic effect (Harris and Hochhauser, 1992; Lewis *et al.*, 1992). Certain phase I cytochrome P_{450} enzymes are involved in this role *e.g.* CYP3A potentiates toxicity of some anthracyclines (Lewis *et al.*, 1992). These enzymes are generally poorly expressed in most tumour cells *in vitro* are so there are very few reports citing the involvement of such enzymes in multiple drug resistant cell line models. However, decreased expression of CYPIA1 in adriamycin resistant MCF-7 cell lines has been reported (Ivy *et al.*, 1988). It has been suggested that CYPIA1 may possibly be involved in the etiology of lung cancer, especially that associated with cigarette smoke. This is due to the reported elevated levels of its mRNA in lung tissue of smokers in comparison to non-smokers (McLemore *et al.*, 1989, 1990; Kawajiri and Fujii-Kuriyama, 1991). This enzyme is known

to catalyse the activation of benzo[a]pyrene, a precarcinogen found in cigarette smoke, to its tumorigenic form. Such enzymes may have an important role to play *in vivo* in drug resistance.

Glutathione-S-transferases (GSTs) form a family of enzymes important in the phase II metabolism of xenobiotics. The three major classes of cytosolic GSTs are termed α, μ and π. GST enzymes catalyse the conjugation of reduced glutathione with many different electrophilic molecules, resulting in more polar and possibly more readily excreted products. The significance of GSTs in multiple drug resistance is uncertain. Premature detoxification or excretion of the anti-tumour drugs is a possibility. Many anti-cancer drugs are substrates for GSTs including VP-16, mitomycin C, cyclophosphamide, melphalan, chlorambucil, nitrogen mustards, 6-thiopurine, mitoxantrone and cisplatin (Clynes, 1993). Conjugation and accelerated accumulation of azathioprine and methotrexate, respectively, by these enzymes can in some cases enhance toxicity (Wolf *et al.*, 1987; Hosking *et al.*, 1990).

Different GST isoenzymes show different tissue distribution. Overall, GST π is the most prominent form found in human tumours (Toffoli *et al.*, 1992), with a 2 to 4 - fold increase in its mRNA transcript levels in tumours of the colon, bladder, ovary and stomach relative to normal tissue (Harris and Hochhauser, 1992). Elevated levels of GST π expression have also been reported both in adenocarcinomas and squamous cell carcinomas, but not in small cell carcinomas of the lung (Tsuchida and Sato, 1992). GST π has been implicated in drug resistance (Moscow *et al.*, 1989; Ali-Osman *et al.*, 1990; Hayes *et al.*, 1991; Tidefelt *et al.*, 1992, Peters *et al.*, 1992) and showed good correlation with smoking history, p-glycoprotein over-expression and *in vitro* sensitivity to adriamycin in non-small cell lung cancer patients (Volm *et al.*, 1991). Wang *et al.* (1989a), reported a study where cell lines were selected for resistance using alkylating agents. Levels of GST π were elevated in all cell lines, but the cell lines were resistant only to their selective agent, and did not express the multiple

drug resistant phenotype. Furthermore, transfection studies of GST π cDNA did not suggest a major fundamental role for GST π in multiple drug resistance (Fairchild et al., 1990; Nakagawa *et al.*, 1990). Increased protection against adriamycin, although small, has been consistently found to correlate with GST π (Harris and Hocchauser, 1992). The role which GST π plays in this case may be the conjugation of free radicals, formed as part of the cytotoxic effect of adriamycin (Booser and Nortobagyi, 1994) enabling cells to survive in the presence of this drug.

1.1.5 Topoisomerases

DNA is repeatedly wound and relaxed within a cell cycle. Since the coiled state is necessary for recognition by other complexes, the role of this unwinding is obviously well controlled within the cell (Zhou *et al.*, 1992). The topological and conformational changes in DNA molecules which are required for DNA replication and transcription are regulated by two classes of Topoisomerase enzymes. These enzymes act by binding to the substrate DNA, inducing DNA single (Topoisomerase I) and double (Topoisomerase II) strand breaks and then binding covalently to the 5' of the break. This allows the strands to pass through one another, unwinding and relaxing the supercoiled DNA (Sander and Hsien, 1983; Liu *et al.*, 1983).

Topoisomerase I is a monomeric protein, encoded by a 4.2 kb mRNA transcript resulting from a gene located on chromosome 20 (Zhou *et al.*, 1992). Two sub-classes of the Topoisomerase II enzyme have been realised which exist as homodimers, termed Topoisomerase IIα and Topoisomerase IIβ, respectively (Drake *et al.*, 1987), associated with different stages of the cell cycle (Drake *et al.*, 1989; Prosperi *et al.*, 1992). These enzymes share extensive sequence homology (75 %) within the first two-thirds of their transcripts (Chung *et al.*, 1989). Topoisomerase IIα has been located on chromosome 17q21-22 (Tsai-Pflugfelder *et al.*, 1988) encoding a 6.2 kb mRNA, resulting in the formation of a

170 kDa protein. Topoisomerase IIβ has been mapped to 3p24 (Jenkins *et al.*, 1992; Tan *et al.*, 1992). Two forms of Topoisomerase IIβ (IIβ-1 and IIβ-2) have more recently been realised and seem to exist as a result of differential splicing (Davis *et al.*, 1993).

Topoisomerase enzymes are an important target for a number of chemotherapeutic drugs. Camptothecins (Liu, 1989; Kaufmann, 1991) and possibly some anthracyclines (Foglesong *et al.*, 1992) effect Topoisomerase I; whereas Topoisomerase II is a target for a number of intercalating agents (including adriamycin and daunorubicin) and the epipodophyllotoxins, VP-16 and VM-26. These drugs seem to act by stabilising a ternary complex that is formed between the cleaved DNA and the covalently linked enzyme, resulting in permenant cell damage (Liu, 1989).

Cross-resistance to chemically unrelated Topoisomerase II inhibitors seems to involve altered (generally reduced) Topoisomerase IIα levels or activity, genetic mutations or post-transcriptional modifications. Reduced levels of Topoisomerase II protein, mRNA and activity have been associated with VM-26 resistance in KB cells (Matsuo *et al.*, 1990). Topoisomerase II protein levels and enzyme activity were reduced in adriamycin-resistant chinese hamster ovarian cells, although no changes in DNA (Southern blot) or mRNA (Northern blot analysis) were realised (Deffie *et al.*, 1992). Alternate splicing of Topoisomerase II mRNA, resulting in alternative mRNA transcript lengths possibly encoding for non-functional proteins, have been reported (Deffie *et al.*, 1989(a; b).

Topoisomerase II mediated multiple drug resistance has been the only mechanism of resistance identified in some cultured cells. As this form of multiple drug resistance does not involve efflux pumps it has been termed "atypical" MDR. Alterations in Topoisomerase II levels in MDR cell lines, however, generally do not occur in isolation. Reduced levels of Topoisomerase II have been found to co-exist with alternative mechanisms, including over-expression of p-glycoprotein, alterations in levels of

activity of Topoisomerase I and or GST π (Cole *et al.*, 1991; Friche *et al.*, 1991; Lefevre *et al.*, 1991). Modifications in Topoisomerase I are generally associated with resistance to a range of drugs (most notably, camptothecin and its analogues) not included in the typical profile of multiple drug resistance associated drugs (Liu, 1989; Pessina, 1993). Topoisomerase I and Topoisomerase II frequently seem to be affected in such a way that when levels of one is decreased, the other is increased (Ferguson *et al.*, 1988; Sugimoto *et al.*, 1990(a, b); Lefevre *et al.*, 1991), thereby balancing the physiological state of Topoisomerases. Although modification in Topoisomerase II is often associated with changes in Topoisomerase I, this is not always the case. In studies of VM-26 selected KB cells, Matsuo *et al.* (1990) reported a decrease in Topoisomerase II mRNA, protein and enzyme activity, by comparison to the sensitive form of the cell line. Topoisomerase I, however, was unaffected, remaining constant in resistant and sensitive cells. Similarly, de Jong *et al.* (1990) observed a reduction in Topoisomerase II in an adriamycin-selected human small cell lung carcinoma cell line, with no changes in Topoisomerase I (or p-glycoprotein) detected.

In clinical investigations, alterations in Topoisomerase II have been found to exist in a large range of tumours. The levels of this enzyme seem to be a good indicator of the susceptibility of the tumour to chemotherapy. Topoisomerase II levels were found to correlate well with clinical responsiveness in studies of many cancers including breast cancer, hepatocellular carcinomas, liver mestastic breast cancer, gastric cancer, colon cancer and oesophageal cancer (Kim *et al.*, 1991, 1992). In general, Topoisomerase II alterations do not occur solely in cancers, but co-exist with other genetic alterations. Increased p-glycoprotein and GST π expression and down-regulation of Topoisomerase II has been reported from a study of 38 human renal cell carcinomas (Volm *et al.*, 1993). Drug resistance in human brain tumours has been associated with altered Topoisomerase II expression (Mousseau *et al.*, 1993). Decreased Topoisomerase II catalytic activity has also been found in

ovarian tumours which have been subjected to platinum/cyclophosphamide chemotherapy (Van der Zee, 1994). However, gene amplification resulting in elevated Topoisomerase IIα expression has been reported in a study of breast cancer biopsies (Keith *et al.*, 1993).

1.1.6 Other Possible Mechanisms of Multiple Drug Resistance

Many other changes, as well as the previously mentioned, have been associated with multiple drug resistance in cultured cells and tumours. The existence of membrane proteins of various sizes have been putatively associated with this phenomenon. These include 24.5 - 34.5 kDa (Mirski and Cole, 1989), 42 kDa (Nakagawa *et al.*, 1992), 85 kDa (Sugimoto *et al.*, 1993), 170 kDa, 180 kDa, 300 kDa (Harris and Hochhauser, 1992), 65 kDa (Kawai *et al.*, 1994), 110 kDa (Scheper *et al.*, 1993) membrane proteins. Altered expression of non-membrane associated proteins (36 kDa, 47 kDa and 55 kDa) have also been observed in resistant cell lines (Mirski and Cole, 1989). Elevated expression of the 36 kDa Annexin protein in a human non-p-glycoprotein expressing resistant lung cell line has also been reported (Cole *et al.*, 1992b).

Amplification of other juxtaposed gene classes mapped with the p-glycoprotein amplicon on chromosome 7q21-31 (Jongsma *et al.*, 1990) have also been detected in multiple drug resistant cells, including that of a cytoplasmic calcium binding protein, sorcin (Jongsma *et al.*, 1987). No correlation has been found, however, with resistance patterns (Harris and Hocchauser, 1992).

Alterations in epidermal growth factor (EGF) receptor levels have also been associated with the multiple drug phenotype in some cultured cells. However, in studies of human lung cell lines, Reeve *et al.* (1990) reported reduced levels of the EGF receptor, whereas Shin *et al.* (1991) and Meyers *et al.* (1993) observed simultaneous amplification of EGF receptor and

MDR 1. Elevated levels of Nuclear Protein Kinase C has been observed in a human drug resistant breast cancer cell line (Lee *et al.*, 1992). Higher intracellular pH has also been associated with drug resistance in some cell lines (Marquardt and Center, 1992).

One of the most basic criteria for effective cytotoxicity to be achieved by anti-cancer drugs is that they enter the cell and reach their specific intracellular target. Inefficient drug uptake by cells and / or alterations in the intracellular distribution of these agents may be responsible, at least in part, for drug resistance in some cell types. Alterations in intracellular drug accumulation, (*i.e.* a shift in adriamycin location from the nucleus to a cytoplasmic or golgi location, have been observed in both "typical" and "atypical" multiple drug resistant cell lines (Coley *et al.*, 1993). Differential intracellular drug distribution was also reported by Keizer *et al.*, 1989; Gervasoni *et al.*, 1991; Schuurhuis *et al.*, 1991 and Mulder *et al.*, 1993.

1.2 Importance of mRNA Analysis

Gene expression, leading from DNA to protein, is a complex multi-step process, with each portion under regulatory control. Such regulation may occur at the level of gene amplification, mRNA transcription or protein translation. The phenomenon of multiple drug resistance has been associated with both gene amplification and increased levels of mRNA transcription. Clynes *et al.* (1992), when studying a multi-drug resistant variant of a human lung squamous carcinoma cell line, reported the presence of double minute chromosomes with homogenous staining regions, both of which are indicative of gene amplification and are a common manifestation of multiple drug resistance. Such gene-associated cytogenetic abnormalities have also been observed in other MDR cell lines (Biedler *et al.*, 1980; Baskin *et al.*, 1981), both in cell lines selected for vincristine-resistance (Meyers *et al.*, 1985; Fojo *et al.*, 1987; Benard *et al.*, 1989)

and adriamycin-resistance (Fairchild *et al.*, 1987; Slovak *et al.*, 1987; Redmond *et al.*, 1990).

In cells with low-level acquired multiple drug resistance, however, p-glycoprotein may be over-produced at the mRNA or protein level, without concominant gene amplification (Bradley *et al.*, 1989), suggesting that MDR 1 gene expression may be transcriptionally or translationally regulated. Induction of MDR 1 mRNA by transient exposure to chemotherapeutic drugs (Kohno *et al.*, 1989; Chaudhary and Roninson, 1993), heat-shock or arsenite (Chin *et al.*, 1990) serum starvation (Tanimura *et al.*, 1992) and certain differentiation agents has been documented. This effect of chemotherapeutic drugs may be of significance from a clinical point of view.

Putative multiple drug resistance-related parameters may, therefore, be studied at a DNA, mRNA and protein level. Since changes in both gene amplification and transcription are reflected in the resulting mRNA levels, understanding the regulation of gene expression depends in part on the ability to accurately measure mRNA species in defined cell populations. Traditionally, levels of individual mRNAs have been analysed by procedures such as Northern blot (Alwine *et al.*, 1977; Thomas, 1980), RNA slot/dot blot (Kafatos *et al.*, 1979), RNase protection assay (Reyes and Wallace, 1987) and *in situ* hybridisation (for details of method see Ausubel *et al.*, 1991a)).

Expression of eukaryotic mRNAs, in the past, has generally been studied using the Northern blot technique (Alwine *et al.*, 1977; Thomas, 1980). Northern blotting is semi-quantitative and does permit mRNA to be sized enabling, for example, the study of splicing patterns of mRNA. However, this method is insensitive, 5-10 μg quantities of purified polyadenylated RNA (poly $(A)^+$ RNA is required to produce a signal. It is also cumbersome, frequently necessitating the use of specific ^{32}P-labelled probes for detection following electrophoresis and transfer of mRNA to filters.

Another method used in the analysis of RNA is the slot/dot blot technique described by Kafatos *et al.* (1979). Using this technique, a semi-quantitative estimation of amounts of particular mRNAs present in a sample can be made. However, it is limited by its lack of sensitivity (1-10 μg of purified poly $(A)^+$ RNA is necessary to produce a signal). In addition, the method is not dependable for detecting extremely rare sequences because of background problems.

RNase protection assays are more sensitive than Northern blotting, (100 ng to 1 μg of poly $(A)^+$ mRNA is required for each assay). The main disadvantages of this method is that, like Northern blotting, it requires hybridisation with a specific probe for each mRNA, as well as the use of radioactive detection methods and sequencing gels.

A more sensitive technique for the analysis of mRNA is *in situ* hybridisation (see Ausubel *et al.* (1991a) for details of method) in which the temporal and spatial expression patterns of mRNA can be determined within complex cell populations and tissues. Using this method 10 to 100 molecules of mRNA can be detected in a single cell and information about transcript distribution and cellular localization can be obtained. However, *in situ* hybridisation can be technically difficult and does not lend itself well to the processing of a large number of samples. In addition, it is not possible to quantify mRNA amounts using this method.

It is obvious, therefore, that the above mentioned methods are not always sensitive enough to detect mRNA in samples limited by either low cell number or low copy number per cell. In addition, they permit only crude quantitation of mRNA.

Application of the PCR technique provides another method of RNA analysis (Wright and Wynford-Thomas, 1990; Larrick, 1992). This PCR based technique has been variously termed RNA-PCR (Kawasaki, 1991), RT-PCR (Rappolee *et al.*, 1988a), RNA

phenotyping (Rappolee *et al.*, 1988b) or message amplification phenotyping (MAPPing) (Brenner *et al.*, 1989). This technique has many advantages over some of the methods mentioned previously. Adaptation of PCR to the detection of RNA has increased the sensitivity of detection of a particular mRNA species by several orders of magnitude (with the exception of *in situ* hybridisation). RT-PCR is 1,000 to 10,000 times more sensitive than the traditional RNA blot techniques which are not sensitive enough to detect mRNA in samples limited by either low cell number or low copy number per cell (Byrne *et al.*, 1988; Wang *et al.*, 1989; Rappolee *et al.*, 1989; Mocharla *et al.*, 1990; Singer-Sam *et al.*, 1990). It has been claimed that RT-PCR can detect actin mRNA from a single cell, or less than 100 mRNA molecules (Rappolee *et al.*, 1989). Mocharla *et al.* (1990) have reported that RT-PCR amplified DNA was obtained from as little as 5 pg of human pancreatic or parotid total RNA, yet transcripts were not detected on Northern blots unless at least 15-30 ng of pancreatic total RNA was used.

Improved sensitivity over the RT-PCR method over Northern blotting is further supported by reports by Clifford *et al.* (1994) where MDR 1 transcripts were detected in 100 % of samples analysed compared to < 17 % by Northern blot analysis. Gilliland *et al.* (1990a) reported a study where no signal could be detected, after stimulation, using Northern blot analysis of GM-CSF mRNA in 10^6 MLA-144 cells, whereas successful detection with good reproducibility was achieved from 200 cells analysed by RT-PCR. Small variations in mRNA transcript levels of less than two-fold (Clifford *et al.*, 1994) and less than three-fold (Rappolee *et al.*, 1989) have been reported. As a result of this sensitivity, RT-PCR can be used to analyse RNA of extremely rare abundance (Chelly *et al.*, 1988), mRNAs in small numbers of cells (1 to 1000 cells (Kawasaki *et al.*, 1988; Rappolee *et al.*, 1989) or in small amounts of RNA (as little as 6 pg of total RNA (Rappolee *et al.*, 1989; Wang *et al.*, 1989b; Mocharla *et al.*, 1990; Kawasaki, 1991).

RT-PCR can be used for the detection of transcripts that display variable expression patterns in tissues and during development (Chelly *et al.*, 1988; Rappolee *et al.*, 1988a; Chin *et al.*, 1989). The dystrophin gene, defective in patients with muscular dystrophy is expressed at very low levels (representing only 0.01-0.001% of total muscle mRNA) making it difficult to study by conventional methods. RT-PCR was successfully used to study levels of dystrophin mRNA in clinical samples (Chelly *et al.*, 1988). More recently, RT-PCR has been used for virology diagnosis *e.g.* Hepatitis C virus (Colucci, pers. comm.), Hepatitis B virus, HIV, papillomaviruses, enteroviruses (Best, pers. comm.).

Expression of multiple mRNAs can be determined simultaneously from a single sample of RNA, a difficult task by traditional methods. Purification of poly $(A)^+$ mRNA is rarely necessary for RT-PCR analysis. RT-PCR should be of particular benefit in clinical studies, where biopsy samples may not provide enough material for accurate analysis by traditional immunological and biochemical analysis.

RT-PCR is generally considered to be a more safe and rapid method for RNA studies than Northern Blot analysis. Radio-labelling (^{32}-p) is frequently used in Northern blotting, however, this is not necessary for RT-PCR. Results from this technique are also also available more quickly than from Northern blot analysis - involving a day instead, possibly, days, weeks or months. This means that many more samples and many more repeats may be analysed by RT-PCR than Northern blotting, in a given time period.

The sensitivity of PCR can be one of the disadvantages of the system. As a result of the exponential nature of PCR, even trace contamination of reagents, samples and apparatus, with cloned or PCR derived sequences, may lead to false positives. Unlike the results obtained using Northern blotting, RT-PCR does not provide information on the size of the transcripts of interest. Although RT-PCR provides an ideal method to survey the

expression of multiple genes simultaneously in diverse populations of cells, subpopulations of cells may provide unacceptable background signals. Expression of genes in cells should be treated with caution as it may not indicate physiological significance *e.g.* using RT-PCR Chelly *et al.* (1988) detect expression of several tissue specific genes in cells not expected to express these genes; this phenomenon has been termed "illegitimate" or "leaky" transcription. This leads to the question as to whether levels detected by PCR are clinically or physiologically relevant and whether in all cases functional protein expression results. For example, in the case of MDR 1 low level expression may be as a result of leaky transcription or possibly may represent subpopulations of p-glycoprotein expressing cells within a tumour. A positive result may be obtained from an RNA preparation from a tumour which may only represent the expression of a gene in a subpopulation of tumour cells. These cells would have a growth advantage in the course of chemotherapy. Low level expression may be significant for some tumour types. Clinical correlative studies are necessary to establish whether low levels of MDR 1 expression would be prognostically significant for different tumours. Recent results by Holzmayer *et al.* (1992) suggest that such correlations may indeed be clinically relevant.

1.2.1 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Polymerase chain reaction (PCR) is an *in vitro* technique for the selective amplification of defined target DNA sequences. This technique was developed by Mullis and co-workers at the Cetus Corporation (Mullis and Faloona, 1987). In order to amplify a specific target sequence using PCR, some prior DNA sequence information about the target DNA locus is normally required. This information is needed to design two oligonucleotide primers which, when added to denatured DNA, will specifically bind to their complementary sequences on opposite strands, immediately flanking the desired target *i.e.* the region

to be amplified. The primers anneal to opposite strands in such a way that the extension reaction directs the synthesis of DNA towards each other, both occurring in the 5' to 3' direction (see Fig. 1). In the presence of a suitable thermostable DNA polymerase enzyme and DNA precursors (the four deoxynucleotide triphosphates - dATP, dCTP, dGTP, dTTP), the synthesis of new strands of DNA, complementary to the individual primed DNA strands, is initiated.

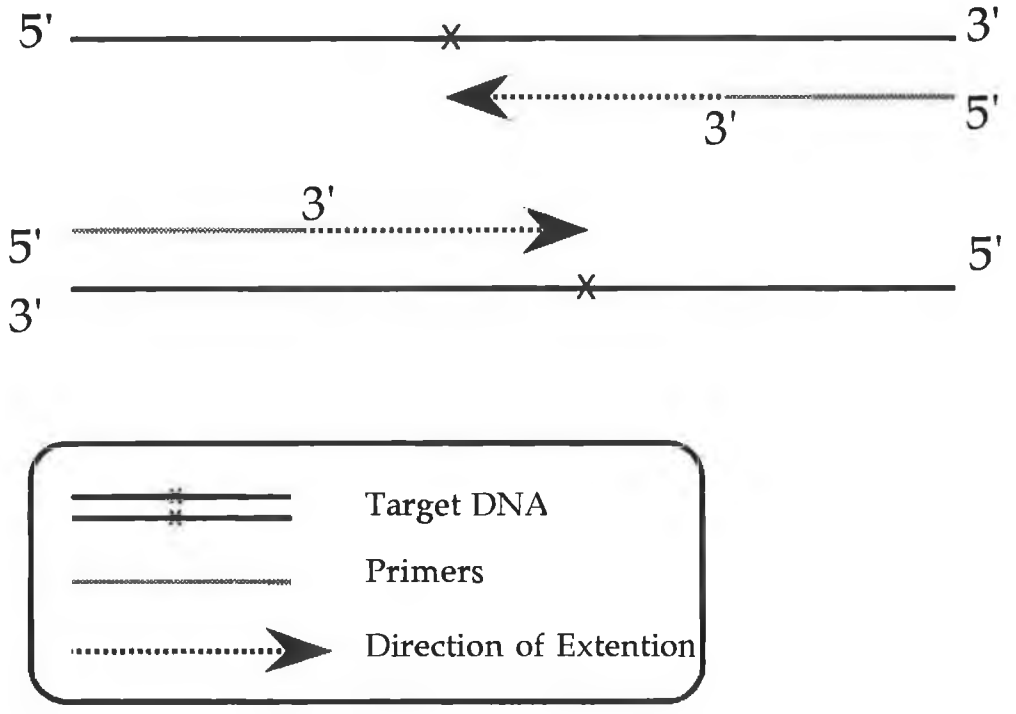


Fig. 1 Primers anneal to their specific sites of recognition on opposite strands, flanking the target DNA region to be amplified. The DNA polymerase enzyme catalyses the incorporation of the free deoxynucleotide triphosphates, resulting in extension of the primers and so the synthesis of new complementary strands of DNA.

DNA generally exists as a double stranded molecule. This must be denatured to single strands for the primers to recognise and bind to their complementary sequences. Denaturation occurs at 90-95 ^{o}C and the temperature is then lowered to allow the primers to anneal. The optimum annealing temperature may range from 37 ^{o}C to 65 ^{o}C; increasing the temperature improves the

primer stringency. (The optimum temperature will depend on the A + T and G + C make-up of the primers, as will be discussed later). Extension of the annealed primers by the incorporation of free deoxynucleotide triphosphates is catalysed by a DNA polymerase enzyme at approximately 72 ^{o}C.

This completes one cycle of the polymerase chain reaction. PCR is a *chain* reaction because newly synthesized strands from one cycle act as templates for further DNA synthesis in subsequent cycles (Fig. 2). This cycle is repeated many times resulting in an exponential increase in the amount of DNA synthesized, until eventually it reaches a plateau.

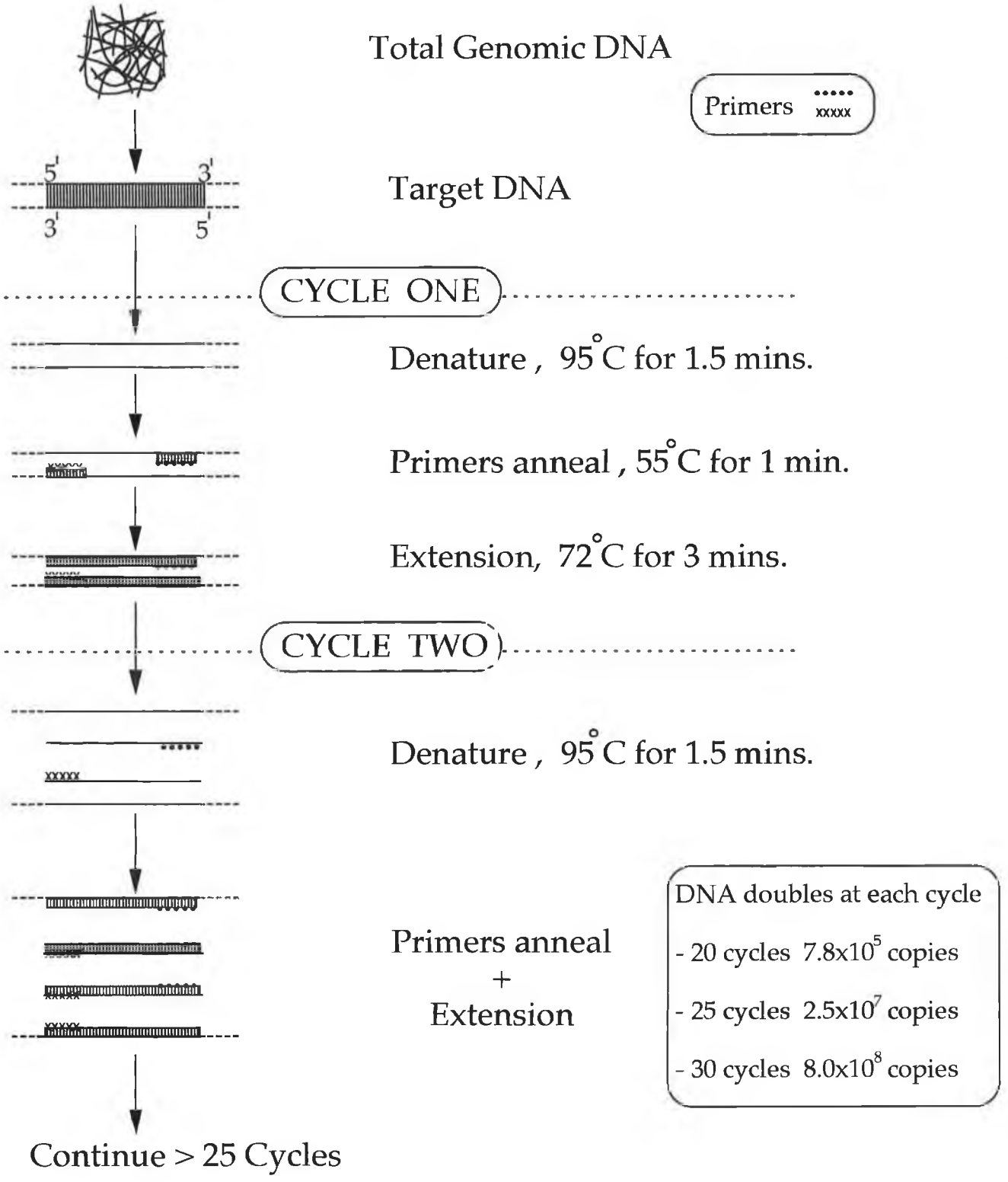


Fig. 2 Each of the primers is chosen so that it recognises one specific DNA sequence and does not bind to any other DNA sequence present in the DNA specimen. Each cycle of the polymerase chain reaction consists of a denaturation step where double-stranded DNA is melted to single strands; an annealing step, where the primers bind to their specific recognition sites on the template; and an extension step, where new strands of DNA are formed, complementary to the primed single strands, by DNA polymerase enzyme extension of the primers. The primers become incorporated into the newly synthesised strands of DNA and so become the boundaries of the amplified region. New strands of DNA formed at each cycle act as template for the next cycle, and so for a finite time DNA strands are synthesised exponentially.

RT-PCR, an adaptation of the basic PCR method enables mRNA be studied in a similar way to DNA. This technique was first described by Seeburg *et al.* (1986). The first published results using this technique were obtained by Veres *et al.* in 1987, when it was used to study point mutations in the mouse ornithine transcarbamylase gene.

mRNA is produced by transcription from DNA and this in turn is translated into proteins. More than twenty years ago reverse transcriptase (also known as RNA-directed DNA polymerase), an animal retro-viral enzyme capable of synthesizing DNA on an RNA template was discovered by Termin (1972). Reverse transcriptase enzymes have been shown to have three enzymatic activities; they can :

(i) copy an RNA molecule to yield double-stranded DNA-RNA, using a primer and joining deoxynucleotide triphosphates in a 3'- 5' linkage;

(ii) degrade RNA in a DNA-RNA hybrid; and

(iii) copy a primed single strand of DNA to form double-stranded DNA (Freifelder, 1983a). The RT-PCR method is based on these properties of reverse transcriptase enzymes.

mRNA is extracted from cells, either in isolation or as part of a total RNA extraction. However, the extraction of undegraded RNA from cells can often prove difficult. This is due to both the labile nature of RNA itself and the presence in the cells of active ribonuclease (RNase) enzymes. The life-time of mRNA is short compared to other types of RNA molecules. This has an important regulatory function in the cell. Degradation of mRNA (to control the formation of a particular protein) proceeds primarily from the 5'-P terminus by RNase enzyme activity (Freifelder, 1983b). Ribonuclease enzymes are formed on ribosomes on the cytoplasmic surface of the endoplasmic reticulum (ER). They then pass into the internal compartments of the ER where they are packaged in secretory granules. This causes the synthetic pathways occurring in the cytoplasm to be separated from the degradative activity of the RNase enzymes. However, the disruption of the cell which occurs when attempting

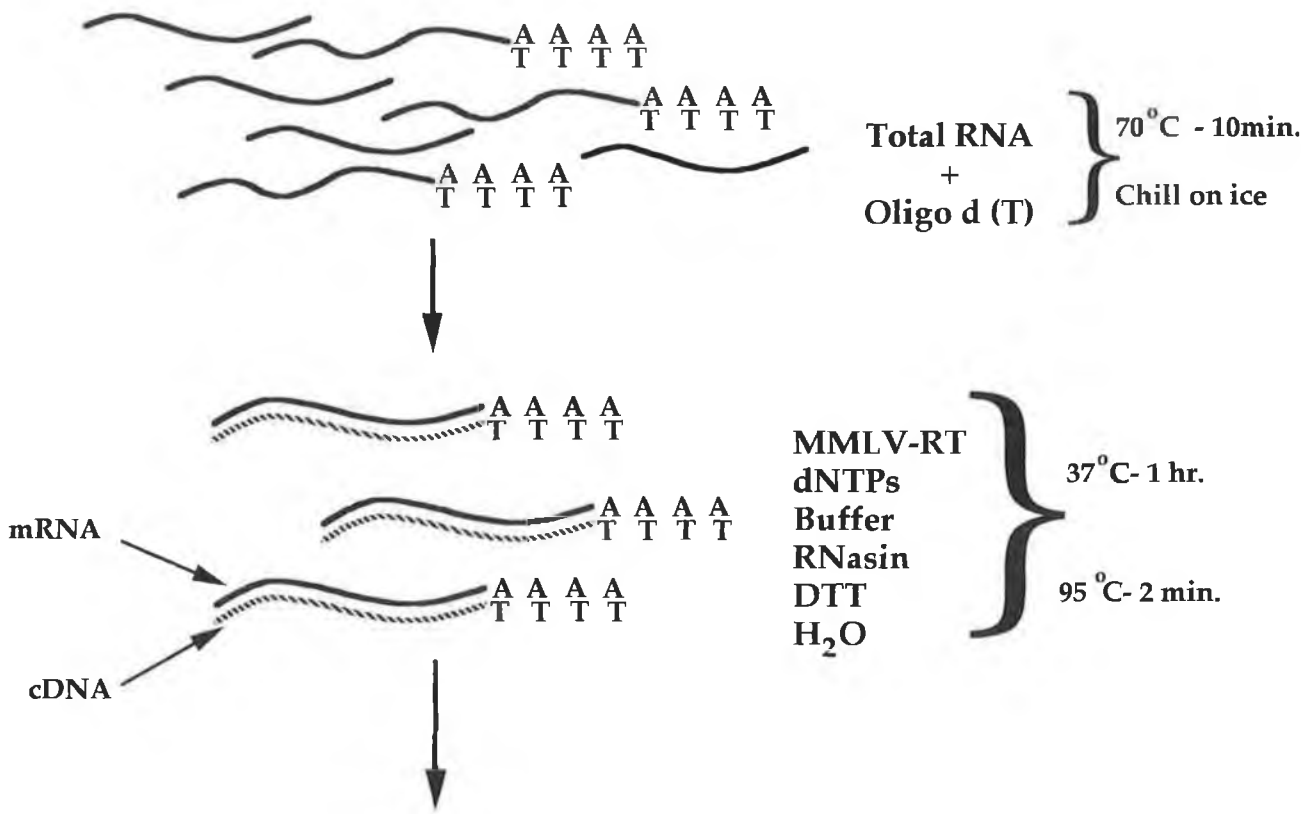
to extract RNA liberates RNases and subjects the RNA to degradation by these enzymes (Jackson *et al.*, 1991). To alleviate this problem, cells are generally lysed in a chemical environment containing guanidium thiocyanate.

Guanidium thiocyanate is one of the most effective protein denaturants known. The use of guanidium to lyse cells was originally developed to allow purification of RNA from cells high in endogenous ribonucleases (Cox, 1968; Ullrich *et al.*, 1977; Chirgwin *et al.*, 1979). Whereas RNases can recover activity after many forms of treatment (such as boiling), they are inactivated by 4 M guanidium thiocyanate and reducing agents such as β-mercaptoethanol (Sela *et al.*, 1957).

After disruption of the cells using guanidium thiocyanate, β-mercaptoethanol and a mild detergent, RNA can be recovered by sedimentation through a cesium chloride gradient (or by organic extraction and ethanol precipitation). This takes advantage of the fact that RNA can be separated from DNA and protein by virtue of its density. To achieve this, the cell lysate is layered on a cushion of a dense solution of cesium chloride. The buoyant density of RNA in cesium chloride (>1.8 g/ml) is much greater than that of other cellular components (Glisin *et al.*, 1974). During centrifugation, RNA forms a pellet on the bottom of the tube, DNA is suspended at the interface and protein floats in the supernatant solution.

mRNA must be selectively primed for it to act as a template on which double-stranded DNA, known as complementary DNA (cDNA), can be formed by a reverse transcriptase enzyme. The cDNA area of interest, which is a direct copy of the mRNA can then be amplified and studied as in a typical PCR. Oligo (dT) primers (for eukaryotic RNA), random hexanucleotide primers, or the 3' antisense gene-specific primer used in the PCR reaction, may be used for this purpose. In choosing the primers for the RT reaction the following should be considered:

Oligo (dT) primers recognise and bind to the poly $(A)^+$ tail (3') of mRNA allowing it to be copied to cDNA, selectively (see Fig.3). (A polyadenylated tail occurs on the 3' end of most, but not all, eukaryotic mRNAs (Ausubel *et al.*, 1991b). The possibility of having long stretches of cDNA or even full-length cDNA made from mRNA have been found to be more likely using oligo (dT) primers. Random primers, as their name suggests, bind randomly to the mRNA template and, again, the reverse transcriptase enzyme catalyses the formation of cDNA strands. These primers, rather than oligo (dT), are claimed to minimise the effects of mRNA secondary structure and the distance of the amplified sequences from the poly $(A)^+$ tail. This is suggested to be of particular importance for the analysis of partially degraded samples of cellular RNA, such as are commonly isolated from tumours and other clinical specimens (Noonan and Roninson, 1991). Because of their random binding, a mixture of short and long cDNA stretches may result. The 3' gene-specific complementary primer binds selectively to the 3' region flanking the mRNA locus of interest. Some advantages of using the downstream gene-specific primer include the fact that cDNA including the specific area of interest should result. It is also convenient as an aliquot of this primer will be used in both the RT and the PCR reactions. Alternatively, the advantages of using oligo (dT) primers or hexanucleotide primers over gene-specific primers for cDNA synthesis is that the products of a single RT reaction can be used for amplification of multiple mRNA sequences, either in the same PCR reaction, or separately. In this way, the cDNA of interest can be amplified simultaneously with an internal standard.



Polymerase Chain Reaction

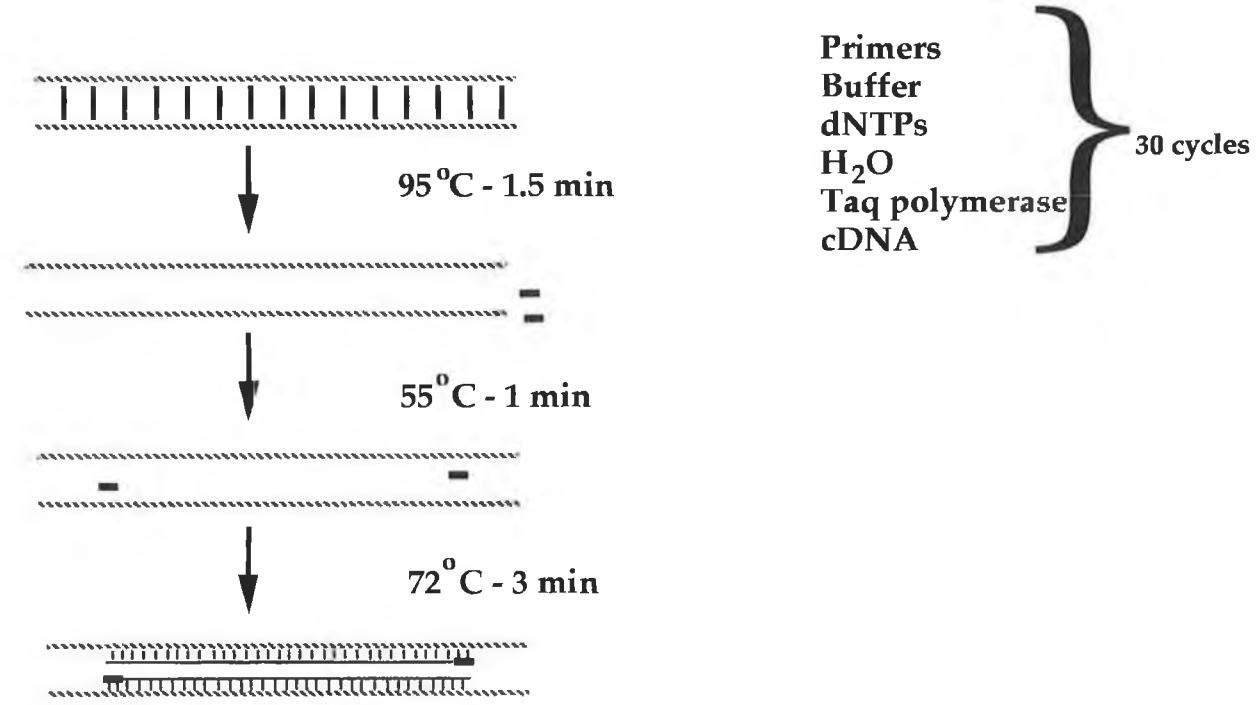


Fig. 3 Oligo (dT) may be used to prime the poly (A)+ tail of mRNA. cDNA is formed on this template by reverse transcription. Specific cDNA regions of interest can then be amplified by PCR for their analysis.

1.2.1.1 Criteria to follow when choosing Primers

One of the many advantages of RT-PCR as a method for mRNA analysis is its specificity *i.e.* mRNA sequences which are highly homologous can be analysed independently. This specificity is determined by the oligonucleotide primer sequences. The success of such a study is, therefore, dependent on the correct choice of primers for the specific application. The following is a list of guidelines for the design of oligonucleotide primers. Due to the uniqueness of each situation's requirements it may not be possible, or indeed necessary, to conform to all of the guidelines listed.

Complementarity to template: The fundamental requirement for a primer is that it should hybridise efficiently to the sequence of interest with negligible hybridisation to other sequences present in the sample. (If sufficient amounts of template are available, this can be tested by performing oligonucleotide hybridisation, Ausubel *et al.*, 1991b).

Target Length: The distance between the primers for which optimum amplification can be achieved is generally considered to between 180 and 500 bp. However, much longer targets may be amplified efficiently. Amplifications of sequences up to 10 kbp in length are sometimes attempted successfully (Ausubel *et al.*, 1991b) but long sequences may be difficult to amplify consistently. A considerable drop-off in synthesis efficiency has been found with distances > 3 kbp (Jeffreys *et al.*, 1988).

The sequence length amplified will obviously be determined by the requirements of the study. For the purpose of RFLP analysis, for example, it is desirable to have a reasonably short distance between primers with the restriction site approximately central (Ivinson and Taylor, 1991). Alternatively, small distances between primers lessens the ability to obtain much sequence information or to re-amplify with nested internal oligonucleotides, should that be necessary (Ausubel *et al.*, 1991b). The fidelity of the DNA polymerase enzyme used in the

PCR reaction (*i.e.* the number of errors produced per nucleotide synthesized) should be taken into consideration when deciding on the target length. *Taq* DNA polymerase does not have a 3' - 5' proofreading exonuclease activity to remove nucleotides that have been misinserted during polymerisation. The longer the sequence being amplified with *Taq* DNA polymerase, the more mismatches will be present. Other DNA polymerase enzymes *e.g.* the T4 and native T7 DNA polymerases are proofreading-proficient and are very accurate with regards to base substitution and one base frameshift mutations. The Klenow fragment of *E. coli.* DNA polymerase I also contains 3' - 5' exonuclease, but the proofreading activity is much weaker than that of T4 or native T7 DNA polymerase. However, the T4, native T7 and Klenow DNA polymerase are heat labile and operate optimally at 37 ^{o}C. Using such low temperatures increases the probability of non-specific annealing of primers to other regions of the DNA and also the likelihood of secondary structure formation being a problem (Eckert and Kunkel, 1991).

For many applications, primers are designed to be exactly complementary to the regions flanking the target sequence. For other purposes, such as engineering of mutations, introducing new restriction sites or for efforts to detect or clone gene homologs when sequence information is lacking, base-pair mismatches will be intentionally or unavoidably created. As far as is possible, it is advisable to include these mismatches as a "tag" near the 5' end of the primer. This should have no effect on the amplification reaction. When incorporating a modification at an internal point in the primer, the fewer the number of modifications, the better *i.e.* 1 - 2 changes (Clarkson *et al.*, 1991).

Overall, it is best to include any changes as far from the 3' end of the primer as possible; at least 10-12 bases from the 3' end of the primer (Clarkson *et al.*, 1991). A mismatch in the 3' area could prevent extension. When only the protein sequence is available, mixed oligonucleotide primers, known as degenerate oligonucleotide primers, derived from an amino acid sequence may

be used. This has had some, but in many cases, limited success (Compton, 1990). Again, the less degenerate the oligonucleotides, especially at the 3' end, the better (Ausubel *et al.*, 1991b). This can be achieved by choosing primers with conserved amino acids which have nondegenerate codons (e.g. Trp and Met) at the 3' end.

Primer Length: The length of a primer contributes to its specificity. It is generally considered ideal to choose oligonucleotide primers between 18 and 30 bases in length; however, shorter and longer primers will work. Low complexity DNA *e.g.* plasmids or previously amplified DNA, can be successfully amplified with shorter primers. This is because the template itself is more "selective" than, for example, a total genomic DNA sample, with a small proportion of the sample consisting of the template of interest. For this reason, the chances of the primers annealing to and amplifying an unwanted template are minimised. Longer primers are often favoured to improve specificity. (However, it is unlikely that primers longer than 30 bases will help improve specificity significantly - Ausubel *et al.*, 1991b). The length of a pair of primers should be similar, if not equal.

Annealing Temperature: The annealing temperature of primers is determined by their length and base composition. Increasing this temperature enhances discrimination against incorrectly annealing primers and so reduces the extension of incorrect nucleotides at the 3' end of the primer. It is considered advisable to choose primers whose melting temperature (*Tm*) (*i.e.* the temperature at which half of the duplex is dissociated (Kimmel, 1987) are between 55 ^{o}C and 75 ^{o}C (Rappolee, 1990). The appropriate PCR annealing temperature may be equal to the melting temperature or 5 ^{o}C below this temperature (Innis and Gelfand, 1990). The *Tm* for a given primer can be estimated using the following equation (Bej *et al.*, 1991):

$$Tm = 2\ ^{o}(\text{no. of A+T residues}) + 4\ ^{o}(\text{no. of G+C residues})$$

Base Composition: Whenever possible, primers should have a balanced G/C and A/T concentration. Primers with a random base distribution should be selected, avoiding stretches of polypurines or polypyrimidines. This, again, is to try to prevent non-specific binding *e.g.* stretches of T's binding to poly $(A)^+$ tails, and to inhibit secondary structure formation. A pair of primers should not be complementary to each other, especially at their 3' end, to avoid the formation of "primer-dimers". (This results when the 3' end of one primer anneals to the 3' end of the other primer, forming an amplified product which will compete with the target of interest in the PCR; as well as being, basically, a waste of primers). Individual primers should not be self-complementary *e.g.* they should not possess palindromes. This is to avoid secondary structure formation.

Location of primers on template:

- Primers should frame a sequence as far 3' as possible so that cDNA produced by oligo (dT) priming need not be full-length to act as template in the PCR (Rappolee, 1990). However, when the genomic structure is not known, primers separated by 300 to 400 bases in the 5' portion of the coding region should be chosen. This is because exons larger than 300 bases in this area are fairly rare in vertebrates. Avoiding areas which may possibly be degraded in mRNA should be a main consideration (Hawkins, 1988). For polyadenylated mRNAs, removal of the poly $(A)^+$ tail appears to precede mRNA degradation (Carter and Malter, 1991). Avoiding the extreme 3' region in close proximity to the poly $(A)^+$ may, therefore, increase the chances of avoiding areas of degradation. (However, if the poly $(A)^+$ tail is degraded, oligo (dT) primers cannot be used successfully in the RT reaction and so random or gene specific primers must be used).

- Where possible, areas that are likely to present problems of secondary structure (as detected by a number of computer programs *e.g.* OLIGO (Rychlik and Rhoads, 1989) should be avoided.

- Primers should flank a sequence that crosses an intron so that DNA contamination can be diagnosed.

- Primers should span introns so that primer-DNA annealing will not occur.

- Primers should frame a sequence with a diagnostic restriction site for validation purposes.

- Primers should frame a sequence that covers a cDNA insert possessed by the laboratory, whenever possible, so that the cDNA can be used as a positive control (discussed later) and/or for validation by Southern blot analysis.

- Primers can be chosen specifically for a single member of a gene family by choosing an area that is unique for that member. Alternatively, a region conserved by a particular gene family may be selected for a more general study of the family. Primers can be chosen for regions of interspecies homology so that the same set of primers can be used for each species or so that primers are sufficiently complementary to hybridise with cDNA from a species with unknown sequences of the gene of interest. Alternatively, primers unique to a particular species may be chosen.

Sequence Banks: Primers should be selected in such a way as to minimise the possibility of their binding to other templates present in the specimen. If one primer binds to an extra template this will result in a waste of primers, *etc.* However, if both primers recognise and bind to the extra template, not only will reaction components be wasted, but an extra region may be amplified unintentionally.

To prevent this happening, the primer sequences can be sent to a DNA data-base *e.g.* EMBL data bank. (See 2.12). At EMBL, the submitted sequence is compared to all the stored sequences, and the first fifty sequences for which it shows strongest homology are listed. For the first thirty of these, the sequences are aligned, their percentage homology calculated and the position on the template shown.

It must be remembered, however, that this is not a comprehensive list, but *only* the first fifty sequences of common homology. Other templates not listed could possibly share enough sequence homology to allow the primers anneal.

1.3 Endogenous Internal Controls

RT-PCR, although renowned for its high sensitivity, specificity, ease and speed, can also be a technique of variable efficiency between preparations and between RNA species. Amplification of replicate samples of plasmid, results in product yield which can vary by as much as six-fold (Gilliland *et al.*, 1990a). Considerable differences in efficiency of amplification between tubes was also reported by Wiesner (1992). This variability may be due to a number of reasons including the fact that the procedure consists of a number of individual techniques - extracting RNA; quantifying the RNA spectrophotometrically; forming cDNA on the mRNA template; and amplifying the region of interest. Taking into account the extraction efficiency, the number of very small volume additions *etc.*, it is understandable that some amount of variability may occur even when extreme care is exercised. For this reason, it is important to include an internal control in each RT-PCR.

In general, "house-keeping" gene products including transcripts encoding metabolic enzymes, ribosomal proteins or translation elongation factors are considered useful as internal controls, as these tend to be ubiquitously expressed (Foley *et al.*, 1993). Obviously, the level of expression of the endogenous control must be the same in each sample to be compared and must not change as a result of the experimental treatment. As such RNAs are extracted at the same time, by the same procedure, using the same solutions, from the same population of cells, quantified also as part of the total RNA and included in the same RT and PCR reactions as the mRNA to be analysed, the products of the two should be directly comparable. The internal control PCR product should differ enough in size to be resolved from the product of interest, but close enough so as to minimise

the probability of differences in amplification efficiency. It should also be distinguishable in size from artifactual "primer-dimers" that may be produced.

The uses of such a control are two-fold. If no signal from the sequence of interest is obtained in a given sample, the internal control will verify whether this is a true (if internal control is present) or false (if both are absent) result. It is not uncommon for amplifications to fail, especially since some experimental samples contain contaminants that interfere with DNA replication (Ausubel *et al.*, 1991b) or inhibitors of *Taq* DNA polymerase. The internal control also allows for quantitation since it normalises for several factors including variation in the amount of sample RNA; efficiency of the RT reaction; efficiency of amplification and the amount loaded on the gel. The fact that such a control minimises differences in RNA yields between samples is of particular importance when studying small tissue samples where the quantities of RNA may be too small to measure by UV spectrophotometry.

Chelly *et al.* (1988) were the first group to use this approach when they used aldolase A mRNA as an internal standard to determine relative levels of dystrophin gene expression in different muscle tissues. β-actin (Horikoshi *et al.*, 1992), β_2-microglobulin (Noonan et al., 1990; Murphy *et al.*, 1990), δ-actin (Zaman et al., 1993); Esterase D (Cole *et al.*, 1991), glyceraldehyde-6-phosphate dehydrogenase (GAPDH) (Dukas *et al.*, 1993) S14 ribosomal protein (Leonard *et al.*, 1993), 18S rRNA (Clifford *et al.*, 1994), 28S rRNA (Khan *et al.*, 1992) histone 3.3 (Futscher *et al.*, 1993) are other examples of such documented controls. Highly expressed genes such as β-actin and β_2-microglobulin are often chosen, based on the fact that they are frequently used to confirm the comparability of RNA loading on gels in conventional Northern analysis. GAPDH has also been used in this capacity (Murphy *et al.*, 1990; Phillips *et al.*, 1993). Even these "house-keeping genes", however, are not completely invariant in their levels of expression (Mansur *et al.*, 1993). The level of the mRNA used as the endogenous control

must, therefore, be examined to ensure its constancy among all of the experimental conditions studied. The ideal control will, therefore, depend upon the specific application.

1.4 Quantitative PCR/RT-PCR:

In the last decade, the polymerase chain reaction (PCR) has been developed for DNA analysis and has been adapted for the study of mRNA (RT-PCR). It is now being advanced to a quantitative level for both DNA and mRNA studies. The aim of quantitative PCR is to deduce the initial amount of target template from the final amount of PCR product. Quantitative PCR can be sub-classed as "semi-quantitative" and "absolutely quantitative" or simply "semi-quantitative" and "quantitative", respectively. Semi-quantitative PCR enables the relative expression of a gene be compared between different samples, whereas using absolutely quantitative PCR, the number of molecules of target mRNA being expressed can be calculated.

The opinions in published literature seem to vary as to how PCR / RT-PCR can be developed to a quantitative level, what controls are required, how reliable this quantitation is, *etc.* Further work, therefore, seems necessary to establish if this quantitative technique can be made less laborious than has been described in some of the previous studies, so that it can be used reliably with relative ease for the study of numerous samples, simultaneously.

RT-PCR requires two enzymatic steps, synthesis of the cDNA template and PCR amplification. Both steps must be considered in any attempt to quantitate mRNA, as a linear relationship between the quantity of mRNA and PCR product requires both efficient conversion to cDNA and exponential amplification (Murphy *et al.*, 1990).

RT Reaction:

Efficiency of cDNA synthesis (RT reaction) can be monitored by the incorporation of radio-labelled dNTPs into TCA precipitable material. The efficiency of the RT reaction has been reported to vary quite significantly from one research group to another (each studying different mRNAs). In studies of myosin heavy chain mRNA from rat ventricles, Wiesner (1992) found the RT reaction to be 100 % efficient, while only 90 % efficiency of this reaction was achieved by Noonan and Roninson (1991) in studies of MDR 1 mRNA. Berger *et al.* (1983) showed that 50 % of input mRNA was reverse transcribed into full-length cDNA. In their calculations, Phillips *et al.* (1993) made the assumption that the efficiency of cDNA production is 50 %. Babu *et al.* (1993) found the efficiency of cDNA synthesis to range from 23 to 27 % in studies of cytokine production by murine T cells. One obvious reason for 100 % RT efficiency not being achieved would be if the mRNA was in any way degraded. Assuming, however, that the mRNA is completely intact, the most likely reasons are insufficient RT reaction components (including primers, dNTPs and reverse transcriptase enzyme), mRNA substrate excess or mRNA secondary structure formation. Results from previous studies suggest that insufficient RT reaction components may be responsible for this lack of efficiency in some cases. Wiesner (1992) reported 100 % RT efficiency when using at least a 50,000 fold excess of primer over template, whereas Berger *et al.* (1983) used only a 2 - 3 fold excess of primer when 50 % efficiency was realised.

From these results if seems important to consider the efficiency of the RT reaction. If this proves inadequate, assays should be done to establish the right concentrations of RT reaction components to result in 100 % RT efficiency for a particular mRNA.

PCR Reaction:

PCR amplification is an exponential process and so PCR product concentration is proportional to the starting target DNA as long as its accumulation remains exponential (Chelly *et al.*, 1988; Singer-Sam *et al.*, 1990). The number of cycles needed to reach the plateau phase varies, depending on the sequence and the original amount of the mRNA. Accurate quantitation requires working within an exponential range, which varies among samples.

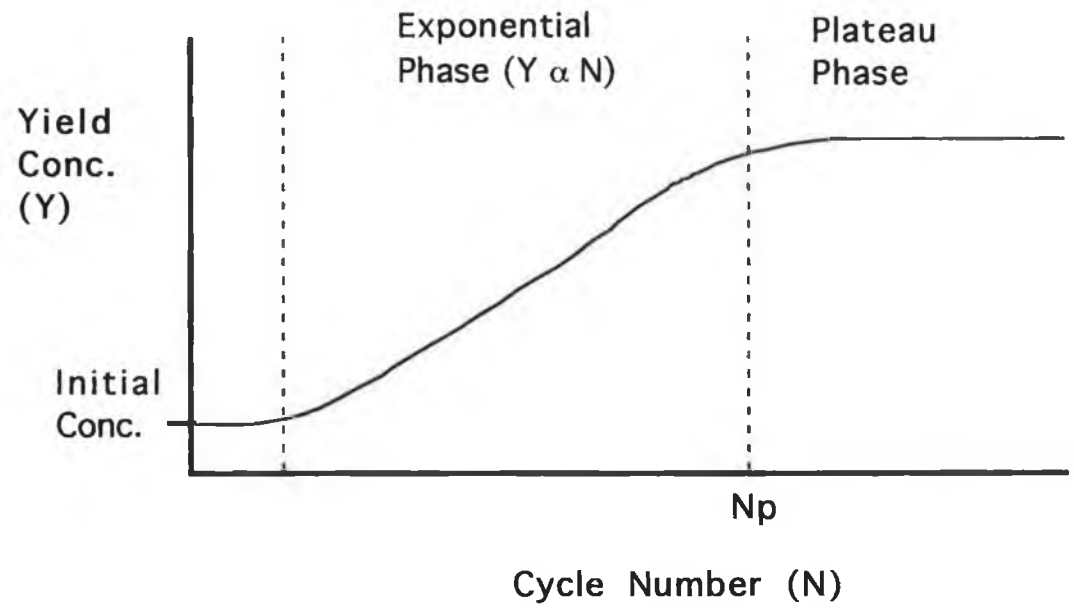


Fig. 4 Exponential and plateau phases of PCR amplification

The exponential range can be determined by carrying out the PCR for a fixed number of cycles on serial dilutions of the RNA reverse transcription product or by performing the reaction with a varying number of cycles on a fixed quantity of cDNA. Murphy *et al.* (1990) reported similar results using these two techniques (however, assays by termination of the reaction at sequential cycles requires greater amounts of input RNA). The point at which exponential accumulation plateaus can be estimated by noting the point at which continued cycles do not produce significantly increased product yield. The efficiency of PCR decreases at the plateau stage due to depletion of reaction components and generation of inhibitors such as

pyrophosphate, diminished enzymatic activity and accumulation of products (Gilliland et al., 1990b; "RT-PCR. Methods and Applications", (Clontech Labs) 1991).

As illustrated in Fig. 4, starting DNA and product concentrations are proportional only during the exponential amplification phase. After Np cycles, the product yield does not increase and, therefore, yield and cycle number are no longer quantitatively linked. In the exponential phase, the yield at any given cycle number can be measured and the initial concentrations can be calculated. Under ideal or theoretical conditions, the amount of product doubles during each cycle of the PCR reaction, according to the equation:

$$N = N_o\ 2^n$$

where N = product yield, N_o = initial concentration of cDNA, n = cycle number. This suggests that a linear relationship exists between the number of amplified target molecules and the initial number of target molecules. The efficiency of amplification (E) *i.e.* the fraction of the template replicated during each reaction cycle, (ideally E = 1), is determined mainly by the sequence of the primers, but may also be influenced by factors such as the nucleotide make-up of the sequence being amplified, the length of this fragment, *etc.* Despite careful selection of primers (see " Criteria to follow when choosing primers"), all PCR reactions are not 100 % efficient *i.e.* E < 1. The value of E has been reported to range from 0.46 to 0.99 for different genes (Chelly *et al.*, 1988; Wang *et al.*, 1989b; Choi *et al.*, 1989). The value of E also varied, from 0.8 to 0.99, when the same gene was amplified in independent tubes under ideal conditions (Wiesner, 1992). As this must also be taken into account, the following equation results (Siebert, 1993):

$$N = N_o\ (1 + E)^n$$

or

$$\text{Log } N = [\text{Log } (1 + E)].n + \text{Log } N_o$$

As PCR amplification is an exponential process, small variations in amplification efficiency can drastically affect the yield of products and in so doing obscure differences in the initial amounts of target sequences (Gilliland et *al.*, 1990b; Bloch, 1991). Diviacco *et al.* (1992) proposed that the exponential range is strictly dependent on the abundance of the starting material (the more abundant the starting material, the shorter the range) and that it is strongly influenced by differences in sample preparations, machine performance, reaction conditions and the presence of inhibitors. PCR product yield, therefore, does not quantitatively reflect the amount of initial template DNA after the reaction has reached the plateau. To overcome these problems when attempting to quantitate mRNA levels by PCR, only products generated during the exponential phase of amplification should be analysed and internal controls should be used.

1.4.1 Semi-Quantitative PCR / RT-PCR:

Semi-quantitative PCR, the simpler of the two approaches for measuring RNA levels by PCR, involves an estimation of the relative (but not the actual) initial amounts of cDNA (or target mRNA in different samples. An endogenously expressed RNA may be used as an internal control template, by which the target gene expression can be normalised. This control also acts as a control for sample-to-sample variations in RT and PCR reaction conditions and monitors the extent of degradation and recovery of RNA. The internal control is co-amplified along with the target RNA(s) of interest and is also evaluated in the exponential range. As these control genes are generally expressed at higher levels than most messages under study and so plateau quickly, a false appearance of comparable loading would occur if care was not taken to study within the exponential range of amplification. In their studies, Murphy *et al.* (1990) reported that their internal standard mRNA, β_2-microglobulin, has reached the plateau phase before the target, MDR 1, was even detectable. To prevent this problem arising, Khan *et al.* (1992)

synthesised cDNA from the control and target mRNAs in separate tubes and then mixed dilutions of the control and target cDNAs before carrying out the PCR reaction. Kinoshita *et al.* (1992), similarly, overcame this problem by waiting until a later stage in the PCR amplification before adding the primers for the endogenous standard. An alternative, and possibly simpler method, may be to add a lower concentration of the endogenous control primers than the target primers when setting up the PCR reaction. If the internal standard mRNA is expressed at the same level in two samples, the ratio of PCR products generated from the target and standard should indicate the relative level of expression of the target mRNA in those samples (Horikoshi *et al.*, 1992). After normalisation with respect to the internal control, target RNA levels in separate samples can, in theory, be compared directly.

The perceived significance of different amplification efficiencies between the control and target DNA seems to differ from one research group to another. Foley *et al.* (1993) suggested that as the internal control and target templates are amplified by different primer pairs and may differ significantly in size, sequence or secondary structure, it may also be necessary to control for differences in amplification efficiencies. To overcome this, they proposed standardising the reaction protocol to a set number of cycles, so that even if the RNA concentrations in the two samples are slightly different, the ratios between the control and target products can still be compared. According to Horikoshi *et al.* (1992), however, when relative gene expression is compared, it is not necessary for the target and internal control DNA to be amplified with the same efficiency; only that the efficiency of the PCR amplification of the same DNA segment remains constant among different samples. It is then possible to use the same internal standard cDNA for comparison of the relative expression levels of any gene whose sequence is known.

When co-amplifying two or more segments, competitive effects result even when different primers are used, thus

limiting the amount of PCR product generated (Horikoshi *et al.*, 1992). In their studies of low abundance genes in small tumour biopsies, Horikoshi *et al.* (1992) used a simpler form of this technique. In these experiments, the relative levels of gene expression were determined by comparing the ratio of product generated by amplification of target DNA and an endogenous internal standard gene in separate reactions. Linear amplification regions were determined by serial dilutions of the cDNA without the need for quantifying the input RNA. Results were calculated from measurements of radio-label incorporation. Using this technique, Horikoshi *et al.* (1992) claimed that less than a two-fold difference in gene expression could be discriminated. Wiesner (1992) and Gilliland *et al.* (1990a), however, have reported significant differences in efficiencies of amplification among duplicate tubes, independent of cycle number. If this is so, it seems that the standard and target DNAs need to be co-amplified in the same tube in order to overcome poor reproducibility of the PCR step.

Semi-quantitative determination of relative target mRNA / cDNA in two or more samples can be more accurately achieved using either titration or kinetic analysis, when the amplification efficiencies are the same for the two samples and analysis is limited to the exponential phase of amplification.

Titration analysis is performed by making a dilution series, or titration, of mRNA or cDNA, amplifying by PCR and quantifying the signal produced. As the amount of target mRNA or cDNA is a constant proportion of the total starting material for each of the various dilutions in a given sample, the amplification efficiencies are the same and the analysis is done within the PCR amplification phase, the relative difference in initial material can be estimated. This is done by extrapolating from a plot of the log of the starting material (*e.g.* in μg) against the log of the product (cpm or $O.D._{260}$ units) for target and control (Siebert, 1993).

An alternative to titration analysis for semi-quantitative PCR is comparative kinetic analysis. To perform a kinetic analysis, the amount of accumulated product is determined for a number of consecutive amplification cycles for each sample under study. This can be achieved by either preparing larger PCR volumes and removing aliquots after the respective number of cycles, or, alternatively, more individual PCR reactions can be prepared and whole tubes removed after the required number of cycles, respectively. To determine the relative difference in target input, using this method, between two co-amplified samples, the results are plotted as cycle number (n) v. log N (the amount of product accumulated, cpm or $O.D._{260}$ units), corresponding to each cycle number. A PCR cycle number is then chosen from the plot at a point where the two curves are parallel (suggesting equal reaction efficiencies) and the amount of product is extrapolated from this cycle number for each curve. At this point, the difference between the accumulated product values is directly proportional to target input differences between the samples (Siebert, 1993).

1.4.2 Absolute Quantitative PCR / RT-PCR

Considerable effort has been devoted to designing PCR assays for determining absolute RNA concentrations. This technique generally involves the use of one of two standards *i.e.* an RNA standard template or an PCR standard template. As with endogenous controls, the theory behind the use of exogenously added standards is that the amount of amplified standard can be quantified after the experiment and the change in the amount of standard is proportional to the change in the amount of target. However, one advantage of using an exogenously added sequence as the control is that the initial amount of standard used in the PCR reaction can be precisely measured. This makes it possible to calculate the absolute level of target mRNA or cDNA present in the original sample.

The RNA standard is included in the RT reaction, simultaneously reverse transcribed with the sample RNA and coamplified by PCR with the target cDNA specific primers. In this way, the RNA standard serves as an internal standard both in the RT and PCR reactions. This approach, using a synthetic standard was first reported by Wang *et al.* (1989b) and subsequently by others including Kanangat *et al.* (1992); Funk and Fitzgerald (1991) and Bouaboula *et al.* (1992). The PCR standard template, on the other hand, is usually DNA not normally in the target sample. This is added only to the PCR reaction. (The RT step is either assumed to be 100 % efficient in this case, or the efficiency, if estimated, is taken into account). Quantitative PCR using such an exogenous standard can be achieved by either forming a standard curve with the control from which the quantity of target can be extrapolated or by titrating the standard against the target (or *vice versa*).

One method of obtaining quantitative results from PCR with an exogenous standard involves generating a standard curve from the collected data (Wang *et al.*, 1989b). Using this approach, a known amount of the RNA standard is mixed with a known quantity of the RNA sample (measured spectrophotometrically) and reverse transcribed. A series of PCR reactions is then set up with dilutions of the cDNA. Similarly, if a DNA control is going to be used, known quantities are included at this stage with dilutions of the target cDNA. The titration is thus being performed on a defined mixture of the target and standard, from which two titration curves can be generated - one for the standard mRNA / DNA and one for the mRNA. The amount of mRNA in the initial sample can then be intrapolated from the standard curve of the exogenous standard. Using this method, Wang *et al.* (1989b) realised changes in mRNA levels of three-fold or less.

An important requirement of this experiment is that the efficiency of amplification be the same for both the target and standard mRNAs. In many cases, this is achieved by designing the standard to contain the same primer binding sequences as the corresponding target mRNA. Additional requirements for using

such exogenous standards are that the PCR products be of similar size and under 1 Kb (Siebert, 1993).

In many of the reported quantitative PCR studies, radio-label was incorporated into the PCR product. Radio-labelling is sometimes favoured because of its sensitivity, as frequently the reaction has started to plateau when PCR products are first visualised on a gel stained with ethidium bromide (Siebert and Larrick, 1992). Incorporation of radio-label can be achieved by either the addition of a radio-labelled dNTP in the mix of dNTPs, or an end-labelled primer in the PCR reaction. These can then be evaluated by forming an autoradiograph which could be scanned by densitometry or by excising the bands of interest and performing scintillation counts. The following formula could then be used to estimate product formation (Wiesner, 1992):

$$\mathbf{N_n\ (moles/\mu l) = cpm/\mu l\ /\ cpm/mol\ x\ y}$$

where, N_n (moles/μl) represents the concentration of product accumulation in consecutive cycles; cpm/μl is the incorporated radioactivity; cpm/mol, the specific radioactivity of the precusor dNTP; and y, the number of this particular dNTP which can be incorporated into the newly synthesised stretch of product. (The initial concentration of a double-stranded DNA template at cycle zero, N_O (moles/μl) and the efficiency of amplification (E), can then be calculated by linear regression analysis of the transformed equation describing product accumulation in the PCR (Wiesner, 1992):

$$\mathbf{\log N_n = \log (1 + E).n + \log N_o}$$

Competitive PCR, an alternative method of absolute quantitative PCR also uses an exogenous template as an internal standard and exploits the principle that the two templates compete for the same primers and, therefore, for amplification, and so preferentially amplify by PCR in proportion to their concentrations. This is done by forming a dilution series of the target sequence or the standard sequence and adding a constant

amount of the other component to the reactions. (The standard may be an RNA or DNA standard). After amplification, the internal standard is distinguished from the product derived from the endogenous sequence of interest by either size, restriction endonuclease cleavage or specific hybridisation. As the amount of template added is known, it is assumed that when the PCR products resulting from the standards and the endogenous mRNA are equal, the amount of mRNA in the original sample is equal to the amount of template added. (This is illustrated in Fig. 5).

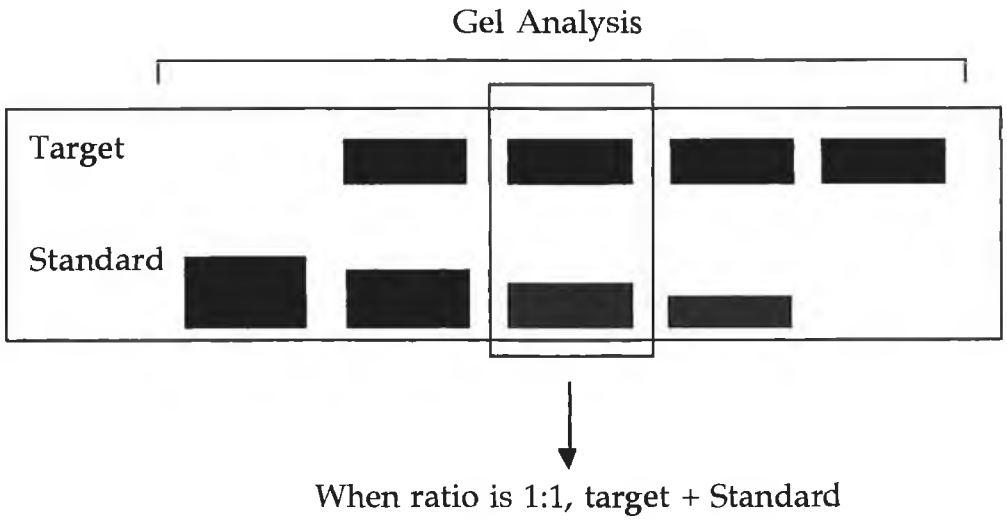


Fig. 5 Relationship between target and control products.

When quantifying mRNA or DNA using a competitive internal control, the ratio of target intensity (Gilliland *et al.*, 1990a) or normalised target intensity (Babu *et al.*, 1993) to that of the competitor may be calculated and the log ratios plotted against the concentration of competitor used in each tube in a log-log scale plot. The amount of target mRNA present in the volume of cDNA taken for quantitation can then be determined from the plot where the target : competitor ratio is 1 (Fig. 5). (When quantifying mRNA, if the RT efficiency has been estimated to be less than 100 %, the amount of target mRNA in the total cellular RNA can be calculated by correcting to 100 % cDNA synthesis). Alternatively, the amount of radioactivity

(scintillation or Cerenkov counts) associated with each band (either target or control) is plotted against the amount of cellular (target) or synthetic (control) input templates. By interpolation, the amount of target mRNA is calculated from the resulting synthetic RNA standard curve (Wang *et al.*, 1989b; Futscher *et al.*, 1993).

Using a derivative of this equation, the relative amount of the target sequence and endogenous standard (*i.e.* the ratio N_{ot} / N_{os}) can be determined from the following equation. Values for the efficiency of amplification (E) for this target and standard are calculated from the slope of a graph of Log N as a function of cycle number (n) (Siebert, 1993):

$$N_{ot} / N_{os} = N_t (1 + E_s)^n / N_s (1 + E_t)^n$$

where:

N_{ot} = Initial number of target molecules;
N_{os} = Initial number of standard molecules;
N_t = Number of amplified target molecules;
N_s = Number of amplified standard molecules;
E_t = Amplification efficiency of target;
E_s = Amplification efficiency of standard;
n = Number of amplified sequences.

When the amplification efficiencies of the target and standard are the same, this equation can be simplified to (Siebert, 1992):

$$N_{ot} / N_{os} = N_t / N_s = A_t / A_s$$

where:

A_t = Amount of amplified target (in cpm or O.D. units)
A_s = Amount of amplified standard (in cpm or O.D. units)

A certain degree of confusion seems to exist as to the significance of differing sizes, sequences and amplification

efficiencies between target and competitive control and whether or not quantitation can be achieved beyond the PCR exponential phase of amplification. According to Diviacco *et al.* (1992) the most reliable approaches to quantitative PCR seem to be those based on the co-amplification of reference templates that share the same primer site and near totality of the amplified sequence, so that the two templates compete for the same primer set and subsequently amplify at the same rate *i.e.* competitive PCR. The target template and competitor should not be very different in size as the efficiency of amplification is inversely proportional to the size of the amplified fragment. Diviacco *et al.* (1992), however, reported competitive PCR to be independent of cycle number, not requiring the exponential phase of amplification.

These views are shared by Gilliland *et al.* (1990a) who found that using a competitive control with a similar sequence to the target of interest, except for the presence of a mutated restriction site or a small intron and so with no significant difference of amplification efficiency, the ratios of unknown product to competitor product remained constant throughout the amplification process, irrespective of cycle number. As competitive PCR was found not to be cycle dependent using this control, amplification could be performed over many cycles so that the product could be visualised and quantified by ethidium bromide staining. In contrast, a competitor containing a large intron or unrelated sequence may have a significantly different efficiency of amplification. Pannetier *et al.* (1993) advised that competitive PCR may not provide accurate results when the sequences of the target and standard molecules are completely different (except for the primer sequences) and analysis is performed after the plateau phase of the reaction has been reached.

Reports by Murphy *et al.* (1990) suggest that with either endogenous or exogenous controls, the PCR reactions of the control and target must both be in the exponential phase of amplification, simultaneously, for results to be quantitative.

The unknown and control should have the same efficiency of amplification. (The length or primary sequence differences between the two templates could have an effect on relative amplification efficiencies). However, results from studies by Wang *et al.* (1989b) and Funk and Fitzgerald (1991) using a multi-specific internal control template show that, at least in the exponential phase of amplification, no significant differences in amplification efficiencies resulted when amplifying sequences differing, to some extent, in either length or nucleotide make-up. Similarly, Futscher *et al.* (1993) reported PCR amplification of a cellular (401 bp) and a synthetic control RNA (487 bp) proceeding with the same efficiencies, despite the fact that the control contained an 86 bp "stuffer" region, so as to enable it be distinguished from the cellular target product. Furthermore, according to Cottrez *et al.* (1994), the amount of a specific cDNA, in the total cDNA, can be calculated by comparison with the internal standard at any cycle number, despite a difference in primary sequence, size or initial quantities.

1.4.2.1 Competitive Controls

Competitive controls for quantitative PCR can be formed in a number of ways. Controls are formed in such a way that they contain the same primer template sequence as the target and so compete with the target for primer binding during amplification. The remainder of the sequence, however, may be a homologous fragment containing an intron or deleted region (yielding a PCR product slightly larger or smaller, respectively, than the mRNA of interest) or an engineered restriction enzyme site - all of which would allow it be distinguished from the target product. To form such a control, the genomic DNA form must be available so that it can be subcloned. Alternatively, the competitive RNA / DNA fragment may have the same primer template sequence, but a completely different (longer or shorter) intervening sequence (Becker-Andre and Hahlbrock, 1989; Gilliland *et al.*, 1990a; Siebert and Larrick, 1992).

Relatively simple methods for forming competitive controls which are homologous to the target, but longer (Diviacco *et al.*, 1992) or shorter (Celi *et al.*, 1993) have recently been described. As illustrated in Fig. 6, a "nearly-homologous" competitor template can be formed by inserting a small "stuffer" fragment within the target region using the "overlap extension method". This is achieved by selecting four primers - two of which span the region to be amplified and are those for which the competitor and target will compete (These are the normal primers which are used to amplify the target). These are termed the out-side primers (indicated as in (1) and (2) in Fig. 6(a). The upper and lower inside primers (numbered (2) and (3) in the diagram) are approximately 40 nucleotides long - 20 nucleotides of which are identical to non-overlapping inner regions in the upper and lower target strands, respectively. The remaining 20 nucleotides at their 5' ends (blackened boxes in diagram) are complementary to each other and are unrelated to the target sequence to be amplified. The aim is to include these two sequences (which are complementary to each other) in the middle region of the final control product. This involves performing a total of three PCR reactions.

Using the four primers and some target template (on which the competitive control is formed), two separate PCR amplifications are carried out using primers (1) and (4) and primers (2) and (3), (see fig. 6(b) and 6(b'), respectively. The resulting products are two fragments each with a single overlapping region of 20 bp at its end. These two products are then combined and re-amplified using the two out-side primers (Fig. 6(c)). The final product is a double-stranded sequence (Fig. 6(d)), homologous to the target, except for an extra 20 nucleotides internally, which can be amplified with the same primers when used as a competitive control (Diviacco *et al.*, 1992).

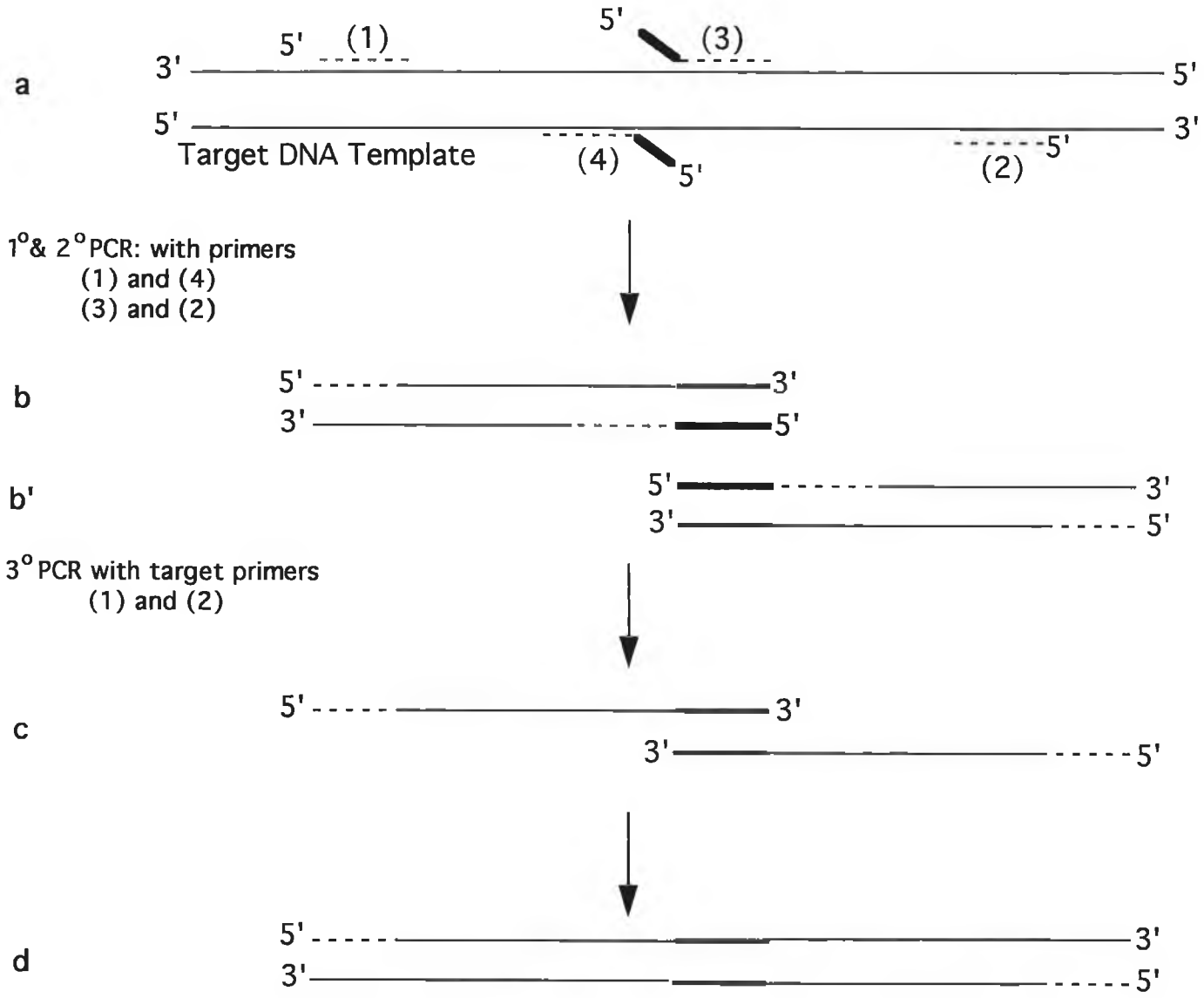
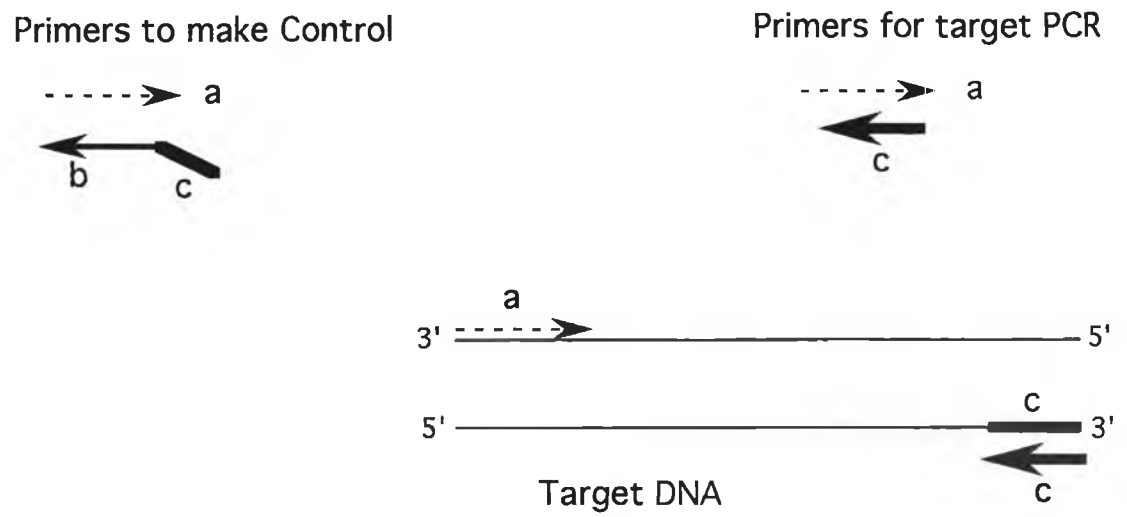


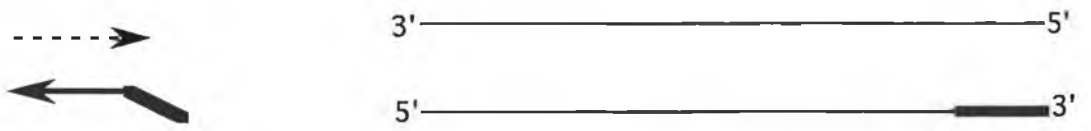
Fig. 6 Formation of an exogenous control, which when co-amplified with the cellular target region will result in a product of a pre-selected length (longer) than the target product. (The exogenous control and target sequences are identical, except for the internal insert).

Using a similar method, competitive controls can also be formed which are "nearly-homologous" to the target, but when amplified with the same primers result in a shorter product. Such controls can be formed as shown in Fig. 7. To make such a control, two primers are synthesised. One primer, (a), is a conventional primer (which is used with primer (c) to amplify the normal target region. The second is a composite primer, (bc) made up of approximately 20 nucleotides at the 3' end (segment b) complementary to the opposite strand of the target sequence, a predetermined distance from (a) (depending on the length of the control sequence required) the other half is complementary to the target (c) region. The target region (being used as a template on which the control is formed) is amplified using the primers (a) and (bc).

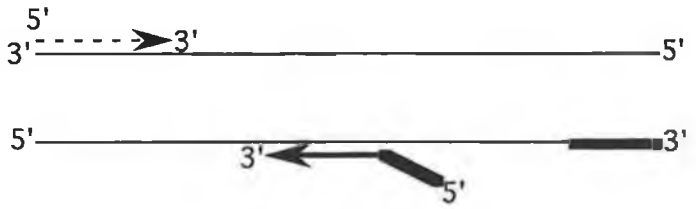
The (c) segment of the (bc) primers may also anneal to the complementary (c) region of the target (which is where the second conventional primer binds). However, extension will not occur because the 3' (b) region of this primer is not complementary to the sequence proximal to the target (c) region. The resulting product, after amplification with the (a) and (bc) primers, is double stranded except for the (c) segment over-hang. A second PCR reaction is performed in which the product of the first reaction is amplified with the (a) and (c) primers, resulting in the required competitive control (Celi *et al.*, 1993).



To Form Control



1° PCR:
One Gene Specific
+
One Composite Primer



2° PCR:
Gene Specific Primers

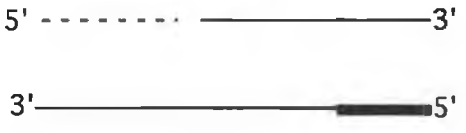


Fig. 7 Formation of an exogenous control, which when co-amplified with the cellular target region will result in a product of a pre-selected length (shorter) than the target product. (The exogenous control and target sequences are identical, except for the lack of the internal region).

Competitive controls with the same primer sequence as the target, but with a different intervening sequence can be formed as in Fig. 8. This involves amplifying a fragment of foreign/neutral DNA using two composite primers. The primers are selected in such a way that the sequence of their 5' end (approximately 20 nucleotides) is that of the primers which amplify the target. Their 3' ends (approximately 20 nucleotides) are complementary to opposite strands of the neutral DNA, respectively (as shown in Fig. 8(a)). Neutral DNA is amplified using these primers. This results in the formation of a product of the desired length, (selected by the location of the inner portions of the primers), with over-hangs complementary to the conventional primers used to amplify the target. (The target-specific primer sequences are incorporated into the PCR product). Using the target-specific primers in a subsequent PCR reaction (as in Fig. 8(b)), a product is formed which consists of neutral DNA with the primer sequences incorporated onto its ends, which can now be amplified with the same primers as the target. Competitive control which are longer or shorter than the target sequence can be formed in this way, as required.

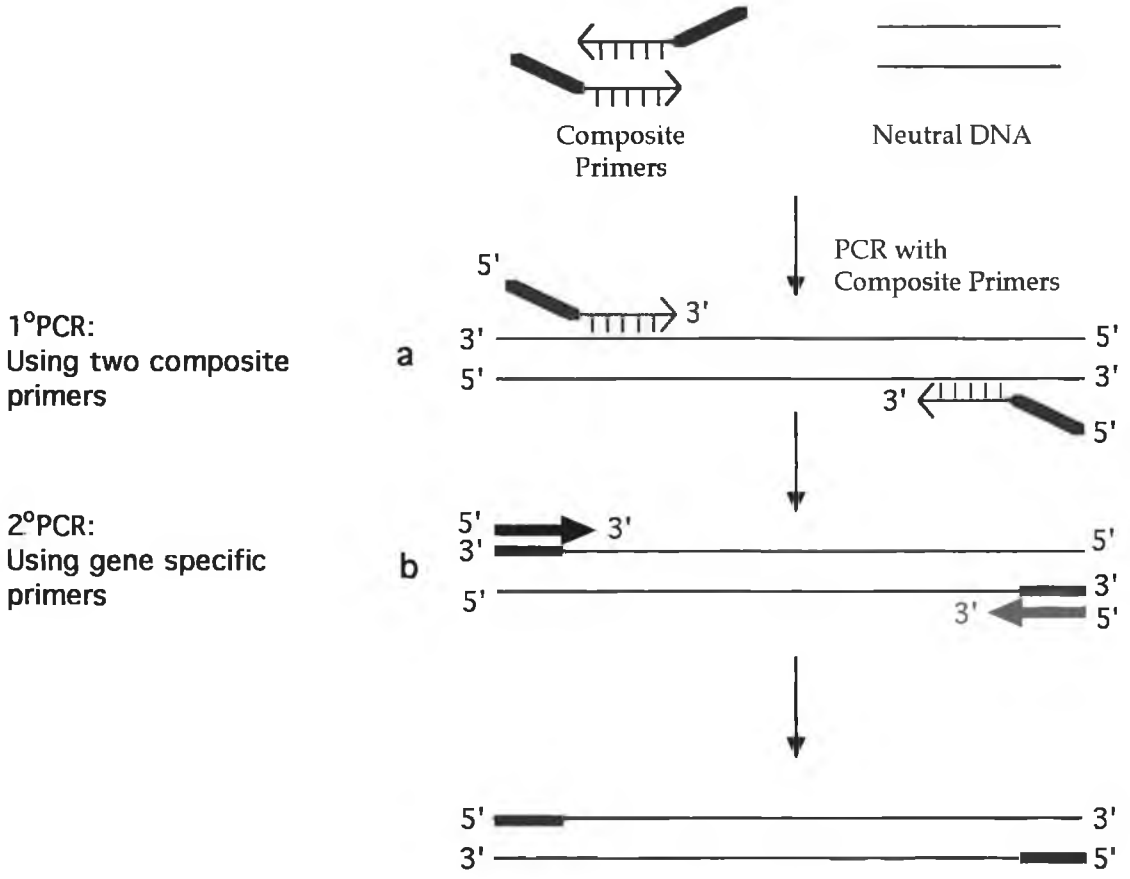


Fig. 8 Formation of exogenous control which is amplified with the primers selected for the target, but whose intervening sequence if different from that of the target. Such a control may be longer or shorter than the target region, as desired.

When deciding which competitive control to create, the following should be considered. If the target and control must amplify with equal efficiencies, this would seem more likely to occur if their sequences were homologous except for a small insertion, deletion or restriction enzyme formation; by comparison to a foreign DNA sequence. However, Siebert and Larrick (1992) reported that unless there are significant differences in denaturation or DNA polymerase extension characteristics due to high G/C content or secondary structure, amplification efficiency is primarily determined by the primer sequences. This was also realised by Wang *et al.* (1989b) who reported that the amplification efficiency of an RNA standard was the same as that of its corresponding target, even though the sequence between the shared primer binding sites was completely different. If sufficient care is taken, therefore, when selecting the foreign DNA source (to prevent problem areas), the latter of the three methods described (fig. 8) may be the method of choice. The reasons for this include the fact that it is probably the simpler of the three methods to perform. Unlike the other methods, each specific DNA target genomic sequence is not required to form its corresponding control - many sources of foreign DNA will suffice. This DNA, once purified, can be used with many different composite primer pairs to create any controls required.

If internal RNA standards are required (instead of DNA standards), an SP6 or T7 RNA polymerase recognition sequence may be incorporated onto the 5' end of the primer for subsequent *in vitro* transcription. Alternatively, the PCR product may be subcloned into an appropriate vector that contains an RNA polymerase recognition sequence (Celi *et al.*, 1993).

1.4.2.1.1 Endogenous Controls v. Exogenous Controls.

In summary, there are basically two types of controls which can be used with PCR - endogenous controls which are selected from ubiquitously expressed genes and exogenous controls, sub-classes as DNA controls and RNA controls. Exactly which type(s) of controls should be used can be a difficult descision. Each of these controls has slightly different characteristics by comparison to the others. The ideal control will, therefore, depend upon the specific application.

The use of an endogenous control as a standard has one major advantage over any type of exogenous control in so far as it controls for any unforseen problems encountered during experimental procedures, including possible degradation of RNA and variations in the efficiency of cDNA synthesis. Furthermore, if care is taken in the selection of an appropriate endogenous control it may serve as a very valid base-line by which the relative level of expression of a particular target gene between different cells or different experimental conditions can be compared (Phillips *et al.*, 1993). Measurement of expression relative to an endogenous internal reference also avoids the need for addition, titration and quantification of an external reference standard, or the manipulation of products post-amplification to distinguish them (Clifford *et al.*, 1994).

Exogenous controls, on the other hand, can be quantified very accurately and so absolute values for specific cellular mRNA levels can be extrapolated. Unlike endogenous controls, which by chance may be degraded or have more complex secondary structures than the target, the exogenous control should be pure and intact so its efficiency of amplification should be predictable. Furthermore, whereas the levels of a so-called ubiquitously expressed gene may differ from one cell type to another, or even at different stages of the cell cycle in a particular cell type, a stock of exogenous control of known concentration can be stored for use in many experiments. According to Horikoshi *et al.* (1992), however, competitor DNA is

often amplified more efficiently than target DNA. This may be due to a less complicated tertiary structure of the synthetic fragment compared to the target fragment. If this is the case, the point where the amplification products are of equal intensity on the gel may not actually represent equal concentrations of the two competing DNA segments. Errors arising from incomplete digestion and heteroduplex formation where restriction enzyme digests are used to distinguish the competitive standard from the target PCR product can add further complications to this procedure.

1.4.2.1.2 Exogenous RNA v. Exogenous DNA Controls

When forming an exogenous control, the choice of RNA or DNA control must also be considered. Again, opinions in the published literature seem to conflict in this area. RNA controls are more laborious to create, as generally a DNA control is formed and this is then used as a template to form cRNA by *in vitro* transcription. According to Foley *et al.* (1993) however, because of the variable efficiency of cDNA synthesis, RNA controls are vastly preferable to DNA controls.

Although a competitive RNA control is more reliable than a DNA control as it accounts for variation in the RT and PCR reactions, care must be taken to ensure that control RNA and template mRNA are not in disproportionate concentrations in the RT reaction, to avoid competition. To prevent this occuring, preliminary simultaneous RT-PCR titration studies with the standard RNA and target RNA for each mRNA species in a sample to be quantified, should be done. This calibration requires the use of more sample RNA and may restrict this approach where extensive quantitation with a limiting sample source is needed. It has, therefore been suggested that these problems are bests overcome by using a DNA control and taking into account the RT efficiency (Babu *et al.*, 1993).

1.5 Ribozymes and their Role in Multiple Drug Resistance

The existence of introns ("int"ervening non-coding sequences) in DNA was first reported in 1977 (Berget *et al.*, 1977; Chow *et al.*, 1977). These introns plus exons ("ex"pressing) regions were found to be transcribed to form precusor mRNA (pre-mRNA). This pre-mRNA then undergoes specific cleavage-ligation reactions, termed RNA splicing, which results in the introns being removed and the exons ligated. This process occurs after 5' capping and the addition of the 3' poly $(A)^+$ tail, in the nucleus (Freifelder, 1983b). That splicing and ligation reactions may be catalysed by RNA molecules was first reported by Cech in 1982, in studies of the protozoan *Tetrahymens thermophila*. Preceding this discovery, RNA molecules were primarily considered as passive carriers of genetic information. Such RNA molecules have been termed ribozymes and are defined as molecules that undergo intramolecular catalysis (*e.g.* self-splicing or self-cleaving) or that act as enzymes (Cech, 1988). Certain ribozymes can cleave substrate RNAs in a sequence-specific manner *i.e.* they behave as an RNA restriction endonuclease, although no such enzymes have been found to date. In many cases, these essentially RNA-catalysed reactions can be further modulated by small nuclear ribonuclear proteins (Cech, 1988).

For an intron to be self-splicing (act as a ribozyme) it must meet the following criteria. It must recognise and cut at its own beginning, where the preceding exon ends. Similarily, it must be able to distinguish its ends from the beginning of the following exon and cleave at this point. Following this, the intron must join together the ends of the two exons which surround it (by "q" ligation) (Maddox, 1989). This is achieved by specific recognition sequences in the pre-mRNA.

Following the initial discovery of the existence of ribozymes, a number of catalytic RNAs have been identified. These are characterised by containing stretches of nucleotides that base-pair with a complementary RNA region. The catalytic

region then cleaves the bound RNA, while the base-pairing holds it in position. Based on their variety of three-dimensional shapes, these ribozymes have been termed hammer-head, hair-pin and axe-head (Barinaga, 1993).

The possibility that ribozymes could be used in bio-engineering and could be specifically designed to manipulate the function of genes or even entire cells was realised (Maddox, 1989). However, reservations existed as to how these ribozymes could be transported into cells, their stability in the cells and the precision of their actions (Gibbons, 1991). Hammer-head ribozymes recognise any GUX (where X can be C, A or U) at the target mRNA and so can bind and cleave the target sequence (Uhlenbeck, 1987; Haselhoff and Gerlach, 1988; Cameron and Jenkins, 1989). The potential role of ribozymes as therapies for diseases such as AIDS and chronic hepatitis has been illustrated, by the successful application of hammer-head ribozymes to cleave human immunodeficiency virus type 1 (HIV-1) in cultured cells. This has been achieved by transfecting expression plasmids, containing DNA sequences encoding the ribozyme framed by other sequences required for the initiation and termination of transcription into the cells (Sarver *et al.*, 1990; Weerasinghe *et al.*, 1991; Dropulic *et al.*, 1992).

The potential of such an approach to gene therapy in cancer is also under investigation *in vitro*. H-ras oncogene is thought to be involved in tumorigenesis, invasion and metastasis. Ribozyme-mediated reduction of H-ras oncogene expression in a cultured human bladder carcinoma cell line (EJ) resulted in a reduction in tumour formation and invasion when these cells were implanted in nude mice (Kashani-Sabet *et al.*, 1992). This supports the proposed role of H-ras. Ribozymes directed against c-fos, which is thought to mediate its effect through transcriptional activation and which is over-expressed in multiple drug resistance to a number of anti-cancer drugs including cisplatin, 5-fluoruracil and azidothymidine, have been transfected into cultured cells. The resulting reduction in c-

fos gene expression was shown to reverse resistance to a number of chemotherapeutic drugs (Scanlon *et al.*, 1991).

P-glycoprotein, encoded by the MDR 1 gene, is associated with "typical" multiple drug resistance in many cell types. To overcome or reduce the level of multiple drug resistance, the activities of p-glycoprotein can theoretically by blocked at the DNA, mRNA or protein level. Although MDR 1 gene amplification is often associated with p-glycoprotein over-expression, this is not a pre-requisite to p-glycoprotein-related resistance in human tumour cells. mRNA or protein, therefore, seem to be more obvious choices as potential targets for therapy. A hammer-head ribozyme directed against MDR 1 mRNA has been engineered and transfected into cultured human hepatic carcinoma cells, EPP86-181P selected for resistance to daunoblastin (Holm *et al.*, 1994). Two hammer-head ribozymes which cleave the GUC sequence in codon 179 and 196 of MDR 1 mRNA were transfected into a human acute leukemia cell line resistant to trimetrexate (MOLT-3/TMQ_{300}) which displayed MDR 1 over-expression (Kobayashi *et al.*, 1994). Holm *et al.* (1994) observed a 75 - 80 % decrease in resistance in their studies and Kobayashi *et al.* (1994), approximately 65 %. Kobayashi *et al.* (1994) reported that the level of resistance and the amount of MDR 1 mRNA expressed appeared to correlate inversely with the amount of ribozyme expression. They proposed that the lack of complete resistance reversal may be due to the fact that the amount of ribozyme transfected into the cells was not sufficient to cleave the constantly over-produced MDR 1 mRNA.

Results from *in vitro* studies suggest ribozymes to have great potential in clinical situations. This form of therapy is very soon to undergo clinical trial in HIV-1 infected patients (Barinaga, 1993). Unlike the situation with MDR 1 mRNA, HIV-1 virus RNA is foreign to human cells and does not have a physiological role to play. Unfortunately, because of the desperation of the situation with HIV-1 positive patients, any form of potential therapy may be worth trying despite the fact

that its side-effects *in vivo* may not yet have been documented completely.

To overcome multiple drug resistance in cancer, further work may be required to ensure that the optimal ribozyme is created. Kobayashi *et al.* (1994) reported that their MDR 1 mRNA-directed ribozymes had different efficiencies and that the transfected cells had various degrees of drug sensitivity. For ribozymes to be effective, they must enter the cell, locate and cleave the mRNA at their specific target. A hammer-head ribozyme directed against HIV-1 RNA has been designed which worked in theory, but not in practice, due to the fact that it was not reaching its target inside the cell (Barinaga, 1993). If MDR 1 mRNA directed ribozymes are to seriously be considered as potential anti-cancer agents, ways in which their transfection, selectively, into tumour cells can be achieved will have to be developed. This is because p-glycoprotein is known to be present in certain normal tissues and organs and so may be indiscriminately destroyed by the actions of the ribozyme. Furthermore, all of the cells of the entire tumour must be transduced simultaneously if this system is to be successful (Kobayashi *et al.*, 1994).

A potential role for ribozymes as therapy in human pancreatic carcinomas (Holm *et al.*, 1994) and acute leukemia (Kobayashi *et al.*, 1994) has been suggested by *in vitro* studies on appropriate cultured cells. Further work is necessary, however, to establish if similar results are produced when studying multiple drug resistance (including "typical" multiple drug resistance) in cells from other organ tumours.

1.6 Aims of this Thesis

Multiple drug resistance has been associated with changes in expression of a number of genes. In order to accurately assess such changes in a range of normal and MDR cell lines, and in human tumour tissue samples, a method that could be used routinely to assess mRNA transcript levels of a number of genes associated with MDR is required. Minimal alterations in expression of certain genes may have a great influence on the physiological state of the cell, so the method of choice must be very sensitive. Furthermore, a very specific method of analysis is required since, for example, the cDNA sequences of two of the gene transcripts under study, MDR 1 and MDR 3, are highly homologous. RT-PCR was, therefore, selected as the method to be evaluated in this thesis. As RT-PCR analyses of some of the mRNAs of interest - MDR 1, MDR 3, MRP, GST α, GST π, CYPIA1, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ - had not been documented in the literature, oligonucleotide sequences had to be selected, using the relevant cDNA sequences and following defined criteria, which would act as PCR primers. As the analysis was to be performed on normal cells, drug sensitive tumour cells and MDR tumour cells, a gene that is ubiquitously expressed in each cell type and apparently not affected by chemotherapeutic drugs used in this study, had to be selected.

mRNA transcript levels of a number of MDR-related genes (listed above) were assessed in a number of MDR cell lines, in comparison to their parent cell lines, to establish their respective mechanisms of MDR. The reproducibility of such experiments needed to be assessed from two points of view: reproducibility of the RT-PCR reaction using different aliquots of the same RNA extract; and reproducibility of the entire procedure starting with two different batches of the same cell line.

This technique needed to be developed to a quantitative level so that the relative levels of gene expression between

cell lines could be assessed. As PCR product concentration is proportional to the starting cDNA only as long as the product accumulation remains exponential, the PCR cycle range of exponential product accumulation and plateau formation for each primer pair had to be established. An "optimum"/suitable cycle number specific for amplification with each primer pair was selected. Whether or not such an "optimum" cycle number could be considered suitable for analysis of the relevant mRNA templates extracted from different cell types was also to be assessed. The accuracy of the semi-quantitative method, and the way in which the results were analysed was assessed. The limitations of the technique will be evaluated, and from the results a proposal made as to how best this technique can be used for routine studies. This technique will also be developed for quantitative analysis, to allow absolute levels of gene expression be determined. The reproducibility of all RT-PCR techniques will to be evaluated.

Control of expression of certain genes may be at the level of translation, rather than transcription. Alterations in the expression of certain proteins (p-glycoprotein and Topoisomerase IIα) associated with MDR were also investigated to establish whether or not the protein levels reflected the mRNA levels.

The MDR cell lines studied in this thesis developed resistance as a result of continuous exposure (up to 18 months) to a chemotherapeutic drug (some to adriamycin, others to VP-16). The ability of such a drug to induce expression of MDR-related genes after short-term exposure (possibly more representative of the clinical situation), was assessed.

The possibility of extracting intact RNA from clinical specimens and assessing for the presence of MDR-related gene transcripts was evaluated, using "fresh" tumour biopsies. A method was also developed which would enable intact RNA be extracted from paraffin-embedded archival tissue, which would allow retrospective studies be performed.

To assess the significance of the MDR 1 gene product in multiple drug resistance, an MDR 1-specific ribozyme was transfected into MDR cells and its affect on the MDR 1 gene transcript levels was monitored. Possible changes resulting in the MDR profiles were also to be assessed by performing toxicity assays on the cells.

Chapter 2

Materials & Methods

MATERIALS AND METHODS

CELL CULTURE

2.1 Water

Water used in the preparation of media and solutions was purified by passing it through a Millipore Milli-RO Plus system with an Elga Elgastat UHP. This involves a pre-treatment which utilises activated carbon, pre-filtration and anti-scaling, followed by a reverse osmosis step. This was followed by organic adsorption, ion exchange, ultra micro-filtration, photo-oxidation and ultra-filtration. The quality of water was monitored on-line. A measure of 18 megaohms/cm reactivity at 25 ^{o}C was considered acceptable.

2.2 Glassware

All glassware and bottle-caps used were soaked, for 1 - 2 hours, in a 2 % (v/v) solution of RBS (AGB Scientific; RBS-25) in warm water. Following this, the bottles were scrubbed manually, bottles and bottle-caps were rinsed in warm water and machine washed using Neodiser detergent. These were then rinsed twice in double-distilled water, once with ultra-pure water and autoclaved before use. Glassware containing spent medium from cells (see 2.6 for disposal of media containing chemotherapeutic drugs) was autoclaved, rinsed with tap water and then treated as above.

2.3 Sterilisation

Water, glassware, and solutions containing thermostable compounds were sterilised by autoclaving at 120 ^{o}C for 20 min., at 15 p.s.i. pressure. Temperature labile compounds were

filtered through a 0.22 μm sterile filter (Millipore; millex-gv).

2.4 Medium Preparation

Growth media for cell culture were prepared as indicated in Table 2.1. The pH was adjusted to 7.45 - 7.55, by the addition of sterile 1.5 M NaOH, and the volume adjusted to 5 litres. The medium was then filtered through a sterile 0.22 μm bell filter (Gelman; G.1423S) into sterile 500 ml bottles. Samples were taken from each bottle for sterility checks to be performed as follows:

1) 3 ml sample was placed in a sterile universal to check for turbidity;
2) 1 ml sample was streaked onto a Columbia (Oxoid; CM331) blood agar plate;
3) 1 ml sample was added to 5 ml of sterile Sabauraud (Oxoid; CM421) dextrose;
4) 1 ml sample was added to 5 ml of sterile Thioglycollate (Oxoid; CM173) broth.

All samples were placed at 37 °C, for 7 days, checking every 24 hrs. to determine the sterility of the individual media bottles.

The medium containing bottles were labelled, dated and stored at 4 °C until required. ATCC medium was prepared by mixing Dulbecco's Modified Eagiles medium (DMEM) and Hams F12 medium in a ratio of 1 : 1. All media were supplemented with 2 mM L-glutamine (Gibco; 043-05030) and 5 % fetal calf serum (FCS) (Seralab; 101024), prior to use. (Throughout the text all references to medium will indicate ATCC fully-supplemented medium, unless otherwise indicated).

ATCC medium into which tumour biopsies were placed for transport from surgery to the laboratory was further supplemented with 2 % Penicillin/Streptomycin (Gibco; 5,000 IU/ml penicillin/5,000 Ug/ml streptomycin).

Table 2.4.1 Preparation of Growth Media

COMPONENTS	**DMEM**	**Hams F12**
10X Medium	500 ml 10X DMEM (Gibco; 042-02501M)	Hams F12 powder (Gibco; 074-01700N)
Ultra-pure H_2O	4300 ml	4700 ml
1M Hepes (pH 7.5)* (Sigma; H9136)	100 ml	100 ml
7.5 % $NaHCO_3$ (BDH; 30151)	45 ml	45 ml

* The weight equivalent of 1M N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (Hepes) was dissolved in an 80 % volume of ultra-pure water and autoclaved. The pH was then adjusted to 7.5 with 5 M NaOH.

2.5 Routine Management of Cells in Culture

Routine maintenance of cultured cells involved feeding, sub-culturing, freezing and thawing. All procedures were performed aseptically in a down-flow re-circulating laminar flow cabinet (Holton or Gelman Cytoguard) which had been swabbed with 70 % IMS (industrial methylated spirits). All articles entering the laminar flow were also swabbed with 70 % IMS to ensure that a sterile atmosphere was maintained. Gloves were worn at all times during these procedures.

2.5.1 Cell Lines

All cell lines used throughout this thesis are outlined in Table 2.5.1.1. These cell lines are all anchorage dependent. Cells were grown in 25 cm^2 flasks (Costar; 3050) or 75 cm^2 flasks (Costar; 3075). Cells were grown at 37 ^oC in the presence of 5 % CO_2 and fed every 2 - 3 days or when a medium pH change was observed (as determined by a change in the colour of the medium due to the presence of a phenol red indicator). This involved removing waste medium into a sterile waste bottle and replacing it with fresh complete medium, as indicated in Table 2.5.1.1 Separate waste and medium-containing bottles were kept exclusive to each cell line to prevent cross-contamination.

Table 2.5.1.1 Cell lines used throughout the course of this Thesis.

CELL LINE	GROWTH MEDIUM	DETAILS OF CELL LINE	SOURCE
DLKP	ATCC	Human Lung Squamous Cell Carcinoma	G. Grant NCTCC
DLKP-4	ATCC	Clone from DLKP	S. Mc Bride NCTCC
DLKP/VP-3	ATCC	DLKP adapted to MDR by increasing stepwise exposure to VP-16*	M.Heenan NCTCC
DLKP/VP-8	ATCC	DLKP adapted to MDR by increasing stepwise exposure to VP-16*	M.Heenan NCTCC
DLKPA	ATCC	DLKP adapted to MDR by increasing stepwise exposure to Adriamycin^	A. Redmond NCTCC
DLKPA-2B	ATCC	Clone from DLKPA	M.Heenan NCTCC
DLKPA-6B	ATCC	Clone from DLKPA	M.Heenan NCTCC
DLKPA-5F	ATCC	Clone from DLKPA	M.Heenan NCTCC
OAW42	ATCC	Human Ovarian Carcinoma	ATCC
OAW42A	ATCC	OAW42 adapted to MDR by increasing stepwise exposure to Adriamycin^	A. Redmond NCTCC
HEp-2	ATCC	Human Epidermoid Carcinoma of the Larynx (HeLa markers)	ATCC
HEp-2A	ATCC	HEp-2A adapted to MDR by increasing stepwise exposure to Adriamycin^	A. Redmond NCTCC

Table Continued Over

CELL LINE	GROWTH MEDIUM	DETAILS OF CELL LINE	SOURCE
SKMES-1	ATCC	Human Squamous Lung Carcinoma	ATCC
SKMES-1A	ATCC	SKMES-1 adapted to MDR by increasing stepwise exposure to Adriamycin^	A. Redmond NCTCC
SKMES-1ADR	ATCC	SKMES-1 adapted to MDR by increasing stepwise exposure to Adriamycin^	I. Cleary NCTCC

Note:

^ Adriamycin (Farmatalia)

* VP-16 Bristol Myers Pharmaceuticals

DLKP-4 is a clone from the DLKP cell line. DLKPA-2B, DLKPA-6B and DLKPA-5F are clones from the adriamycin-selected cell line, DLKPA. DLKP/VP-3 and DLKP/VP-8 are MDR cell lines selected by continuous exposure to concentrations of VP-16, ranging from 0.3 μg/ml to 3.0 μg/ml in the case of DLKP/VP-3, and from 0.3 μg/ml to 8.0 μg/ml in the case of DLKP/VP-8. The fold-resistance, with respect to DLKP, of these MDR cell lines to a range of chemotherapeutic drugs are shown in Table 4.1. HEp-2A possesses IC_{50} values in regard to its toxicity to adriamycin, vincristine, VP-16 and 5-fluorouracil which are 87.87-, 14.1, 2.16 and 1.12-fold, respectively greater than that exhibited by HEp-2. The fold-resistance of SKMES-1ADR to adriamycin, vincristine, VP-16 and carboplatin, with respect to SKMES-1 are 25, 50, 3 and 2.16, respectively.

2.5.2 Sub-Culture of Cell Lines

The cells used throughout the course of this study grow as a monolayer, attached to the bottom of flasks. Upon reaching confluency, or when required for experiments, the cells were sub-cultured by enzymatic detachment. This involved removing waste medium from the flask of cells, rinsing the cells with 2 ml of trypsin/EDTA (0.25 % trypsin (Gibco; 043-05090), 0.01 % EDTA (Sigma; EDS) solution in PBS (Oxoid; BR14a), which had been pre-incubated at 37 ^{o}C, for 15 mins, and then incubating with a further 2 ml of the trypsin/EDTA solution for 7-10 mins., or until a single cell suspension had been obtained. An equal volume of medium was then added. The cell suspension was transferred to a 30 ml sterile universal (Sterilin; 128a) and centrifuged at 120 g., for 5 mins. The medium was poured from the universal into the waste bottle and the cell pellet was resuspended in 5 ml medium. A cell count was performed as outlined in Section 2.5.3. Approximately 2 X 10^4 - 10^5 cells were added to 10 ml of medium in a 25 cm^2 flask, or 20 ml of medium in a 75 cm^2 flask, respectively. These were then incubated at 37 ^{o}C, in 5 % CO_2.

2.5.3 Cell Counting

A sample of a single cell suspension was mixed in a ratio of 4 : 1 with trypan blue dye (Gibco; 525). This was incubated for 2 mins., after which a sample of the cell mixture was applied to a haemocytometer, such that it was held in the area between the haemocytometer and the cover-slip. Cells in the 16 squares of the four outer corner grids were counted, and the average of the four squares was multiplied by 10^4, to determine the cell number per ml. Cells which stained blue were considered non-viable while those unstained were viable.

2.5.4 Large-Scale Cell Culture

Cells required in large numbers were cultivated in roller bottles. Approximately 100 ml of growth medium was allowed to equilibrate in a roller bottle at 37 ^{o}C, for 30 mins., after which a single cell suspension, of approximately 2 X 10^7 cells, was added. The roller bottle was incubated at 0.25 r.p.m. overnight, then the rotor speed was increased to 0.50 r.p.m. The cells in the roller bottle were fed as required until they reached approximately 80 % confluency, at which stage they were ready for use.

2.5.5 *Mycoplasma* Detection

All cell lines used in this study were routinely tested to ensure that *Mycoplasma* contamination had not occurred. These procedures were performed in isolation from the routine cell culture designated areas (by Cathy Halligan, Una Gilvarry or Dr. Roisin Weedle), to avoid possible contamination of clean cell stocks. This analysis was done using the Hoechst 33258 indirect staining method and *Mycoplasma* culture methods. The cell lines to be tested were grown in a drug-free medium for a minimium of three passages following thawing. A 5 ml aliquot of conditioned medium *i.e.* medium which had been exposed to a flask of nearly-confluent cells for 2 - 3 days, was then removed and tested for the presence of *Mycoplasma*.

2.5.5.1 Hoechst 33258 Indirect Staining

Indicator cells (NRK) were grown (2 X 10^3) overnight on sterile cover-slips in 1 ml DMEM medium supplemented with 5 % FCS and 2 mM L-glutamine, in individual 35 mm sterile petri-dishes. 1 ml aliquots of the conditioned media (2.5.5), from each cell line to be tested, were added to duplicate cover-slips of NRK cells and incubated for 5 days (at which stage the NRK cells should have reached approximately 50 % confluency). The

media was removed from the cover-slips, which were washed twice with PBS, once with a 1 : 1 solution of ice-cold PBS : Carnoy's fixative (a freshly prepared 1 : 3 solution of glacial acetic acid (Sigma; A0808) : methanol (BDH; 101584W) which had been stored at -20 °C for 30 mins. prior to use), and fixed for 10 mins. in Carnoy's fixative. Following this, the cover-slips were allowed to air dry. Then 2 ml of Hoechst 33258 stain (Sigma; B2883) at a concentration of 50 ng/ml in PBS, was added to each cover-slip and incubated in darkness for 10 mins. The cover-slips were washed with water and mounted on a glass slide, using 50 % glycerol (BDH; 101184K) in 0.1 M citric acid (Sigma; C2916), 0.2 M disodium phosphate (Sigma; S9390), pH 5.5 as the mounting solution (with the cells between the two surfaces). The slides were then examined for *Mycoplasma* contamination under oil immersion using a mercury fluorescent lamp. Hoechst 33258 stains nucleic acids. Therefore, the NRK cell nuclei stained brightly. Any extra-nuclear staining was an indication of the presence of *Mycoplasma*. Both positive (a sample of medium known to be contaminated) and negative (medium not exposed to cells) controls were included in this procedure.

2.5.5.2 *Mycoplasma* Culture Method

The substrate for the *Mycoplasma* culture method of detection consisted of 90 ml of *Mycoplasma* agar (Oxoid; CM401) and *Mycoplasma* broth (Oxoid; CM403) bases, which were supplemented with 16.33 % FCS, 0.002 % DNA (BDH; 42026), 2 μg/ml fungizone (Gibco; 05290), 2 X 10^3 units penicillin (Sigma; Pen-3) and 10 ml of a 25 % (w/v) yeast extract solution (which had been boiled for 10 mins. and filtered through a 0.2 μm filter). An 0.5 ml aliquot of sample medium from the cell line being tested was incubated with 3 ml of the broth for 48 hrs. at 37 °C in a CO_2 environment. A aliquot of the broth was then streaked onto a 10 ml agar plate which was incubated for up to 3 weeks at 37 °C in 5 % CO_2, with constant microscopy analysis for colony formation. The presence of "fried egg" type colonies were indicative of *Mycoplasma* contamination of the cell line.

2.5.6 Serum Batch Testing

One of the main problems associated with the use of FCS in cell culture is its batch to batch variation. In extreme cases, this variation may result in a lack of cell growth, whereas in more moderate cases, growth may be retarded. It is very important to avoid this happening, particularly where cell growth is the characteristic being studied in an assay. Therefore, a range of FCS batches were screened for growth of each cell line. A suitable FCS was then purchased in bulk for a block of work, to avoid artifactual variations. Results were recorded as a percentage of a control *i.e.* cells grown in a FCS with a known acceptable growth pattern.

Cells were pre-treated, to ensure that all cells were at the same stage of growth, by seeding into flasks at low density four days prior to assessment. On day 4 the cells were fed with complete medium. On day 5 when the cells were approximately 75 % confluent, they were seeded from a single cell suspension into a 96 well plate (Costar; 3599), at a cell density of 10^3 cells/well, in 100 μl medium void of FCS. 100 μl volumes of medium containing 2 %, 10 % and 20 % (v/v) of the FCS being evaluated were added to respective wells on the 96 well plate, resulting in final dilutions of the FCS to 1 %, 5 % and 10 %, respectively. The first column of each plate was maintained as a control where FCS resulting in a known acceptable growth rate was used. Plates were placed at 37 ^{o}C in a 5 % CO_2 environment, for 5 days, after which growth was assessed by acid phosphatase enzyme activity (Connolly *et al.*, 1986; Martin and Clynes, 1991). After incubation for the required length of time, medium was removed and the assay plates rinsed twice with (100 μl volumes/well) PBS. To each well of the 96 well plate, 100 μl of 10 mM *p*-nitrophenyl phosphate (Sigma; 104-0) in 0.1 M sodium acetate, 0.1 % triton X-100 (Sigma; X100), pH 5.5 was added and the plate incubated at 37 ^{o}C for 2 hrs., in darkness. Following this, the enzyme reaction was stopped by the addition of 50 μl of 1.0 M NaOH. The plates were then read in a dual beam plate

reader at 405 nm (reference wavelength 620 nm) (Titertek; Multiskan).

2.5.7 Freezing Cells in Culture

To ensure that adequate stocks of all cell lines were maintained and that all studied could be done within a given passage number range, stocks of cells were frozen to allow their long-term storage.

A single-cell suspension was prepared (Section 2.5.2) from a sub-confluent large-scale culture of cells (Section 2.5.4). The cell pellet was resuspended in FCS. An equal volume of 10 % (v/v) DMSO (Sigma; D5879) in FCS was added drop-wise, with constant shaking of the cell suspension, to result in a final concentration of 10^7 (viable) cells/ml. Aliquots of 1.5 ml volumes of the resulting cell suspension were then placed in cryovials (Greiner; 122 278) (labelled with the cell line, passage number, date and operators initials). These were placed in the vapour phase of liquid nitrogen (as soon as possible) for 2.5 hrs., and were then stored in the liquid phase until required. A vial of cells was thawed 2 - 5 days after freezing to determine the sterility and viability of the stock.

2.5.8 Cell Thawing

The required vial of cells was removed from the liquid nitrogen storage and thawed in a 37 ^{o}C water-bath. The thawed cell suspension was quickly transferred to a universal containing 5 ml of medium and was centrifuged at 120 g., for 5 mins. The medium was gently removed and the cell pellet was resuspended in a 5 ml volume of medium. This cell suspension was then transferred to a 25 cm^2 flask containing 5 ml of medium and was incubated at 37 ^{o}C. Following cell attachment, the cells were re-fed with fresh medium.

2.6 Safe Handling of Cytotoxic Drugs

Because of the potential risks involved in the use of cytotoxic drugs, extreme care was exercised in their handling and disposal. All work with such drugs was performed in a Gelman "Cytoguard" laminar air flow cabinet (CG Series), face masks and double gloves were worn when dealing with concentrate stocks and all drug waste (pure or diluted in medium) was disposed of as recommended by its manufacturer (outlined in Table 2.6.1).

Table 2.6.1 Storage and Disposal of Chemotherapeutic Drugs

CYTOTOXIC DRUG	SUPPLIER	DISPOSAL	STORAGE
Doxorubicin (Adriamycin) 2 mg/ml	Farmatalia	Inactivate with 1 % hyperchlorite	Store at 4°C in darkness
Vincristine 1 mg/ml	David Bull Laboratories, Ltd.	Inactivate by autoclaving and dispose with excess water	Store at 4°C in darkness
VP-16 (Etoposide) 20 mg/ml	Bristol Myers Pharmaceuticals	Incinerate	Store at room temp. Working stock at -20°C, in darkness

INDUCTION OF GENE EXPRESSION

2.7 Induction of MDR 1 mRNA Expression

Two days prior to induction, all cells (DLKP and OAW42) were pre-treated, to ensure that they were healthy and in a logarithmic phase of growth. This involved sub-culturing into 75 cm^2 flasks at a density of 2 X 10^4 cells/flask. The cells were allowed to attach overnight. The following day, the cells were fed with fresh medium. On the day of experimentation, duplicate flasks of each cell line, which were approximately 50 % confluent, were treated as follows:

1) Cells were fed with 20 ml of medium containing no drug (to act as a control);

2) Cells were fed with 20 ml of medium containing 0.1 μg/ml adriamycin;

3) Cells were fed with 20 ml of medium containing 1.0 μg/ml adriamycin.

After 48 hrs., the cells were re-fed as above. Physiological features of the cells were assessed frequently, by microscopy, to identify any signs of cell stress. After a total of 72 hrs., visible signs of cell stress were apparent in the cells treated with drug. RNA was, therefore, extracted at this time and the induction of expression of a number of MDR-related genes was studied, using RT-PCR techniques (Section 2.10).

2.8 RIBOZYME TRANSFECTION

2.8.1 Transfection of MDR 1 Ribozyme into Mammalian Cells

Plasmid containing the MDR 1 Ribozyme (received as a gift from Dr. Kevin Scanlon, City of Hope Medical Center) was transfected into DLKPA-5F cells (a cloned population of DLKPA) and DLKP-4 cells (cloned population of DLKP) using a calcium phosphate transfection technique.

2.8.1.1 Pre-treatment of Cells

DLKP-4 and DLKPA-5F cells were pre-treated in a similar way to ensure that they were healthy and in a logarithmic phase of growth prior to experimentation. This involved sub-culturing the cells into a 75 cm^2 flask at a density of 2 X 10^5 cells/flask, 3 days prior to being required. The cells were allowed to attach overnight and the next day the cells were fed with fresh medium. The day before transfecting, cells from a sub-confluent population were seeded (from a single cell suspension) at 5 X 10^5 cells per 10 cm-diameter petri-dish, to ensure that the cells were approximately 70 % confluent the following day. For each plate of cells to be transfected, 10 μg of plasmid DNA (*i.e.* containing the ribozyme) was diluted with 410 μl sterile ultra-pure H_20 in a sterile 3 ml tube and was stored overnight, at 4 ^oC. The following day, 480 μl 2 X HBS, pH 7.12 (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, filtered through a 0.22 μm filter and stored at 4 ^oC, until required) were placed in a sterile tube and left at room temperature. The tubes containing the DNA were incubated at 37 ^oC, for 1 hr. When this incubation period was completed, 60 μl of 2 M $CaCl_2$ was added drop-wise into these tubes with continual mixing using a vortex. Immediately, this DNA-$CaCl_2$ mixture was added to the 2 X HBS dropwise, again with continual mixing using a vortex, after which this solution was allowed stand at room temperature, for 30 mins., in a laminar flow cabinet. After 30 mins. this mixture was added to the cells, drop by drop, with constant gentle

aggitation of the universal and swirling of the petri-dish, to ensure that an even mixture came in contact with all cells. The culture plates were then returned to the incubator for 4 hrs. To potentiate the entry of DNA, the cells were glycerol shocked after the 4 hr. incubation. This involved removing the DNA-$CaCl_2$ mixture and adding 5 ml of 10 % sterile glycerol in 1 X HBS. This was left on the cells for exactly 3 mins., removed and the cells were washed three times with serum free medium. The transfected cells were then fed with 20 ml complete medium and returned to the incubator.

The plasmid containing the ribozyme sequence also contains a gene which confers resistance to geneticin (G418) (Sigma; G9516). Geneticin was, therefore, used as a selective agent by which transfected cells could be identified *i.e.* cells which grow in the presence of geneticin are resistant and so stable transfectants.

2.8.2 Selection of MDR 1 Ribozyme Transfected Clones

The transfected cells were grown in the presence of geneticin, starting with 400 μg/ml of geneticin in medium 48 hrs. after transfecting, and increasing the concentration of geneticin to 1 mg/ml over a three week period. The cells were grown with constant monitoring until single cells had formed individual colonies of approximately 50 cells. The colonies were individually sub-cultured, as follows, using cloning rings (stainless steel rings, with an inner diameter of 8 mm) to form individual trypsination chambers.

The medium from the petri-dish was removed and the cells were washed twice with sterile PBS. The PBS was then removed and a sterile cloning ring, with its end dipped in siliconised grease, was placed around an individual colony (which had been marked when viewed under the microscope), isolating it from other cells on the plate and forming a sealed sub-culturing

chamber. Trypsin/EDTA (100 μl) (Section 2.5.2) was added to the colony and the cells incubated at room temperature , for 5 mins. The trypsin/EDTA was then gently pipetted up and down (4 - 5 times), using a Gilson micropipette, to ensure that all cells had detached (this was checked by viewing under a microscope). The cells were transferred to a single well of a 96 well plate (Costar; 3599). Medium (100 μl) was added to the well and the cells were allowed to attach at 37 ^{o}C in 5 % CO_2, overnight. Following attachment, the medium in the well was replaced with 200 μl fresh medium. When the cells had reached approximately 90 % confluency, they were sub-cultured into a single well of a 48 well plate (Costar; 3548). This was repeated through to a 24 well plate (Costar; 3424), 6 well plate (Costar; 3406), 25 cm^2 flask (Costar; 3050) and 75 cm^2 flask (Costar; 3075). The 10 transfectants (5 from DLKP-4 and 5 from DLKPA-5F) resulting were maintained as individual cell lines, similar to all other cell lines used in this study.

To verify that the transfection had been performed successfully, that the ribozyme was being transcribed and to assess the effect of the ribozyme of the MDR 1 mRNA levels and resistance profiles of the cells, a number of studied were performed. DNA from the transfectants (and parent lines as controls) was amplified by PCR (as in Section 2.10.5) with primers specific for the ribozyme DNA, to establish that transfection had occurred. RNA isolated from these cells was amplified by RT-PCR (Section 2.10.4 & 2.10.5) to i) assess if transcription of the transfected ribozyme had occurred, and ii) evaluate its effect on the endogenous MDR 1 mRNA levels.

The primers used for ribozyme DNA amplification (221 bp) were as follows (Scanlon and co-workers, City of Hope Medical Center):

5' GAC CAG TGT TTG CCT TTT A 3'
5' TCT GGA TCC CTC GAA GC 3'

The primers used for ribozyme RNA analysis (118 bp), which would result in an 880 bp product if contaminating DNA was present,

were as follows (Scanlon and co-workers, City of Hope Medical Center):

5' AGC ACA GAG CCT CGC CTT T 3'
5' TCT GGA TCC CTC GAA GC 3'

Primers used for MDR 1 mRNA analysis amplify a 157 bp product and are documented in Table 3.1

Glyceraldehyde-6-phosphate dehydrogenase (GAPDH) was amplified in this studies (380 bp) at both DNA and RNA levels, to act as an endogenous control. The primers used for its amplification are as follows (Dukas *et al.*, 1993):

5' TCC ATG ACA ACT TTG GCA TCG TGG 3'
5' GTT GCT GTT GAA GTC ACA GGA GAC 3'

The effect or the transfected ribozyme on the drug resistance profile of these cell lines was assessed by performing toxicity assays with adriamycin, vincristine and VP-16.

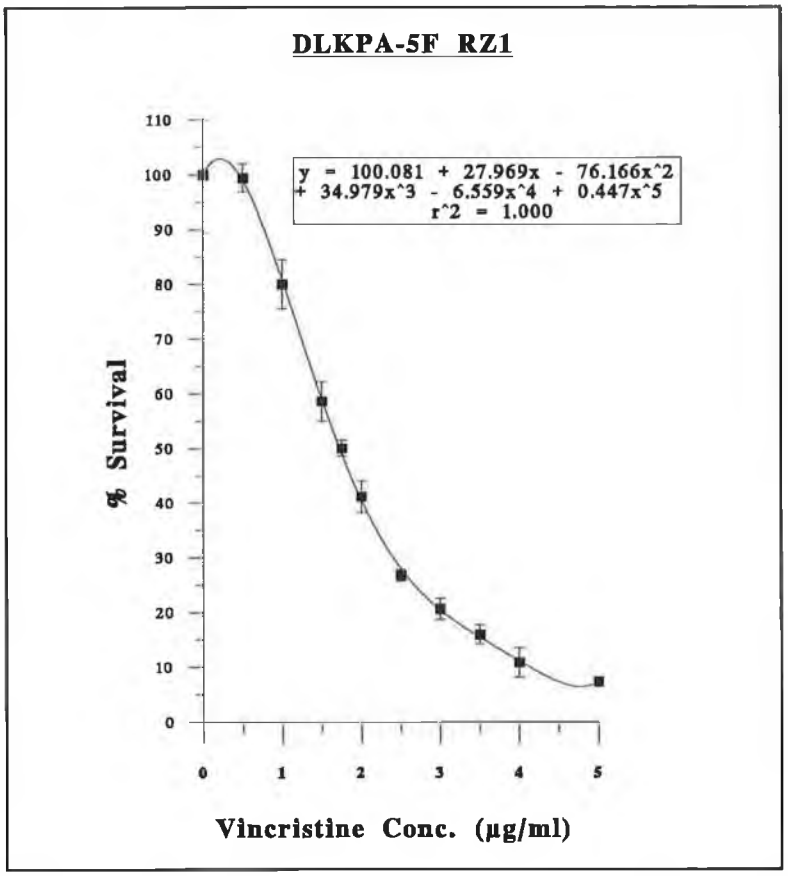
2.8.3 Miniaturised Toxicity Assay

Two days prior to setting up in a toxicity assay, all cells (transfectants and their respective parent cell lines) were pre-treated, to ensure that they were healthy and in a logarithmic phase of growth. This involved sub-culturing into 75 cm^2 flasks at a density of 2 X 10^5 cells/flask. The cells were allowed to attach overnight. The following day, the cells were fed with fresh medium. On the day the assay was set up, the cells were sub-cultured and a single cell suspension was obtained (as described in Section 2.5.2).

2.8.3.1 Toxicity Assay

Cells were seeded, from a single cell suspension, into a 96 well plate (Costar; 3599) at a cell density of 10^3 cells/well in 100 μl medium; except for the first column of the plate which was used as a control, containing only 100 μl medium. The cells were allowed to attach overnight at 37 ^{o}C, in 5 % CO_2. Drug concentrations used in each assay ranged from concentrations which would result in no kill (*i.e.* no drug) to approximately 100 % kill. The required drug dilutions range was prepared (at a twice the final concentration to be assayed) and 100 μl of each drug dilution was added to each well, in replicas of eight. The plates were returned to the incubator and incubated for a further 6 days or until control wells (where no drug was added) reached 80 % confluency. Drug toxicity was then determined by acid phosphatase analysis (Connolly *et al.*, 1986; Martin and Clynes , 1991). Medium was removed and the assay plates rinsed twice with (100 μl volumes/well) PBS. Then 100 μl of 10 mM *p*-nitrophenyl phosphate (Sigma; 104-0) in 0.1 M sodium acetate, 0.1 % triton X-100 (Sigma; X100), pH 5.5 was then added to each well and the plate incubated at 37 ^{o}C for 2 hrs., in darkness. Following this, the enzyme reaction was stopped by the addition of 50 μl of 1.0 M NaOH. The plates were then read in a dual beam plate reader at 405 nm (reference wavelength 620 nm) (Titertek; Multiskan).

2.8.3.2 Determination of the IC_{50} value.



The above diagram represents the toxicity of vincristine to the cell line DLKPA-5F RZ1. The toxicity assay was performed as outlined in Section 2.8.3.1, and the level of acid phosphatase correlated to cell survival in the presence of chemotherapeutic drug. The polynomial equation illustrated in the above diagram, presents the best correlation between cell survival, on the Y-axis and the drug concentration, on the X-axis. This equation has been obtained from the graphical curve fitting carried out by the Cricket Graph computer software package. The correlation coefficient, r, conveys information about the state of the fitness of the equation, in relation to the data obtained from the assay. Thus, the drug concentration corresponding to any percentage of the cell survival, can be calculated by solving the polynomial best fit curve. The determination of the IC_{50}, which denotes the drug concentration corresponding to 50% cell survival, has been carried out by taking Y = 50 and solving the best fit curve equation, with respect to Y. The Newton-Raphson method for numerical solution of *n*th. order polynomial equations has been employed for this determination. A computer program was developed in-house, by Dr. Mohamad Saleh, to execute and perform the calculation. The IC_{50} values, from replica assays, were determined in this manner and used to ascertain the average IC_{50} value and the standard deviation on the average value.

2.9 DNA ANALYSIS

DNA for PCR analysis was "extracted" using the following procedure as described by Higuchi (1989). A single cell suspension was obtained from an approximately 80 % confluent 75 cm^2 flask. These cells were washed twice in PBS, centrifuging at 100 g., for 2 - 5 mins. between each wash. The supernatant was discarded. The cells were resuspended in a volume of "PCR buffer with nonionic detergents and Proteinase K" to result in 6 X 10^6 cells per ml. This buffer consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45 % Nonidet P-40 (NP40) (Sigma; N0896), 0.45 % Tween 20 (Sigma; P1379), which was prepared, autoclaved and stored frozen until required. When required, it was thawed and 0.6 μl of 10 mg/ml Proteinase K (Sigma; P2308) (in water) per 100 μl of solution, was added.

This cell suspension was then transferred to an eppendorf tube and incubated at 50 - 60 °C, for 1 hr. Following this, the Proteinase K enzyme was inactivated by incubating at 95 °C, for 10 mins. DNA quantitation was performed on the cell extracts by reading an aliquot of each sample spectrophotometrically at 260 nm and 280 nm. (An optical density of 1 at 260 nm is equivalent to 50 mg/ml DNA. A A_{260}/A_{280} ratio of 1.8 is indicative of pure DNA). The DNA samples were then stored at -20 °C until required for analysis. 1 μg of this crude DNA extract was included in a subsequent PCR reaction to establish if transfection of the ribozyme had occurred (Section 2.10.5).

RNA ANALYSIS

2.10 Preparation for RNA Extraction

A number of special precautions were taken when studying RNA due to the labile nature of RNA and the existance of RNase enzymes. RNases are ubiquitous in the environment and so care was taken to avoid their introduction while extracting RNA.

- General laboratory glassware and plasticware are often contaminated by RNases. Glassware was, therefore, treated by baking at 180 °C for eight hours, or more. Sterile, disposable plasticware is essentially free of RNases and so was used for the preparation and storage of RNA without pretreatment. Polyallomer ultracentrifuge tubes, eppendorf tubes, pipette tips, *etc.* were all autoclaved prior to use. All spatulas which came in contact with any of the solution components were baked (as for glassware), chemicals were weighed out onto baked aluminium-foil and a stock of chemicals kept separate, for "RNA extraction only", from general stocks.

-Solutions that came in contact with the RNA, after lysing the cells in guanidium solution, were all prepared in baked glassware, made from sterile ultra-pure water and treated with 0.1% diethyl procarbonate* (DEPC) (Sigma; D5758) before autoclaving, with the exception of Tris containing solutions (DEPC reacts with amines and so is inactivated by Tris).

- Gloves were worn at all times to protect both the operator and the experiment. This, again, prevented the introduction of RNases and foreign RNA/DNA into the RT and PCR reactions. Gloves were changed frequently.

* DEPC is a strong, but not absolute inhibitor of RNases. Due care was taken when using DEPC as it is suspected to be carcinogenic.

2.10.1 Total RNA Isolation

2.10.1.1 RNA Extraction from Cultured Cells

The following procedure was conducted in a laminar flow cabinet to maintain sterile conditions and prevent the introduction of contaminants. Cells were grown in 135 mm (tissue culture treated) diameter petri-dishes, until approximately 80% confluent. The medium was removed and the cells rinsed twice with PBS. The cells on 5 replica plates were lysed by the addition of 5 ml of a 4 M guanidinium thiocyanate (GnSCn) solution. The 4 M GnSCn consisted of 50 g of guanidinium thiocyanate (Sigma; G6639), 0.5 g of N-lauroyl sarcosine (Sigma; L5125) and 5 ml of 1 M sodium citrate (RDH; 32320), pH 7.0. The solution was brought up to 100 ml with water which had previously been DEPC-treated and autoclaved and was filtered through a 0.45 μm filter. This was stored at room temperature, in the dark, until required. Immediately before use, 700 μl/100 ml of β-mercaptoethanol and 330 μl/ 100 ml of antifoam A (Sigma; A5758) were added.

The pooled cell lysate from the five plates was centrifuged at 120 g., for 5 mins. This was then layered on 5.5 ml of a 5.7 M cesium chloride cushion in a polyallomer ultracentrifuge tube. The 5.7 M cesium chloride solution had been prepared using 95.8 g of CsCl (Sigma; C3032) and 2.5 ml of 1 M sodium citrate, pH 7.0 per 100 ml of water. The solution was filtered through a 0.22 μm filter. DEPC was added (100 μl/100 ml solution) and this was then left at room temperature for 2 hrs. before autoclaving.

Taking care on route not to disturb the cesium chloride : GnSCn interface, this mixture was spun at 100,000 g. at 15 ^{o}C, for 21 - 24 hrs. in a swinging bucket centrifuge. This resulted in protein layering on top of the GnSCn, DNA at the GnSCn : cesium chloride interface and RNA pelleting at the bottom of the tube. Care was taken to prevent disturbing the RNA pellet or contaminating the RNA with DNA. The guanidinium thiocyanate and

"jelly-like" layer below the GnSCn/CsCl interface was removed by aspiration, until approximately 1 ml of cesium chloride remained. The tube was inverted and the bottom, containing the RNA pellet, cut from the rest of the tube using a heated scalpel blade. The pellet was rinsed with 95 % ethanol at room temperature and resuspended in 200 μl of DEPC-treated water by gently pipetting up and down whilst keeping on ice. The resuspended pellet was transferred to an eppendorf tube and the suspension remains rinsed into the eppendorf with a further 200 μl of DEPC-treated water. The RNA was precipitated out of solution by the addition of 3 M sodium acetate (to result in a final volume of 0.3 M) and 2 volumes of ice-cold absolute ethanol, overnight at -80 ^{o}C. The RNA was then pelleted by spinning at 4 ^{o}C, at maximum speed in a microfuge. The pellet was washed with 70 % ethanol, the supernatant removed and the pellet dried briefly*. The pellet was resuspended in 100 μl of DEPC-treated water.

* Care was taken to ensure that the pellet did not dry completely as this greatly decreases its solubility. The solubility of RNA can be improved by heating to 55 - 60 ^{o}C with intermittent vortexing or by passing the RNA solution through a pipette tip, if necessary.

2.10.1.2 RNA Extraction from Whole Tissue

During the course of this research work, RNA was extracted from whole tissues using an adaptation of this technique (See 3.9.1).

2.10.1.3 RNA Extraction from Paraffin-Embedded Tissues

During the course of this thesis, a method was developed for the extraction of RNA from paraffin-embedded tissues. Using

the techniques described RNA was successfully extracted and amplified from a number of specimens which had been stored for at least ten years (See 3.9.2).

2.10.2 Micropipette Accuracy Tests

Accuracy and precision tests were carried out routinely on all micropipettes used in both DNA and RNA analyses by PCR and RT-PCR techniques, respectively. The accuracy and precision of 1,000 μl and 100 μl pipettes were determined by standard methods involving repeatedly pipetting specific volumes of water, and weighing them on an analytical balance. The specifications for these tests were supplied by Gilson. The 10 μl pipettes were returned to the manafacturer for routine calibration.

2.10.3 RNA Quantitation

RNA was quantified spectrophotometrically at 260 nm and 280 nm. An optical density of 1 at 260 nm is equivalent to 40 mg/ml RNA. A A_{260}/A_{280} ratio of 2 is indicative of pure RNA. Partially solubilised RNA has a ratio <1.6 (Ausubel *et al.*, 1991c). The yield of RNA from most lines of cultured cells is 100 - 200 μg per 90 mm plate (Sambrook *et al.*, 1989). Similar amounts of RNA were retrieved in these studies.

2.10.4 Typical Reverse Transcriptase (RT) Reaction

cDNA was formed on RNA templates using the following procedure, which was incubate on ice unless otherwise indicated. A combination of 1 μl oligo $(dT)_{12-18}$ primers (1μg/μl) (Promega; C1101), 1 μl of total RNA (1μg/μl), and 3 μl of water were placed in an eppendorf, mixed, heated to 70 ^{o}C for 10 mins., and then chilled on ice. (Heating to 70 ^{o}C got rid of any RNA secondary structure formations and allow the oligo (dT) primers bind to the poly $(A)^{+}$ tail of the mRNA). To this, 4 μl of a 5 X buffer (consisting of 250 mM Tris-HCl pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 2 μl of DTT (100 mM), 1 μl of RNasin (40 U/μl) (Promega; N2511), 1 μl of dNTPs (10 mM each of dATP, dCTP, dGTP and dTTP), 6 μl of water and 1 μl of Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) (40,000 U/μl) (Gibco; 510-8025 SA) was added. This mixture was incubated at 37 ^{o}C for 1 hr. to allow the MMLV-RT enzyme catalyse the formation of cDNA on the mRNA template. The enzyme was then inactivated and the RNA and cDNA strands separated by heating to 95 ^{o}C, for 2 mins.

Once the cDNA copy had been created using the mRNA template, the PCR reaction was conducted immediately, as outlined below. Alternatively, the cDNA was stored at -20 ^{o}C, until required for analysis.

2.10.4.1 Reverse Transcription of RNA from Paraffin-Embedded Tissues

cDNA copies of mRNA templated were formed using RNA isolated from paraffin-embedded tissues (as detailed in Section 3.9.2).

RT Reaction Components

5 X Buffer

250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$.

The successful use of the PCR buffer instead of the 5 X buffer in the reverse transcriptase reaction has been reported by Kawasaki (1989). Better results were achieved using the PCR buffer, at least when reverse transcribing short RNA sequences. This, it is suggested, simplifies the protocol by eliminating the possibility of differences in optimal monovalent and divalent ionic strengths between the two enzymes.

RNasin

An inhibitor of ribonuclease enzymes was included in the RT reaction. This was to prevent degradation of RNA by ribonucleases. RNasin is a broad spectrum ribonuclease inhibitor which acts by binding noncovalently to ribonuclease enzymes. RNasin is unstable at temperatures of 50 ^{o}C or above. As ribonucleases are capable of retaining their degradative properties under denaturing conditions, such temperatures were avoided for the RT reaction to prevent release of active ribonuclease enzymes.

Dithiothreitol (DTT)

The concentration of DTT is critical as RNasin requires 1 mM DTT to prevent its dissociation from the RNases.

Deoxynucleotide Triphosphates (dNTPs)

The dNTPs (dATP, dCTP, dGTP and dTTP) were aliquoted as a mix at a concentration of 10 mM each. These were the precursors of the cDNA strand.

Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT)

MMLV-RT was the reverse transcriptase enzyme used to form cDNA on the primed mRNA template. It has been claimed that RT-PCR can be simplified by the fact that *Taq* DNA polymerase also exhibits reverse transcriptase activity at 68 °C (Jones and Foulkes, 1989). RT-PCR using one enzyme was conducted by Shaffer *et al.* (1990) in a study of interleukin-2 mRNA in gibbon T cells.

The use of *Taq* DNA polymerase as the enzyme catalysing the RT reaction may have some advantages over MMLV-RT. The RT reaction in the presence of reverse transcriptase enzyme generally takes approximately an hour. Using *Taq* DNA polymerase, cDNA can be formed in a number of minutes (Shaffer *et al.*, 1990; Singer-Sam *et al.*, 1990). *Taq* DNA polymerase enzyme is much more heat-stable than MMLV-RT, which should allow the reaction to be conducted at higher temperatures. This should increase the primer stringency and help eliminate RNA secondary structure. Optimum reverse transcription using *Taq* DNA polymerase may require the addition of magnesium (Bej *et al.*, 1991).

Trouble-Shooting

Rappolee (1990) reported first-strand cDNA synthesis by reverse transcription to be a technique of variable efficiency between preparations and between RNA species. To overcome secondary structure problems in RNA, a number of recommendations were made. These include: increasing the RT reaction temperature from 37 °C to 55 °C (*Taq* DNA polymerase seems to be more stable at high temperatures than reverse transcriptase enzymes); increasing the reverse transcription enzyme concentration from 1 X to 3 X or more, and using random hexanucleotide primers and/or 3' antisense oligonucleotide priming. To improve sensitivity, the first strand synthesis may be repeated after heat denaturation. Reverse transcriptases have some amount of heat stability, but it is often advisable to add a fresh aliquot of enzyme for greater efficiency if a second cycle of reverse transcriptase is included. (This further addition of enzyme may

not be necessary if *Taq* DNA polymerase reverse transcriptase activity is taken advantage of).

2.10.5 Typical Polymerase Chain Reaction

The details given here are for a typical PCR reactions, set up as 50 µl volumes, using 5 µl of cDNA, and amplifying for 30 PCR cycles. In many cases, variations to this basic protocol were made. Such changes are indicated in the relevant "Results" section *e.g.* when establishing the exponential range of product accumulation and the PCR reaction plateau phase, 100 µl PCR volumes were prepared by doubling the amount of each of the components added and aliquots were removed after 15, 20, 25, 30 and 35 cycles.

Each PCR reaction tube contained 24.5 µl water, 5 µl 10 X buffer (100 mM Tris-HCl, pH 9.0 at 25 °C; 50 mM KCl, 1 % Triton X-100), 3 µl of 25 mM $MgCl_2$, 8 µl of dNTPs (1.25 mM each) (Promega; U1240), 1 µl of first strand target primer (250 ng/µl), 1 µl of second strand target primer (250 ng/µl), 1 µl of first strand endogenous control primer (250 ng/µl), 1 µl of second strand endogenous control primer (250 ng/µl), 0.5 µl of 5U/µl *Taq* DNA polymerase enzyme (Promega; N1862) and 5 µl of cDNA from the reverse transcriptase reaction. (The cDNA was pre-heated to 95 °C to separate strands and get rid of any secondary structure formations, followed by cooling on ice). A drop of mineral oil was added to each reaction tube. The cDNA was then amplified by PCR (Techne; PHC-3) using the following program:

- 95 °C for 1.5 mins. (denature ds DNA);
- 30 cycles: 95 °C for 1.5 mins. (denature);
  55 °C for 1 min. (anneal);
  72 °C for 3 mins. (extend);
- 72 °C for 7 mins. (extend).

All reaction tubes were then maintained at 4 °C until analysed.

The products formed were analysed by gel electrophoresis and densitometry. A 10 μl aliquot of tracking buffer consisting of 0.25 % bromophenol blue (Sigma; B5525) and 30 % glycerol in water, was added to each tube of amplified cDNA products. 10 μl of cDNA products from each tube were then separated by electrophoresis for 4 hrs. at 100 mV through a 4 % agarose (Promega Bioproducts; NuSieve GTG) gel containing ethidium bromide (Sigma; E8751), using TBE (22.5 mM Tris, 22.5 M boric acid (Sigma; B7901), 0.5 mM EDTA) as running buffer. Molecular weight markers "V" (Boehringer Mannheim; 821 705) or "Phi-X" Hae III digested (Promega; G1761) were run, simultaneously, as size references.

The resulting product bands were visualised as pink bands when the gel was placed on a transilluminator (UVP transilluminator), due to the fact that ethidium bromide intercalates with the cDNA forming a product that is readily visible under ultraviolet transillumination. The gels were photographed and the negatives produced were analysed by densitometry (Imaging Densitometer. Bio-Rad. Model GS-670).

PCR Components

$MgCl_2$: (25 mM):

The magnesium chloride concentration has been found to greatly influence the PCR result. It is generally advised to vary the concentration of magnesium chloride (usual range 1 - 8 mM) to determine the optimum concentration of this component.

Deoxynucleotide Triphosphates (dNTPs):

The dNTPs for the PCR were aliquoted as a mix (dATP, dCTP, dGTP and dTTP) at a final concentration of 1.25 mM each. These are the required precursors for the synthesis of new cDNA strands.

Primers: (see Section 1.2.1.1)).

A primer pair was included in the PCR which is specific for the target cDNA region of interest. A primer pair which results in amplification of an ubiquitously occurring cellular component was also included. This served as an endogenous control (see 1.3). The majority of primer pairs used were selected as part of this thesis work (Table 3.1). Other primers used are detailed in Section 2.8.2. A set of primers, not previously listed, which amplified a putative endogenous control, Esterase D (Cole *et al.*, 1991) were also evaluated during this study. Their sequences are as follows:

5' GGA GCT TCC CCA ACT CAT AAA TGC A 3'
5' GCA TGA TGT CTG ATG TGG TCA GTA A 3'

Note: All oligonucleotide primers used throughout the course of this thesis were made to order on an "Applied BioSystems 394 DNA/RNA Synthesiser" by R & D Systems Ltd., 4-10 The Quadrant, Barton Lane, Oxon, England.

Mineral Oil:

The purpose of the mineral oil was to prevent evaporation of the reaction components when subjected to the high temperatures required for melting the double-stranded DNA to single-strands.

2.10.5.1 Restriction Digestion of RT-PCR Products

Products formed after RT-PCR amplification of MDR 1, MDR 3, MRP, GST π, CYPIA1, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ were digested with an appropriate restriction enzyme (Table 3.1/Table 2.10.5.1.1) as follows.

A 10 μl aliquot of the particular RT-PCR product, 10 units of the relevant endonuclease restriction enzyme, 5 μl of 10 X buffer (supplied with each restriction enzyme) and the appropriate volume of water to result in a total volume of 50 μl, were mixed in an eppendorf tube and incubated at 37 ^{o}C, for 2 hrs. A 20 μl aliquot of the resulting products was analysed by electrophoresis through a 4 % agarose gel (as in 2.10.5).

Table 2.10.5.1.1 Restriction Enzymes used to Identify RT-PCR Products

AMPLIFIED PRODUCT	**RESTRICTION ENZYME**	**SUPPLIER**	**CATALOGUE NUMBER**
MDR 1	Mae 1	Boehringer Mannheim	822 213
MDR 3	Ssp 1	Boehringer Mannheim	972 967
MRP	Ban II	Promega	R6561
GST π	Sty 1	Promega	R6481
CYPIA1	Hinc II	Promega	R6031
Topoisomerase I	EcoN 1	S.T.S	521 S
Topoisomerase II	Hind III	Promega	R6041
Topoisomerase IIα	Alu 1	Promega	R6281
Topoisomerase IIβ	Hph 1	S.T.S	158 S
β-actin	Hph 1	S.T.S	158 S

2.11 Exogenous Control Formation

A non-homologous DNA fragment was constructed (as detailed in 1.4.2.1, Fig. 8), by Dr. Carmel Daly, for use as an exogenous competitive control to quantify endogenous MDR 1 mRNA levels after RT-PCR reactions. The exogenous control was amplified during the PCR reaction, using the same primer sequences as those for the amplification of MDR 1, resulting in products differing by 174 bp in size *i.e.* MDR 1 product was 157 bp; control was 331 bp.

In order to prepare such a competitor fragment for use in the quantitation of MDR 1 mRNA, composite primers were chosen. These 40 bp composite primers contained 20 bp of MDR 1 specific primer as well as 20 bp complementary to pGEM4Z. The primers to pGEM4Z were chosen so that they would amplify a fragment of differing size to the product of the MDR 1 specific primers. The MDR 1 specific primers amplify a 157 bp product (Table 3.1) from mRNA. The pGEM4Z primers were chosen to produce a fragment of 291 bp.

Composite primers were used in the first PCR reaction which contained 400 ng of pGEM4Z. The PCR reaction was as in Section 2.10.5, except that only one set of primers was included. The second pair was replaced with extra volumes of water. PCR amplification resulted in the production of a 291 bp DNA fragment incorporating the MDR 1 gene specific primer sequences at the extreme ends. Products of the PCR reaction were separated on a 2.5 % agarose gel (using buffers *etc.* as described in Section 2.10.5). The portion of the gel containing the 291 bp fragment was cut out of the gel and placed in a column (Costar spin X - column; 8169). The column containing the agarose was frozen at -20 ^{o}C, for 1 hr. The DNA was then isolated by spinning the column at 13,000 r.p.m., for 10 mins. The resulting solution which contained the 291 bp DNA fragment was purified by phenol chloroform extraction and ethanol precipitation or using a clean up column (Promega wizard DNA clean up system; A7280). A 400 ng aliquot of this DNA competitor fragment was then PCR

amplified using MDR 1 specific primers to yield a product of 331 bp (*i.e.* combination of pGEM4Z (291 bp) fragment and the two MDR 1 primer lengths ligated onto its ends (20 bp each)). This was isolated from an agarose gel and purified. This DNA was quantified spectrophotometrically at 260 nm and 280 nm. (An optical density of 1 at 260 nm is equivalent to 50 mg/ml DNA. A A_{260}/A_{280} ratio of 1.8 is indicative of pure DNA). This product was then stored at -20 ^{o}C until required as an exogenous control for MDR 1 quantification.

MDR Composite Primers used:

5' CCC ATC ATT GCA ATA GCA GGA CGG CCA GTG AAT TGG ATT T 3'
5' GTT CAA ACT TCT GCT CCT GAT TCC CGA CTC GAA AGC GGG C 3'

The first 20 bases (5') of each are MDR 1 specific and the second 20 (3') are pGEM4Z specific.

2.12 Using cDNA sequence Data-Bases to check Uniqueness of Primers.

The most critical element in the choice of primers for PCR is obviously that the primers are unique *i.e.* that they do not cross-react with sequences likely to be present in the mRNA of the cells/tissues being studied. This involved choosing primers using the criteria described in Section 1.2.1.1, and checking *via* a DNA data-base (*e.g.* EMBL, GenBank) what similar sequences exist. This approach is essential if no references are available on RT-PCR of the mRNA in question. Even where references do exist, there is need for checking because :

- the cDNA data-base is expanding rapidly and new cross-reactivity may have been discovered since the choice of primers was published;

- occasionally, published primers have not been well chosen and cross-reactions have been overlooked; also, occasionally, probably due to typographical errors, incorrect primer sequences have been published.

Because of the excellent search facilities available, checking the sequence uniqueness of primer sequences is a straightforward procedure.

EMBL DNA data bases were accessed easily *via* E-Mail by linking to large mainframe computers (*e.g.* VAX). Using software (KERMIT), IBM-PCs were used as VAX terminals for E-Mail. Once E-Mail had been accessed on the PC (through use of KERMIT commands and the appropriate user number and password), primer sequences were sent to be checked to EMBL by the following procedure:

Note:
Italics = prompts received;
Bold = to be typed in;
[R] = "return" key;
???? = type in actual nucleotide sequence;
Accession No. = type in actual accession number;
CTRL/Z = press "control" key and "Z", simultaneously.

Username : **xxxxxxxx** [R]
Password : **yyyyyyyy** [R]

$ **mail**
Mail> **send**
To: **In%"Fasta@embl-heidelberg.de"** [R]
Subj: (not necessary to choose subject) [R]
Enter your message below. Press CTRL/Z when complete or CTRL/C to quit:
Lib emall [R] (can choose to check a particular library in this way)
Align 20 [R] (can choose how many alignments required. Default is 30)
Seq [R]
CCCATCATTGCAATAGCAGG[R] (The example given here is the MDR 1 primer (a) sequence - see Table 3.1).
end [R]
CTRL/Z [R]

If you have an accession number *e.g.* for a cDNA sequence, and wish to obtain the cDNA sequence, the address and procedure is slightly different:

Mail> **send** [R]
To: **In%"Netserv@embl-heidelberg.de"** [R]
Subj: [R]
Enter your message below. Press CTRL/Z when complete or CTRL/C to quit:
GET NUC:X00351 [R] (The example shown here is the EMBL accession number of human mRNA for β-actin).
CTRL/Z

To access returned mail:

Mail> **dir** [R] (will give a list of your directory of returned messages)

Mail>**3** [R] (chose the number of any of the listed return messages *e.g.* no. 3)

By pressing [R] you can read through the message or

Mail> **print** [R]
CTRL/Z (if want message printed).

After use, when have exited by using CTRL/Z command

$ **lo** (to exit system).

Depending on the pressure on the EMBL facility at the time, a list of related sequences were sent back in minutes or hours.

2.13 PROTEIN ANALYSIS

Protein levels of p-glycoprotein and Topoisomerase II were analysed in a number of cell lines using Western blotting techniques.

2.13.1 P-glycoprotein Analysis

P-glycoprotein is a membrane-associated protein. Cell membranes were, therefore, prepared for its analysis. This involved using 2 X 10^7 cells, during their exponential phase of growth. The cells were trypsinised, pelleted and the cell pellets were washed three times in PBS. The following procedures were performed on ice, except when otherwise indicated. The cell pellet was resuspended in 5 ml of ice-cold 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.4, to which 2 mM PMSF was added immediately prior to use. The cells were then sonicated until lysis was observed in samples viewed under a microscope. The lysed cells were centrifuged at 100,000 g., at 4 °C, for 1 hr. The resulting pellet was resuspended in 500 μl of lysis buffer. This was divided into 100 μl aliquots and lyophilised overnight, after removing an aliquot for protein quantitation. The lyophilised cell membrane samples were then stored at -80 °C until required for Western blot analysis.

2.13.2 Nuclear Extraction of Topoisomerase II

The nuclear DNA enzyme, Topoisomerase II, was isolation from a large culture (10^8)(Section 2.5.4) of healthy cells, during the logarithmic phase of growth. The protocol used for its isolation was an adaption of a method published by Danks *et al.* (1988). To inhibit proteolytic degradation of Topoisomerase II, all solutions used during the isolation procedures contained the following freshly prepared cocktail of inhibitors - phenylmethylsulfonyl fluoride (PMSF)(1 mM), (Sigma; P7626);

leupeptin (50 μg/ml),(Sigma; L2884); soyabean trypsin inhibitor (1 mg/ml),(Sigma; T9003); pepstatin A (1 μg/ml),(Sigma; P6425); aprotinin (20 μg/ml)(Sigma; A1153) and benzamidine (1 mM)(Sigma; B6506). Unless otherwise indicated, all procedures were performed at 4 ^{o}C (or on ice).

Cells were trypsinised, pelleted and washed in PBS. The resulting cell pellet was permeabilised by resuspending in 1.75 ml of hypotonic buffer (5 mM KH_2PO_4, 2 mM $MgCl_2$, 4 mM DTT, 0.1 mM Na_2EDTA, pH 7.0) and incubating for approximately 20 mins. The viability of samples of the cells were tested as in 2.5.3. When approximately 90 % of the cells stained blue (lysed), they were centrifuged at 400 g., for 5 mins. The cells were resuspended in hypotonic buffer containing 0.25 M sucrose, at a cell density of 5 X 10^8/8 ml of buffer. Each 8 ml aliquot was then gently layered over 3 ml of hypotonic buffer containing 0.3 M sucrose and centrifuged at 2,000 g., for 20 mins. The supernatant was removed and the pellet volume estimated. The pellet was resuspended in a half volume of lysis buffer (5 mM KH_2PO_4, 4mM DTT, 1 mM Na_2EDTA, pH 7.0) and was incubated for 15 mins. Following this, nuclear Topoisomerase II was extracted by adding half the pellet volume of a 1.0 M salt extraction buffer (40 mM Tris, 2.0 M NaCl, 4 mM DTT, 20 % glycerol, pH, 7.5). This was then vortexed, incubated for 30 mins., and centrifuged at 100,000 g., for 1 hr. The clear supernatant was removed and the pellet was re-centrifuged. Both supernatants were pooled and were analysed for Topoisomerase II levels. This analysis was either performed immediately or the extracts were stored at -170 ^{o}C, for up to 3 days.

2.13.3 Protein Quantitation

To ensure equal quantities of protein were loaded on the Western blots, the protein levels in a sample of each extract was determined using the Bio-Rad Protein Assay Kit (Bio-Rad; 500-0006). A range of known concentrations of bovine serum albumin (BSA) (Sigma; A9543) were used as standards. A 100 μl

volume of the appropriate standards and test samples, were placed in clean, dry test-tubes. 5 ml of diluted dye reagent (which was provided as a 5-fold concentrate in the kit) was added to each tube, and the mixture vortexed. Between 5 - 60 mins. later, the $O.D._{595}$ was measured on a spectrophotometer, against a reagent blank. A plot of the standard concentrations *versus* their $O.D._{595}$ readings was formed, from which the concentrations of protein in the test samples were extrapolated.

2.13.4 Polyacrylamide Gel Electrophoresis

The proteins present in the cell extracts were seperated, based on size, by electrophoresis through a 7.5 % SDS polyacrylamide gel. The resolving gel was prepared by mixing 3.8 ml of an acrylamide stock (29.1 g acrylamide (Sigma; A8887) and 0.9 g N,N-methylene bis-acrylamide (Sigma; N7256) made up to 100 ml with water), 3.0 ml 1.875 M Tris-HCl, pH 8.8 and 8.0 ml water. To this solution, 150 μl of 10 % SDS (Sigma; L4509), 50 μl of 10 % ammonium persulphate (Sigma; A1433) and 7.5 μl of TEMED (Sigma; T8133) were added. The gel was immediately poured into two clean 10 cm X 8 cm gel cassettes (each consisting of a glass and an aliminium plate seperated by two 0.75 cm plastic spacers, one on either outer edge). The gels were overlaid with saturated butanol and allowed to set. Once set, the saturated butanol was poured off and the gel tops washed with water. The stacking gels were then poured. The stacking gel was prepared by combining 0.8 ml of acrylamide stock, 0.5 ml of 1.25 M Tris-HCl, pH 6.8 and 3.6 ml of water. To this was added, 50 μl of 10 % SDS, 17 μl of 10 % ammonium persulphate and 5 μl of TEMED. This solution was poured onto the resolving gels. A comb of the appropriate thickness and well size was immediately inserted and the gels allowed to set. When the wells had formed, the combs were removed and the gels transferred to a mini-electrophoresis apparatus. The gels were flooded with running buffer (1.9 M glycine (Sigma; G6761), 0.25 M Tris, 0.1 % SDS. The pH of the buffer was 8.3, unadjusted).

Protein samples to be analysed were diluted 1 : 1 with loading buffer (50 mM Tris-HCl, pH 6.8; 1 % SDS; 5 % β-mercaptoethanol (Sigma; M6250), 5 % glycerol and 0.1 % bromophenol blue). These were boiled for two minutes. Equal quantities of protein from each sample, and appropriate molecular weight markers, were then loaded into the wells. The gels were run at 250 mV and 45 mA, for 1.5 hrs.

2.13.5 Western Blot Procedure

Following electrophoresis, the acrylamide gels were equilibrated in transfer buffer (25 mM Tris, 192 mM Glycine, pH was between 8.3 - 8.5 without adjusting). Nitrocellulose filter (Amersham; Hybond C) onto which the protein was to be transferred, was cut to the same size as the gel and was soaked in transfer buffer, for 5 mins. Whatman 3 mm filter paper was also cut to the same size as the gel, four stacked sheets of which were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus. Trapped air was removed from between the filters by rolling a glass pipette over and back across them. The nitrocellulose was then placed on the wetted filter paper, taking care not to trap air bubbles. The acrylamide gel was removed from the transfer buffer and placed on the nitrocellulose. The nitrocellulose was marked at the locations of the gel lanes and molecular weight markers. A further four sheets of Whatman filter paper, which had been soaked in transfer buffer were placed on top of the gel (again, preventing air bubbles being trapped). The protein was transferred from the gel to the nitrocellulose at 0.34 mA/0.15 V, over a period of 30 mins. The nitrocellulose was then placed in blocking buffer (as described in Sections 2.13.5.1 and 2.13.5.2) to prevent non-specific binding of antibody. The nitrocellulose was then exposed to a specific primary antibody directed against the antigen under analysis. Simultaneously, negative control blots were formed where the primary antibody was replaced with antibody diluent, or pre-immune serum, if available.

2.13.5.1 P-Glycoprotein

Following transfer of protein to nitrocellulose, the nitrocellulose was placed in blocking buffer (0.5 % non-fat dried milk (Cadbury; Marvel skimmed milk) in TBS (500 mM NaCl, 20 mM Tris, pH 7.5) at room temperature, for 2 hrs. Following this, the nitrocellulose was exposed to primary antibody (0.25 μg/ml C219 (Centicor Diagnostics) in 10 ml TBS containing 0.1 % Tween 20) at 4 °C, overnight. The negative control was further exposed to antibody diluent for this time. The nitrocellulose was washed three times in TBS containing 0.5 % Tween 20 to remove any unbound primary antibody. The nitrocellulose was then exposed to secondary antibody (1/9,500 dilution of rabbit anti-mouse IgG (Sigma; A1902) in TBS containing 0.1% Tween 20) at room temperature, for 1.5 hrs. The nitrocellulose was washed three times in TBS containing 0.5 % Tween 20. The blot was developed as in Section 2.13.6.

2.13.5.2 Topoisomerase II

Following transfer of protein to nitrocellulose, the nitrocellulose was placed in blocking buffer (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 % non-fat dried milk, 3 % BSA, 0.2 % Tween 20) at 4 °C, overnight. The primary antibody, a polyclonal rabbit serum raised against Topoisomerase II and pre-immune rabbit serum (as a negative control) were each diluted 1/700 in blocking solution. (The antibodies were received as gifts from Dr. W.T. Beck and documented by Friche *et al.* (1991)). The nitrocellulose was incubated with the primary antibody (or pre-immune serum) at 4 °C, for 48 hrs. The nitrocellulose was then washed three times in a washing buffer (10 mM Tris, 140 mM NaCl, pH 7.4) and was exposed to the secondary antibody (1/13,000 dilution of goat anti-rabbit IgG (Sigma; A8025) in blocking solution) at room temperature, for 2 hrs. The nitrocellulose was again washed three time as above. The blot was developed as in Section 2.13.6.

2.13.5.3 Topoisomerase IIα

Following transfer of protein to nitrocellulose, the nitrocellulose was placed in blocking buffer (PBS containing 3 % BSA) at 4 °C, overnight. The primary monoclonal antibody, 6G2, was diluted 1/50 with PBS containing 1 % BSA, at 4 °C, overnight. (This antibody was received as a gift from Dr. G. Astaldi-Ricotti and was described by Negri *et al.* (1992)). Antibody diluent was used in place of 6G2 for the negative controls. The nitrocellulose was washed three times with PBS containing 0.1 % Tween 20. This was then incubated with the secondary antibody (1/9,500 dilution of rabbit anti-mouse IgG (Sigma; A1902) in PBS containing 0.1 % BSA and 0.1 % Tween 20) at room temperature, for 2 hrs. The nitrocellulose was again washed three times with PBS containing 0.1 % Tween 20 and was developed as outlined in Section 2.13.6.

2.13.6 Developing Western Blots

The blots were developed by incubating in a developing substrate until bands and/or background colour appeared. The developing substrate consisted of a mixture of 2.5 ml of Solution A and 1 ml of Solution B in 22.5 ml of a solution consisting of 0.5 M Tris-HCl, pH 8.9 containing 0.1 ml of 1 M $MgCl_2$. Solution A consisted of 0.1 % Nitroblue tetrazolium (Sigma; H5514) in 10 ml Tris-HCl, pH 8.9. Solution B consisted of 5 mg/ml 5-bromo-4-chloro-indolyphosphate (Sigma; B0274) in dimethyl formamide (Sigma; D8654). Once the blots had developed, they were washed in water at room temperature, allowed to dry and photographed. The blots were stored in darkness to prevent fading.

Chapter 3

Results

RESULTS

3.1 Design of Oligonucleotide Primers for RT-PCR Studies of MDR-related mRNAs

Primer pairs were selected to enable mRNA of multiple drug resistant (MDR)-related genes to be analysed by RT-PCR methods (Table 3.1). The primers selected were for the study of MDR 3, MRP, GST α, GST π, CYPIA1, Topoisomerase I, Topoisomerase II, Topoisomerase IIα, Topoisomerase IIβ and β-actin. (The MDR 1 and β_2-microglobulin primers used throughout the course of this study were previously published by other research groups (see 3.1)). All primers were chosen, based on the coding sequence of the specific target of interest and the uniqueness of each primer was checked *via* the EMBL DNA data-base (2.12). A number of criteria suggested for choosing primers (see 1.2.1.1) were used as a guide-line and were adhered to as far as possible. It was realised, however, that choosing primers representing all of the suggested guide-lines was not possible in all situations. The primer sequences selected, therefore, represent the best attempts to optimise, taking into consideration the conflicting requirements of different criteria (see Table 3.1).

3.1.1 Examples of Primer Pairs and how they Fit Specified Criteria

Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ: Primers were chosen which allow amplification of Topoisomerase II cDNA, in general, by selecting a region that is common to both Topoisomerase IIα and Topoisomerase IIβ isozyme. Specific primers were also chosen for Topoisomerase IIα and Topoisomerase IIβ so that their transcript levels could be studied independently. This allows the levels of Topoisomerase IIα and Topoisomerase IIβ to be analysed individually, the overall levels of Topoisomerase II evaluated, and the results compared.

Topoisomerase II: When choosing primers for analysis of Topoisomerase II, in general, the C-terminus and the extreme N-terminal regions were avoided as these are the main areas where Topoisomerase IIα and Topoisomerase IIβ diverge in structure. The chosen primers are both 100 % homologous to specific regions of the Topoisomerase II mRNA template, resulting in a region of 216 bp being amplified. Digesting with Hind III restriction enzyme produced bands of 129 bp and 87 bp. The primers are not equal in length. This seemed unavoidable, however, when attempting to maximise their specificity. Many sequences were chosen as potential primers, but the primer pair 1395-1412 and 1585-1610 (Tsai-Plugfelder *et al.*, 1988) were favoured by comparison to the others as they are independently homologous to fewer other human sequences and they do not share common homologies other than Topoisomerase II. The annealing temperature for Topoisomerase II(a) primer is slightly below the recommended range; the Topoisomerase II(b) primer is within this range. Neither primer contains palindromes or stretches of polypurines or polypyrimidines. Due to the unavailability of the Topoisomerase II genomic sequence, it is uncertain whether or not these primers span or cross introns.

Topoisomerase IIα (IIα): The C-terminal region which is the longest region of divergence between Topoisomerase IIα and Topoisomerase IIβ was considered to be the best area to choose specific primers for Topoisomerase IIα and Topoisomerase IIβ, respectively. As the region of divergence between Topoisomerase IIα and Topoisomerase IIβ is relatively small, primers that span the longest region possible were favoured. This is so that the product and the restriction enzyme digestion products can be easily detected on a gel. A number of potential 3' primers were not chosen for Topoisomerase IIα as they contained stretches of polypurines and polypyrimidines. To avoid these and other problem areas, primers 4052-4072 and 4165-4190 (Tsai-Plugfelder *et al.*, 1988) were chosen. Although the 139 bp region amplified is homologous with that of Topoisomerase IIβ to a small extent the primers are specific for Topoisomerase IIα. Digestion with Alu 1 restriction enzyme resulted in stretches of 87 bp and 52

bp being produced. The annealing temperature for the 5' primer is within the recommended range, but that for the 3' primer is slightly high. Both primers are independently homologous to a number of other human sequences, but do not seem to share common homologies.

Topoisomerase IIβ (IIβ): The Topoisomerase IIβ primers (4335-4364 and 4425-4452, Jenkins *et al.*, 1992; EMBL accession no. X68060) amplify a 118 bp region unique to Topoisomerase IIβ. Digestion with EcoR 1 restriction enzyme produced fragments of 80 bp and 38 bp; Hph 1 restriction enzyme resulted in 69 bp and 49 bp products. The primers are quite long - 28 bp and 30 bp, respectively, to increase their specificity. They have an annealing temperature slightly above the recommended range. Neither primer contains palindromes or lengths of polypurines or polypyrimidines. Both these primers, too, are independently homologous to a number of human sequences, but they apparently do not share common human homologies (with the exception of IIβ).

Glutathione-S-Transferase π (GST π): The cDNA sequence of GST π (Moscow *et al.*, 1989) apparently differs from that of human myocardial fatty acid ethyl esters-III (FAEE-III) (Bora *et al.*, 1991) by only six nucleotides. This has been claimed to reflect an inherent functional difference between the resulting protein products. As primers specific for GST π could not be found, an effort was made to find a restriction enzyme recognition site that is present in the GST π amplified region but not in FAEE-III, or *vice versa* (due to the four nucleotide sequence difference in this region). However, such a site was not identified.

β-Actin: Choosing primers for β-actin amplification was a difficult task as at least six isoforms of actin are known to exist in vertebrates. From the primer sequences published for amplification of β-actin it seems to be very difficult to choose sequences which are specific for β-actin and do not recognise β-actin pseudogene, mutated β-actin, α-actin or γ-actin. The β-

actin primer pair chosen in this thesis were also homologous to mutant β-actin (EMBL accession no. X63432). This was unavoidable when the other actin forms were avoided and the guide-lines for choosing primers were considered, as the normal (EMBL accession no. X00351) and the mutant forms differ only by one nucleotide in the amplified region.

MDR 1: The primers published by Noonan *et al.* (1990) are both 100% homologous to the MDR 1 mRNA template, resulting in a region of 157 bp being amplified. These primers were also assessed in this study to establish how they fit the criteria suggested. A diagnostic restriction enzyme recognition site for this MDR 1 region was chosen in this laboratory. Digestion with Mae 1 results in products of 84 bp and 73 bp. The primers are equal in length and the annealing temperature for both fit into the recommended range of 55 ^{o}C to 75 ^{o}C. Neither primer contains palindromes or stretches of polypurines or polypyrimidines. The region amplified by these primers is from position 2596 to 2752 bp and the poly $(A)^{+}$ tail commences at position 4223 bp. The primers flank a sequence that crosses an intron so that DNA contamination can be diagnosed. If the relevant area of DNA was amplified, a band of 1,257 bp would result in comparison to a band of 157 bp from cDNA. The primers were selected to amplify only MDR 1 (avoiding MDR 3). Both primers share homology with a number of other human nucleotide sequences. However, their only common homologies are MDR 1 mRNA (for which they were selected) and MDR 1 gene. By virtue of the fact that the selected region has an intron present in the DNA, if contaminating DNA is amplified, it should be possible to identify it by its longer size in comparison to the amplified region of cDNA.

Table 3.1: Primers to amplify cDNA formed by reverse transcription on mRNA templates of MDR related factors.

		Primer length (bases)	A+T : G+C	Tm	Amplified mRNA sepuence length (bases)	Diagnostic R.E.	Restriction product length (bases)	Corresponding DNA length (bases)	Location on template
MDR 1	(a)	20	10 : 10	60	157	Mae 1	84 + 73	1,257 *(Chen et al., 1990)*	2596 - 2615
	(b)	20	11 : 9	56					2733 - 2752 *(Chen et al., 1986 ; 1990)*
MDR 3	(a)	24	16 : 8	64	321	Ssp 1	194 + 127	N.K.	2580 - 2603
	(b)	24	15 : 9	66					2877 - 2900 *(van der Bliek et al., 1988)*
MRP	(a)	21	13 : 8	58	203	Bvu 1	129 + 74	N.K.	1317-1337
	(b)	21	11 : 10	62		Ban II	129 + 74		1499-1519
						Apa 1	129 + 74		*(EMBL no. L05628)*
TOPO I	(a)	30	17 : 13	86	180	EcoN 1	115 + 65	2,280 bp *(D'Arpa et al., 1988; Kunze et al., 1991)*	1123 - 1152
	(b)	27	16 : 11	82					1276 - 1302 *(D'Arpa et al., 1988)*
TOPO II	(a)	18	10 : 8	52	216	Hind III	129 + 87	N.K.	1395 - 1412
	(b)	26	16 : 10	72					1585 - 1610 *(Tsai-Pflugfelder et al., 1988)*
TOPO IIα	(a)	21	11 : 10	62	139	Alu 1	87 + 52	N.K.	4052 - 4072
	(b)	26	12 : 14	80					4165 - 4190 *(Tsai-Pflugfelder et al., 1988)*
TOPO IIβ	(a)	30	19 : 11	82	118	Hph 1	69 + 49	N.K.	4335 - 4364
	(b)	28	18 : 10	76					4425 - 4452 *(Jenkins et al., 1992)* *(EMBL no. X68060)*

		Primer length (bases)	A+T : G+C	Tm	Amplified mRNA sepuence length (bases)	Diagnostic R.E.	Restriction product length (bases)	Corresponding DNA length (bases)	Location on template
GST π	(a)	18	8 : 10	56	270	Sty 1	171 + 99	749	58 - 75
	(b)	24	14 : 10	68				*(EMBL no. X08058)*	304 - 327 *(Moscow et al., 1989)*
GST α	(a)	30	18 : 12	84	330	Hind III	206 + 124	4,630	235 - 264
	(b)	30	14 : 16	92		Hsu 1	206 +124	*(Klöne et al., 1992; Tu and Qian, 1986; EMBL no.s X65726, X65734)*	535 - 564 *(Tu and Qian, 1986)*
						Msp 1	228 + 102		
CYPIA1	(a)	21	12 : 9	60	327	Hinc II	246 + 81	755	1021 - 1041
	(b)	21	14 : 7	56				*(Jaiswal et al., 1985 a)*	1327 - 1347 *(Jaiswal et al., 1985b)*
β-Actin	(a)	29	16 : 13	84	383	Hin f 1	193 + 190	590	619 - 647
	(b)	22	12 : 10	64		Hph 1	266 + 117	*(EMBL no. M10277)*	980 - 1001 *(EMBL no. X00351, M10277)*
β_2-Micro-globulin	(a)	20	11 : 9	58	114	Mse 1	68 + 46	1,974	271 - 290
	(b)	20	12 : 8	56				*(Güssow et al., 1987)*	365 - 384 *(Güssow et al., 1987)*

Notes:

* This primer was not selected in our laboratory, but was published by Noonan *et al.* (1990).
When numbering all primers "A" of the start codon was considered as position "1".

^ β_2-microglobulin may be used as an internal control when amplifying this sequence if diagnostic digestion with a restriction enzyme is to be included. This is because the region amplified with the primers chosen for β-actin has also a site present for this restriction enzyme, whereas the β_2-microglobulin region does not.

$ GST α primers were chosen on the human liver cDNA templates (clone pGTH1 and pGTH2) and the human kidney cDNA template (GSTα 12 k), according to the sequences published by Klöne and co-workers (1992). GSTα (a) primer is located on exon 4 and primer (b) spans from exon 6 to exon 7 (Klöne *et al.*, 1992).

N.K. = Not known.

3.1.2 Trial Amplification and Product Restriction for all Primer Pairs Selected

To ensure that each primer pair amplified a specific cDNA region, producing a single band of the predicted size, PCR reactions were set up (as in 2.10.5) including cDNA formed on mRNA templates extracted from the sensitive (parent) form of each of the cell lines included in this study *i.e.* DLKP, SKMES-1, OAW42 and HEp-2. The analysis was performed on two RNA extractions from each cell line. All reactions were set up in duplicate. An endogenous control was also amplified in all reaction tubes; β-actin in the case of MDR 1, MDR 3, MRP, GST π, CYPIA1, Topoisomerase I, Topoisomerase II. β_2-microglobulin was amplified, simultaneously, with the Topoisomerase IIα and Topoisomerase IIβ target regions, respectively. In all cases, 30 cycles of PCR amplification were performed. An aliquot of each amplified product was digested (in duplicate) with an appropriate restriction enzyme. An example of these results (from the OAW42 cDNA analysis) is illustrated in Fig. 3.1.2.1 and Fig. 3.1.2.2. Amplification in all cases was successful, resulting in a single band of the appropriate size. Digestion with a relevant restriction enzyme also produced the predicted sized bands. The levels of internal control transcripts, β-actin remained equal in all reactions (see Table 3.1. for sizes of amplified bands and restriction digest products).

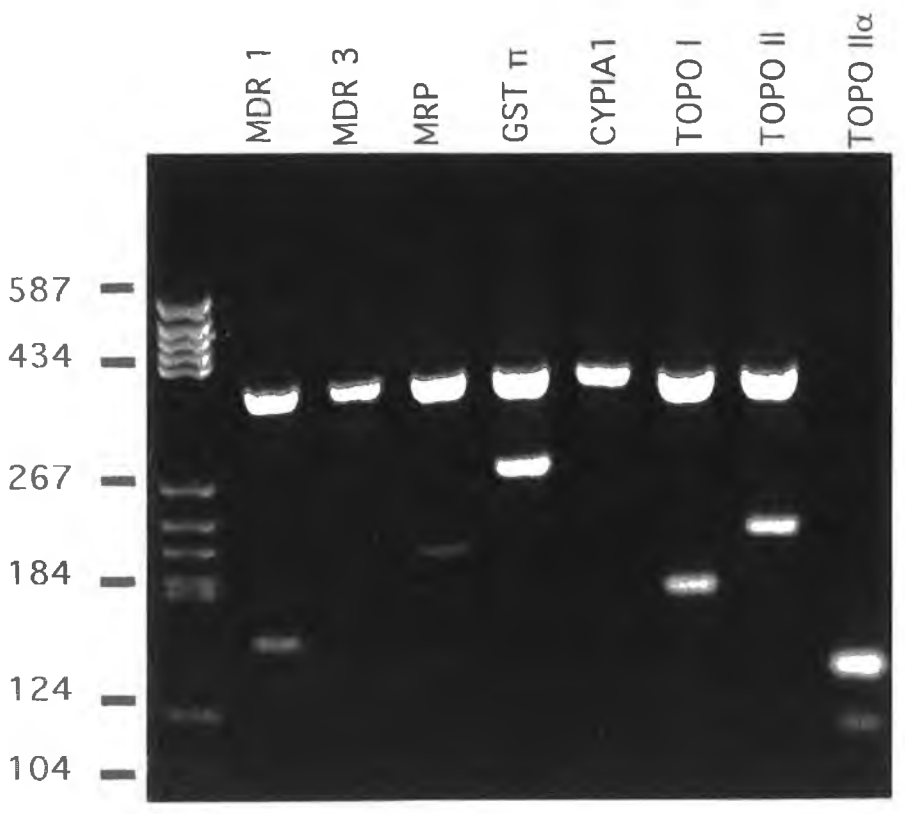


Fig. 3.1.2.1 MDR 1 (157 bp), MDR 3 (321 bp), MRP (203 bp), GST π (270 bp), CYPIA1 (327 bp), Topoisomerase I (180 bp), Topoisomerase II (216 bp) and Topoisomerase IIα (139 bp) gene transcript analysis by RT-PCR, in cDNA derived from OAW42 cells. The left-hand lane contains the DNA molecular weight marker consisting of 22 fragments with the following numbers of base-pairs: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11 and 8 with which the target products were sized. Two bands were produced in all cases. The upper band (in all, except Topoisomerase IIα analysis) represents β-actin (383 bp) which acts as an endogenous control. β_2-microglobulin (114 bp) was amplified as an internal control when studying Topoisomerase IIα. A band of the expected size was also produced in all cases, indicative of the specific target mRNA under analysis.

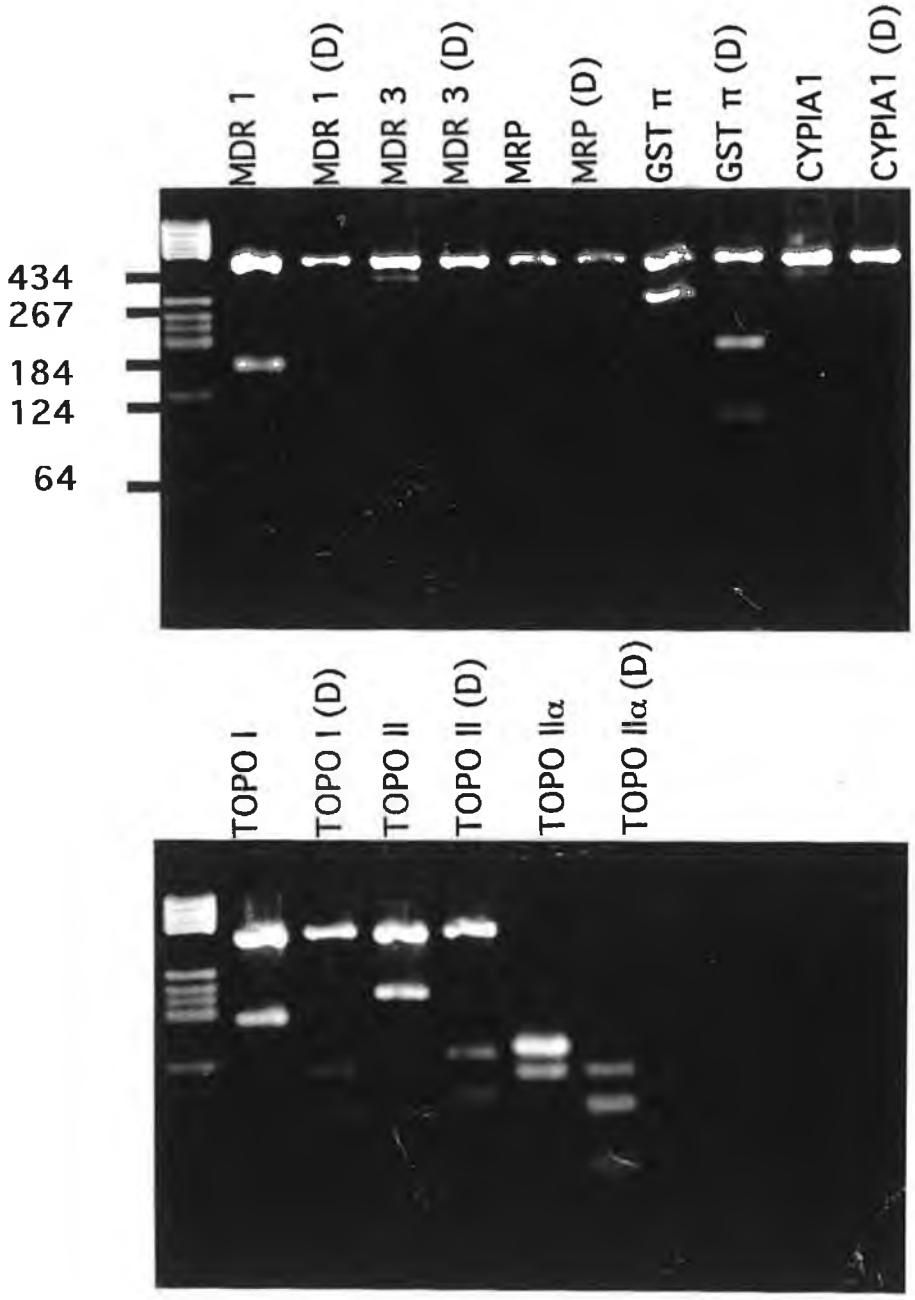


Fig 3.1.2.2 Restriction digestion of target amplified products (as indicated in Fig. 3.1.2.1) with approptriate restriction enzymes: MDR 1 (Mae 1), MDR 3 (Ssp 1), MRP (Ban II), GST π (Sty 1), CYPIA1 (Hinc II), Topoisomerase I (EcoN 1), Topoisomerase II (Hind III) and Topoisomerase IIα (Alu 1). Products of the predicted sizes resulted: MDR 1 (84 + 73), MDR 3 (194 + 127), MRP (129 + 74), GST π (171 + 99), CYPIA1 (246 + 81), Topoisomerase I (115 + 65), Topoisomerase II (129 + 87) and Topoisomerase IIα (87 + 52), when sized against the molecular weight marker ran in the left-hand lanes.

3.2 Selection of Appropriate Endogenous Internal Controls

Experiments were done to select, from a range of potential endogenous internal controls (see 1.3), one such control which could be used routinely to normalise target mRNA transcript levels, throughout the course of this study. Such a control should not be affected by the experimental conditions. This required that the levels of this gene transcript be equal in the sensitive and resistant forms of each cell line and that it be unaffected during, or following, exposure to the anti-cancer drugs - adriamycin, vincristine and VP-16. Based on published literature, the endogenous controls considered were β-actin, β_2-microglobulin, Esterase D and GAPDH. PCR reactions were set up to amplify each of these mRNA transcripts from cDNA derived from two extracts of DLKP, DLKPA, OAW42, OAW42A, HEp-2, HEp-2A, SKMES-1 and SKMES-1A, respectively. All reactions were set up in duplicate. As shown for HEp-2 and HEp-2A (Fig. 3.2, Table 3.2.1), the levels of each of the potential endogenous controls was fairly consistent in both sensitive and drug resistant cell lines, with the exception of Esterase D, which seemed to be slightly eleviated in the MDR cell lines by comparison to the sensitive cell lines. Similar results were produced when studying DLKP, DLKPA, OAW42, OAW42A, SKMES-1 and SKMES-1A. β-actin was, therefore, selected as the endogenous control for future studies as it produced a sharp, clear band which was larger in size than all the other transcripts under analysis. β_2-microglobulin was chosen for studies of Topoisomerase IIα and Topoisomerase IIβ when the resulting product was to be digested by a restriction enzyme as both Alu 1 and Hph 1 restriction enzymes which restrict these products, respectively, also cleave the β-actin product.

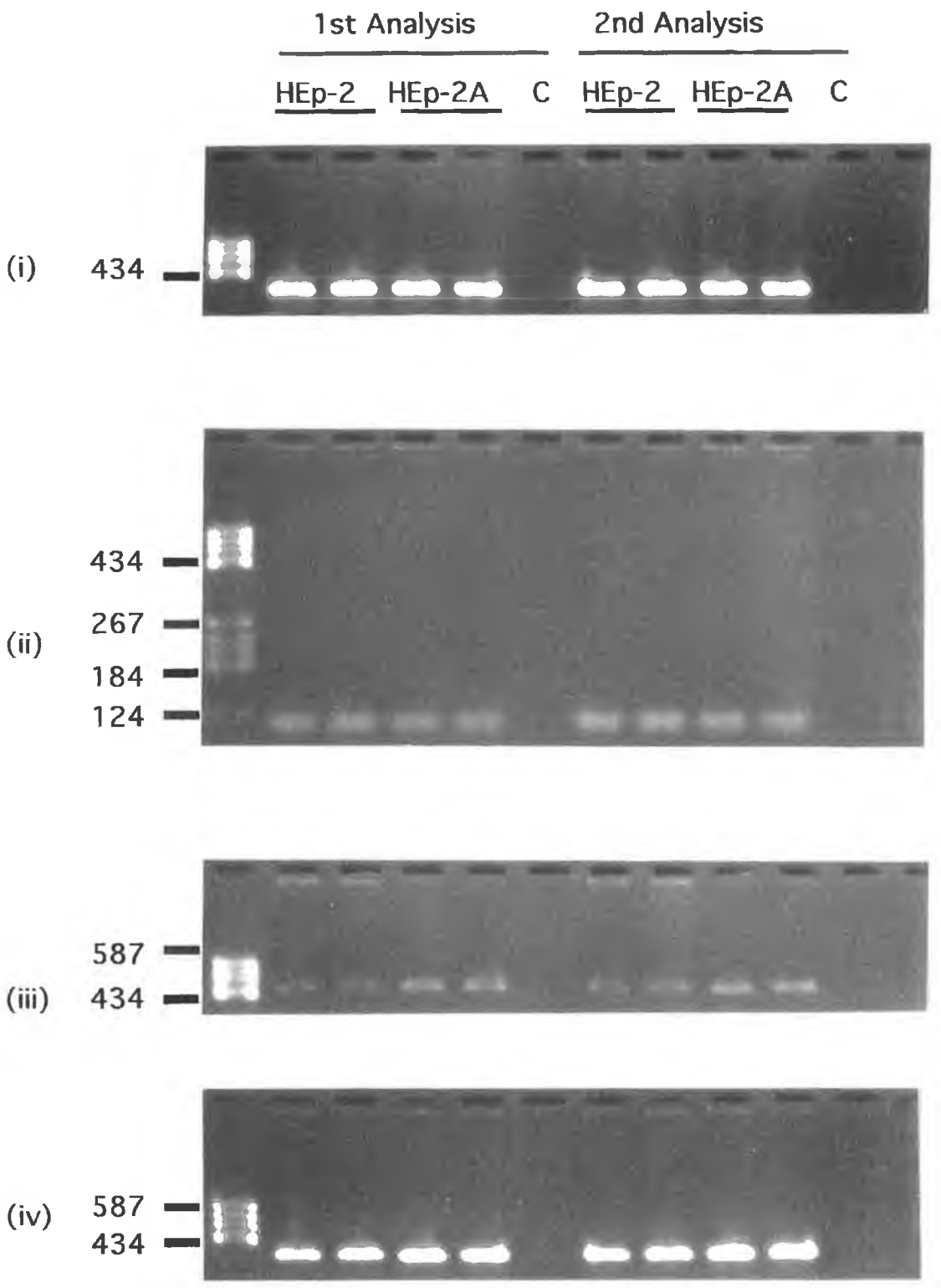


Fig. 3.2 Selecting from a number of putative ubiquitously expressed genes, including (i) β-actin (383 bp), (ii) β_2-microglobulin (114 bp), (iii) Esterase D (453 bp) and (iv) GAPDH (380 bp), a potential endogenous control for analysis of sensitive and MDR cells. This analysis was done, in duplicate, on two independent RNA extracts from HEp -2 and HEp -2A. The left-hand lane contains the DNA molecular weight marker. A negative control (C), where RNA was replaced by an equal volume of ultra-pure water in the RT reaction, was included with each RNA extract analysis (lanes 5 and 10, respectively). The results from each extract (lanes 1-4 and 6-8, respectively) were reproducible for all gene products examined. No significant differences in any of these transcript levels were realised with MDR, by comparison to the drug sensitive cells, with the exception of Esterase D, which was apparently increased in MDR lines.

This analysis of HEp -2 and HEp -2A cells is indicative of results obtained in similar studies of DLKP, DLKPA, OAW42, OAW42A, SKMES-1 and SKMES-1A cell lines.

Endogenous Control	HEp-2	HEp-2A	HEp-2	HEp-2A
β-actin	0.613	0.615	0.609	0.610
β_2-m	0.144	0.141	0.164	0.161
Esterase D	0.093	0.121	0.099	0.132
GAPDH	0.518	0.529	0.576	0.581

Table 3.2.1 Analysis, by densitometry, of band intensities resulting from RT-PCR amplification of (i) β-actin, (ii) β_2-microglobulin (β_2-m), (iii) Esterase D and (iv) GAPDH (Fig. 3.2) when selecting an appropriate endogenous control.

3.3 Qualitative Analysis of mRNA Transcripts in Sensitive v. MDR Cell Lines

Alterations in the expression of genes involved in conferring an MDR profile on cells were investigated at the mRNA level in a number of MDR cell lines, in comparison to their sensitive parent lines. The presence of gene transcripts corresponding to MDR 1, MDR 3, MRP, GST α, GST π, CYPIA1, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ was investigated in DLKP, DLKPA, OAW42, OAW42A, SKMES-1, SKMES-1A, SKMES-1ADR, HEp-2 and HEp-2A. The resulting bands were analysed to establish if any differences in the resistant and sensitive lines were apparent, which may be indicative of MDR. The analysis was performed on two RNA extracts from each cell line. Duplicate batches of cDNA were generated from each of these RNA sources on which the analysis was performed. As always, all reaction tubes were set up in duplicate. 30 cycles of PCR amplification of the cDNA of interest (and β-actin cDNA) were performed. Successful amplification of all gene products, except GST α and CYPIA1 was achieved; the MDR 3 amplified product was generally quite faint. These results are exemplified in Figs. 3.3.1 (DLKP and DLKPA) and 3.3.2 (SKMES-1 and SKMES-1A).

MDR 1 transcript levels were increased in the resistant variant, by comparison to the sensitive form of each of the cell lines studied. This was particularly obvious in DLKP and DLKPA (Fig. 3.3.1). A strong band representing the MDR 1 amplified product was produced on amplification of DLKPA-derived cDNA; DLKP apparently expressed no detectable levels of MDR 1 mRNA. Similar results were obtained for SKMES-1 and SKMES-1ADR. Although a band resulted after amplification of this region in cDNA derived from the SKMES-1 cell line, the intensity of the resulting band was significantly weaker than that produced with the MDR cell line.

Analysis of mRNA encoding Topoisomerase II revealed the presence of this mRNA in all cell lines, with apparent

reductions (*albeit* slight) in the resistant variants of the cell lines. From the independent studies of Topoisomerase IIα and Topoisomerase IIβ it seems that the Topoisomerase IIα levels were slightly reduced and the Topoisomerase IIβ slightly increased, possibly balancing the physiological levels of Topoisomerase, overall.

With the exception of the MDR 1 and Topoisomerase II mRNA levels, no obvious differences in gene expression were apparent from these results. However, as mentioned in 1.4, PCR product concentration is proportional to the starting target cDNA only as long as the product accumulation remains exponential. As the point at which the exponential accumulation plateaus was not assessed prior to this analysis, it is possible that other differences in mRNAs exist between the resistant and sensitive cell lines that had not yet been realised.

Figs. 3.3.1 and 3.3.2 Gel electrophoresis of the products of RT-PCR analysis of MDR 1, MDR 3, MRP, GST π, CYPIA1, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ gene transcripts in MDR cell lines, DLKPA (R) and SKMES-1A (R), and their sensitive parent cell line, DLKP (S) and SKMES-1 (S), respectively. All reactions were set up in duplicate. The analysis was performed after 30 cycles of PCR amplification. The left-hand lane contains the DNA molecular weight marker consisting of 22 fragments with the following base pair numbers: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11 and 8 with which the resulting bands were sized. Two bands were produced in most cases; the upper band, by the β-actin primers (acting as an endogenous control) and a lower band, the product of amplification with specific primers as indicated above each lane. Amplification with β-actin primers resulted in a single band of the appropriate size, verifying that the other products formed are the result of amplification with specific target primers and not bi-products of β-actin amplification. These bands also indicate that the levels of β-actin mRNA are not significantly different in the sensitive and MDR cell lines.

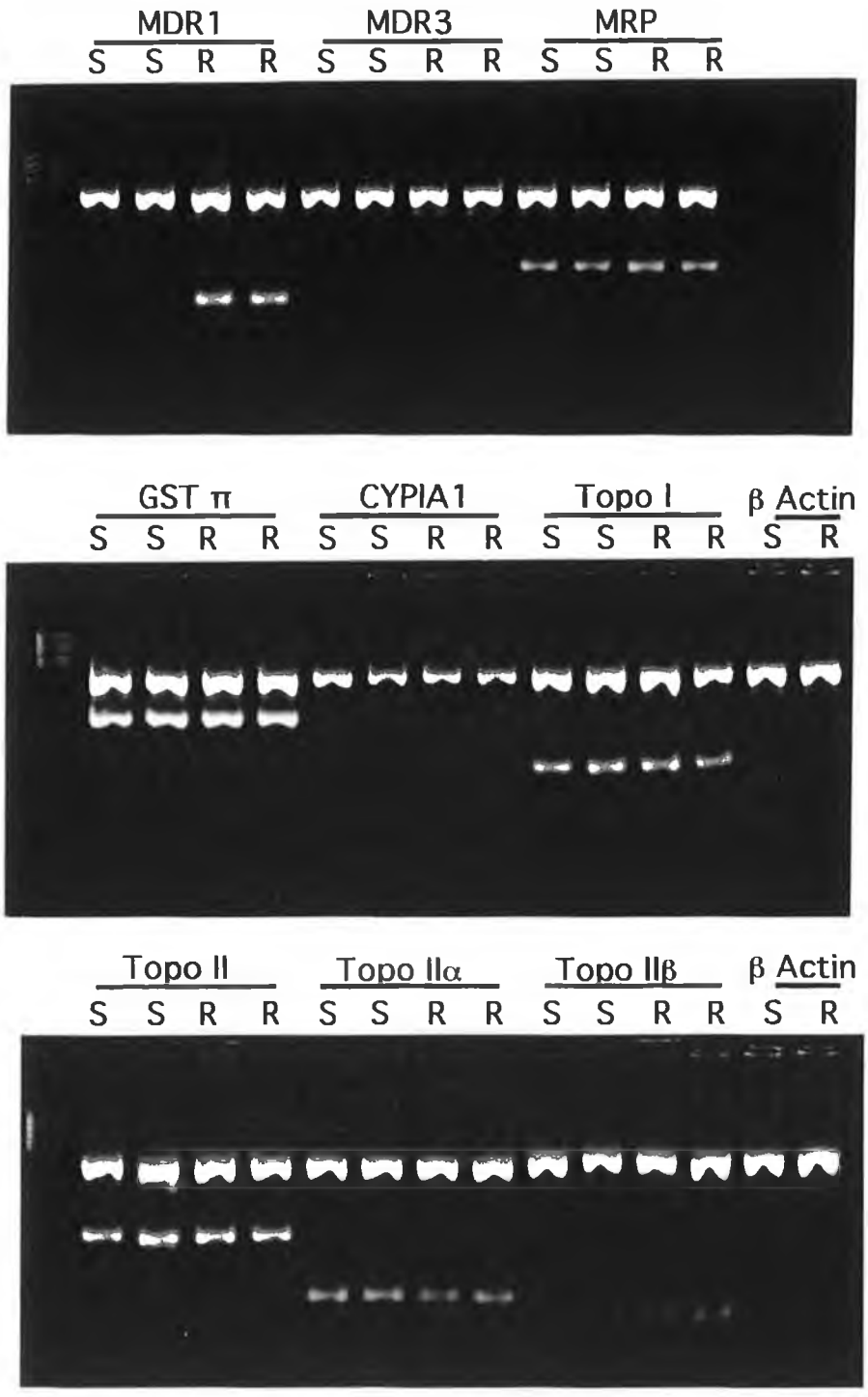


Fig. 3.3.1 Increased levels of MDR 1, MDR 3 and reduced levels of Topoisomerase II (in general) and Topoisomerase IIα are apparent in the DLKPA cell line (R), when compared to the sensitive parent cell line, DLKP (S). Elevated levels of Topoisomerase IIβ (and possibly MRP) mRNA levels were observed. Amplification of the CYP1A1 region seemed to produce a faint double band in both DLKP and DLKPA cells, which may be indicative of degraded mRNA. No other changes in gene transcript levels were observed.

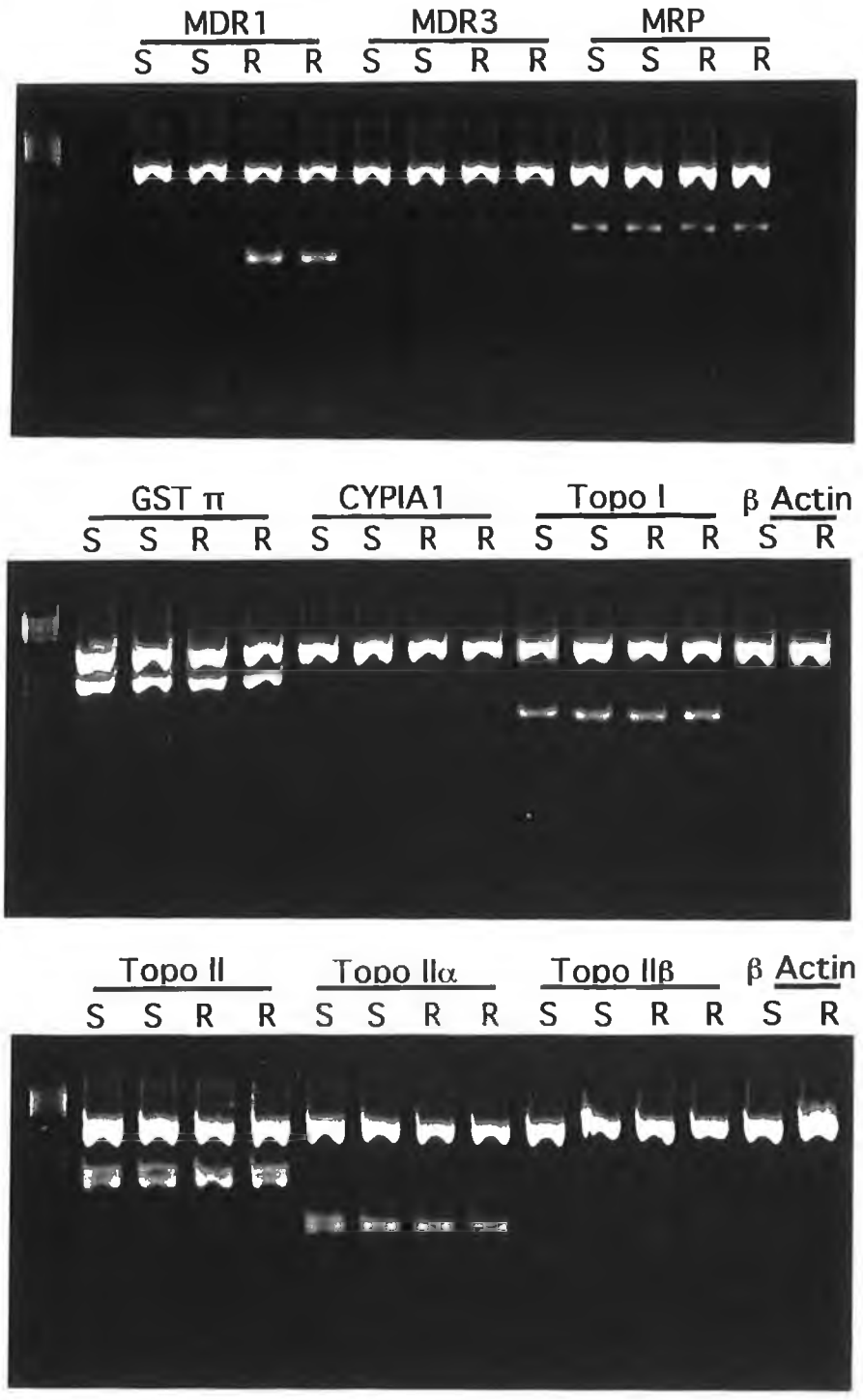


Fig. 3.3.2 The most significant differences in the gene transcript levels studied in SKMES-1 (S) and SKMES-1A (R) cell lines appear to be increased expression of MDR 1 and decreased expression of Topoisomerase IIα genes in the MDR cell line with respect to the parent cell line. No definate changes in MRP, GST π, or Topoisomerase II mRNA, with drug resistance, was observed. Increased expression of MDR 3 (although the resulting bands are quite faint) and reduced levels of Topoisomerase I mRNA seemed to occur with MDR. No detectable bands were produced on amplification of the CYPIA1 region under study, in either SKMES-1 or SKMES-1A. The results from Topoisomerase IIβ analysis although inconsistent on this gel, were generally found to be slightly elevated.

3.4 Establishing PCR Cycle Range where PCR Product Accumulation is Exponential

To develop this study to a semi-quantitative level, the PCR cycle range of exponential product accumulation and plateau formation was established for each primer pair (listed in Table 3.1) when amplifying cDNA formed on mRNA templates extracted from the DLKP, DLKPA, OAW42 and OAW42A cell lines (see 2.10.5). These were selected as representatives of the cell lines included in this study. (Preliminary experiments were performed which proved that results were the same whether a range of 50 µl PCR volumes were set up and entire tubes were removed at each of the cycle time points, or a total volume of 100 µl was prepared and 10 µl volumes were removed at each of these time points - 15, 20, 25, 30 and 35 cycles (2.10.5). The latter was favoured as it prevented any possible tube-to-tube variations. Furthermore, it required less cDNA (and so RNA) and all of the other PCR components).

The analysis was performed on DLKP and DLKPA RNA extracts a total of six times. This involved extracting RNA from three independent stocks of each cell line. Duplicate batches of cDNA were generated, for analysis, from each of these. As usual, all reactions were set up in duplicate. Similarly, OAW42 and OAW42A RNA was analysed a total of four times in this way *i.e.* on duplicate stocks of cDNA formed from two independent RNA extracts. All results were analysed by densitometry. The level of transcripts of interest in each cell line was analysed in three different ways. (a) The intensity of the target mRNA was normalised to the level of expression of the endogenous control mRNA, after correcting for background in both cases. (b) The intensity of the target mRNA bands were corrected for background reading, without taking into the account the internal control and (c) the densitometry readings of intensity for the target were analysed without subtracting background or normalising with the internal control. β-actin mRNA transcripts were also studied, independently, in this way. These were also (a) corrected for background and (b) analysed in their unadjusted

form. In all cases, the cycle numbers were plotted *versus* the intensity (densitometry reading) and the range of exponential accumulation estimated from resulting graphs. Although similar plots were achieved in most cases, whether or not they were adjusted for background and normalised to β-actin, background subtraction and normalising with this endogenous control was favoured as it allows each sensitive cell line and its resistant variant to be compared directly.

Fig. 3.4.1 - 3.4.10 are examples of results (photographs of cDNA fragments separated by gel electrophoresis and the corresponding plotted data after background subtraction and normalising with the ubiquitously expressed internal control) for one analysis of cDNA formed on template mRNA from one DLKP and DLKPA RNA extract. An estimation of the exponential phase of product accumulation, based on these results is presented in Table 3.4.1 (Experiment 1). Similar analysis was performed for the other five cDNA stocks formed on DLKP and DLKPA mRNA templates. These results are shown in Table 3.4.1 also, as experiments 2 - 6. Corresponding data for OAW42 and OAW42A are presented in Table 3.4.2.

MDR 1: The exponential range of amplification for the MDR 1 primers for all cell lines studied seems to extend, overall, from approximately 16 - 33 cycles (see Tables 3.4.1 - 3.4.2). However, the range was quite small when amplifing cDNA from DLKPA extracts (16 - 23) (see Table 3.4.1). 20 cycles was considered to be the optimal for MDR1 mRNA studies in this cell line as it lies within the exponential range for all extracts of DLKPA. No detectable bands were produced with DLKP cDNA, when studying this region, even after 35 cyces (Fig. 3.4.1).

The range of exponential increase in product formation with these primers was similar for both the OAW42 (19 - 33) and OAW42A (19 - 29) cell lines (Table 3.4.2). Therefore, although 20 cycles is, again, within the "acceptable" range for semi-quantitative comparisons to be made; approximately 25 cycles

would possibly be more suitable for amplification of MDR 1 cDNA from these particular cell lines.

The amounts of MDR 1 mRNA were significantly greater in the DLKPA cells by comparison to the DLKP cells, where no detectable bands were realised following gel electrophoresis. A similar situation was realised when studying the OAW42 and OAW42A lines *i.e.* the levels of MDR 1 mRNA were apparently greater in the resistant, by comparison to the sensitive form of the cell line. However, the difference in amounts between the two forms of the ovarian cell lines was not as great as that with the lung cell lines.

MDR 3: The resulting MDR 3 and β-actin amplification products are 321 bp and 383 bp, respectively. As can be seen in Fig. 3.4.2, because of the close proximity of these bands on the gels, the "smearing" effect below the larger, more intense β-actin bands may "mask" the MDR 3 band, making it less obvious. The bands produced on amplification of the MDR 3 region, in cDNA formed from DLKPA extracted RNA, were quite weak and were not visible before approximately 25 cycles of PCR (Fig. 3.4.2). Although Fig. 3.4.2.1 suggests cycles 24 - 33 onwards to be on the exponential increase line, from the other experiments (Table 3.4.2), it seems as if approximately 27 cycles of PCR may be required for a detectable product to result. Product accumulation remained exponential for most, if not all, of the study *i.e.* 20, 25, 30 (and possibly 35 cycles) all seemed to be within the exponential range of amplification.

As indicated in Table 3.4.1 and Fig. 3.4.2, when comparing the results of MDR 3 study in DLKP and DLKPA extracts, no product seemed to result with DLKP. Similarly, no MDR 3 amplification product was detected when studying this region in the OAW42 and OAW42A extracts.

MRP: The exponential range of product formation when amplifying the MRP region of interest seems to be within cycle numbers 20 -

32, for all cell lines studied. 25 cycles was, therefore, chosen as the optimum cycle number (Tables 3.4.1 and 3.4.2).

No definite trends were seen between the MRP mRNA amounts in the DLKP and the DLKPA cell lines (Fig. 3.4.3), although there is apparently a slight increase in level in the resistant *versus* the sensitive cell lines after normalising with β-actin. On the other hand, the results from the ovarian lines do not suggest any significant changes in MRP mRNA levels in the resistant and the sensitive cell lines.

GST π: The overall exponential range of amplification of the GST π region appeared to be between cycle numbers 16 - 22 and 16 - 23, when amplifying cDNA formed on the DLKP and DLKPA mRNA extracts, respectively; and between 16 - 24, for both the OAW42 and OAW42A cell lines. 20 PCR cycles was, therefore, considered as a suitable level of amplification for each of these cell lines.

At 20 cycles, the amount of GST π mRNA was found to be similar in the DLKP cell line by comparison to the resistant form of this line (Fig. 3.4.4). Alternatively, for the ovarian cell lines, the GST-π mRNA levels were apparently slightly greater in the resistant form, in comparison to the sensitive form of the cell line. This difference was not detected, however, in all cases at 25 and 30 cycles. This emphasises the importance of working within the exponential range of product formation.

CYPIA1: No visible bands were often produced for any of the cell lines after amplification with with the CYPIA1 primers when analysing to establish the exponential and plateau phases of amplification, although these primers have previously been shown to amplify this region of cDNA when present in a plasmid and when analysing cDNA derived from the OAW42 cell line (Fig. 3.1.2.1). This suggests that the primers are suitable and that possibly the mRNA levels of this enzyme are undetectable by this method; or, at least, the region for which the primers were

selected. Alternatively, it is possible that because of its relative closeness in size to the β-actin band (β-actin, 383 bp; CYPIA1, 327 bp), the bands have not separated sufficiently for CYPIA1 to be detected. However, this is unlikely as the MDR 3 band, which is only six base pairs shorter, is often distinguishable when amplified with β-actin.

In a number of experiments conducted, faint bands and smears were visible below the β-actin band, in and below the region where the CYPIA1 band should appear (see Fig. 3.4.5). This may possibly be indicative of a CYPIA1 mRNA breakdown product or amplification of regions with secondary structure formations.

Topoisomerase I: The exponential range of amplification of the Topoisomerase I cDNA region was estimated to be between cycle numbers 21 - 31 for DLKP and DLKPA; and 22 - 28 for OAW42 and OAW42A. 25 cycles of PCR amplification was considered to be suitable for each of the cell types (see Tables 3.4.1 and 3.4.2).

Generally, no detectable bands were produced for Topoisomerase I after 15 cycles of amplification, with faint bands often resulting after 20 cycles (Fig. 3.4.6). From the results at 25 cycles, it seems that the Topoisomerase I transcript levels are slightly reduced in the DLKPA *versus* DLKP cells. All experiments conducted on two sets of RNA extracts from these cells (*i.e.* a total of four) suggest greater amounts of this mRNA to be present in the sensitive cell line. However, both experiments done on the third extracts suggest the opposite *i.e.* increased levels in the resistant form by comparison to the sensitive cells. These differences are not consistent at 30 cycles suggesting that this is possibly approaching, or within, the plateau phase of the reaction.

The level of Topoisomerase I mRNA seemed to be slightly decreased in the resistant form of the ovarian cell line

(OAW42A), in comparison to the parent cell line (OAW42), at all PCR time points tested.

Topoisomerase II: The range of exponential amplification of the DLKP and DLKPA cDNA with the Topoisomerase II primers was generally found to be between 20 - 31 cycles. For OAW42 and OAW42A, the exponential range was, apparently, between 16 - 26 cycles. 24/25 cycles was, therefore, considered to be a suitable time point for semi-quantitative comparisons to be made between each of these two sets of cell lines, respectively.

When comparing Topoisomerase II mRNA levels between the DLKP and DLKPA cell lines (Fig. 3.4.7), in all experiments at most time points, the levels seemed to be less in the resistant form by comparison to the sensitive form of the cell line. This difference is most obvious after 20 and 25 cycles of amplification. A similar slight reduction in Topoisomerase II transcript levels was also suggested from the results of the ovarian cell line studies.

Topoisomerase IIα: The exponential range for Topoisomerase IIα amplification of DLKP and DLKPA cDNA was estimated to be between 21 - 30 cycles. Similar results were realised (21 - 31) when studying the OAW42 and OAW42A cell lines. Approximately 24/25 cycles of amplification was, therefore, considered optimum. In all experiments, the levels of Topoisomerase IIα were slightly greater in the sensitive (DLKP) by comparison to the resistant (DLKPA) cells (Fig. 3.4.8). Similarly, the Topoisomerase IIα levels were apparently less in the OAW42A cells compared to the OAW42 cell line.

Topoisomerase IIβ: Although the results suggest that the exponential range of amplification of the Topoisomerase IIβ mRNA region under study in the DLKP and DLKPA cells is between 26 - 35 and 25 - 35 cycles, respectively, it would seem advisable to perform at least 26 PCR cycles. This is because the bands are quite faint at approximately 27 cycles. As the resulting bands are not very intense in many cases, at any of the time points

studied, it seems that approximately 27 - 30 cycles are possibly required to increase the chances of any existing product being detected. This would also seem to be a suitable PCR cycle number for Topoisomerase IIβ analysis in OAW42 and OAW42A cells, as the exponential range for these is apparently between 26 - 35 cycles.

From these results it seems that the Topoisomerase IIβ levels were apparently slightly greater in the resistant cells (DLKPA) by comparison to the sensitive cells (DLKP)(Fig. 3.4.9). This was also found to be the case with the OAW42 and OAW42A cell lines.

β-actin: The range of PCR amplification during which β-actin cDNA product formation is exponential was found to be between cycle numbers 16 - 27, for each of the cell lines studied. This suggests that β-actin should act appropriately as an endogenous control as it should not have reached its amplification plateau prior to any of the target mRNAs under study. No significant difference in β-actin mRNA levels were detected in the DLKP or OAW42 cell lines, by comparison to their resistant variants.

Figs. 3.4.1 - 3.4.10 Establishing the PCR cycle range of exponential product accumulation and plateau formation during MDR 1 (157 bp), MDR 3 (321 bp), MRP (203 bp), GST π (270 bp), CYPIA1 (327 bp), Topoisomerase I (180 bp), Topoisomerase II (216 bp), Topoisomerase IIα (139 bp), Topoisomerase IIβ (118 bp) and β-actin (383 bp) cDNA amplification, respectively. 100 μl sample volumes were set up to amplify the relevant cDNA derived from DLKP (S) and DLKPA (R) cells. β-actin, which acts as an internal positive control and as a means to normalise the intensity of the target bands of interest, was also amplified. A band representing β-actin resulted in all samples, the intensity of which increased with cycle numbers, up to 30 cycles. The band intensities resulting after 30 and 35 cycles were not visibly different, in any case. No differences in β-actin band intensities were observed after any given PCR cycle number, between the sensitive and resistant cells, when co-amplifying with any of the target cDNAs. 10 μl volumes were removed at 15, 20, 25, 30 and 35 cycles. The resulting products were separated by electrophoresis through a 4 % gel and identified with respect to the molecular weight markers (left-hand lane of each gel), consisting of 22 fragments of the following sizes: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11 and 8 base pairs. All analysis was performed in duplicate.

Figs. 3.4.1.1 - 3.4.10.1 Relationship between the PCR cycle number and intensity of bands produced after analysis by densitometry. The densitometry readings represent the results of the target gene transcript analysis (Fig. 3.4.1 - 3.4.10), after correction *i.e.* after background subtraction and normalising with β-actin. From these plots, which generally consist of a log and a plateau phase, an estimation can be made of the cycle numbers for which PCR products accumulate exponentially, before reaching a plateau.

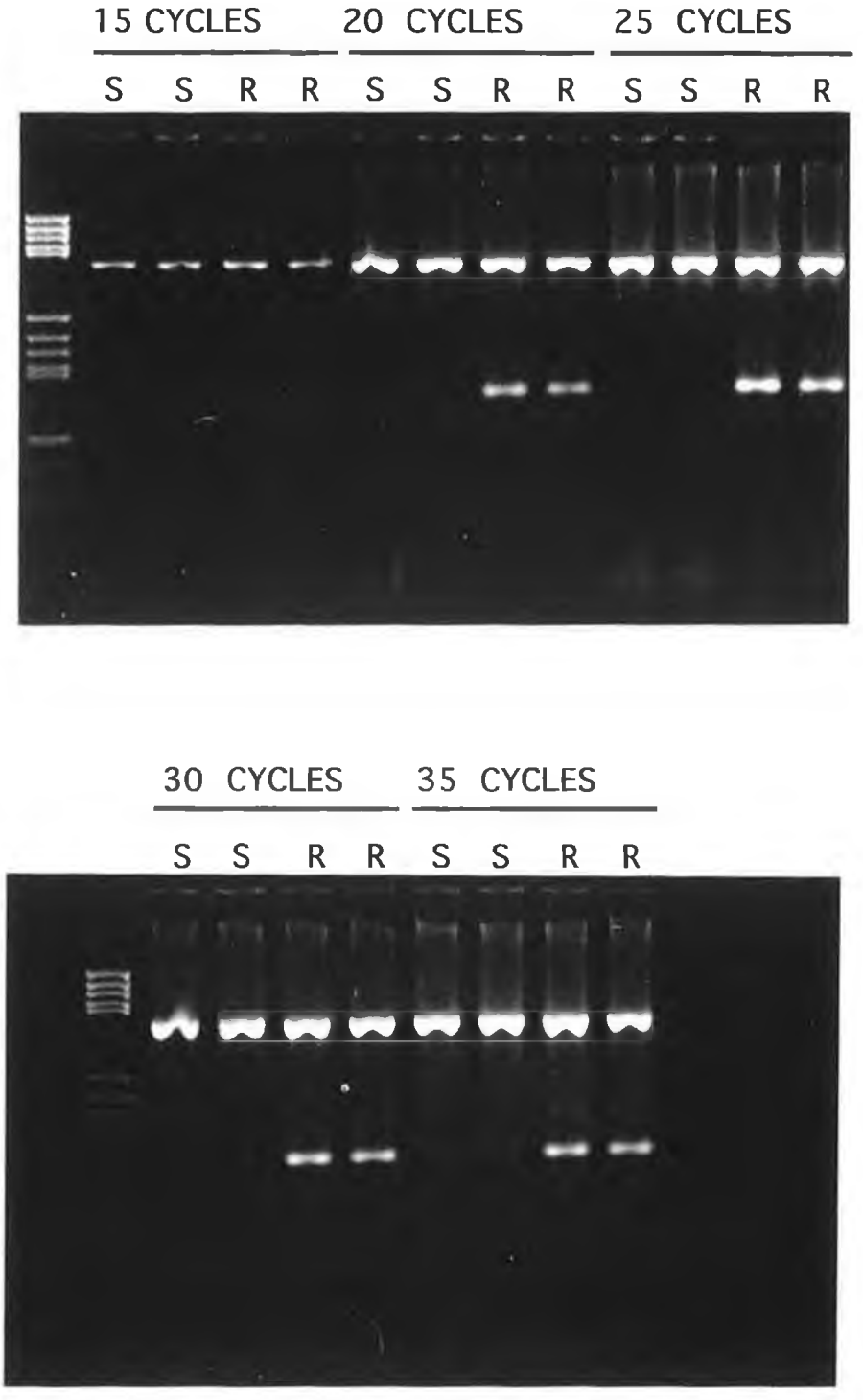


Fig. 3.4.1 No detectable levels of MDR 1 mRNA were produced with DLKP cDNA. On analysis of DLKPA, the MDR 1 band (157 bp) apparently increased with PCR cycle number up to approximately 25 cycles, after which further product yield was not observed. The band intensity increased significantly between 15 - 20, and again, (possibly to a lesser extent), between 20 - 25 cycles. Beyond this, no further increases were observed.

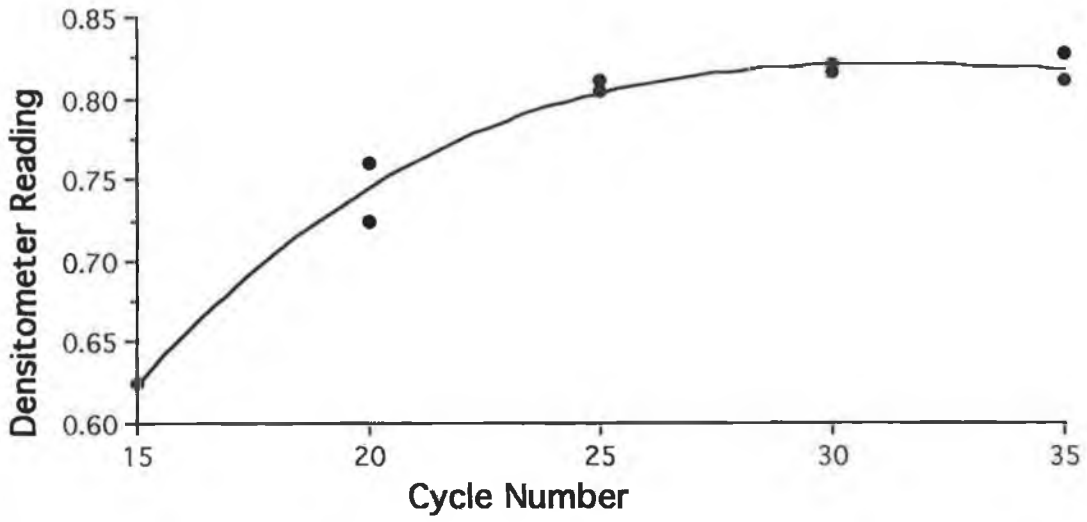


Fig. 3.4.1.1 Plot of the PCR cycle numbers *versus* the densitometry readings of the resulting MDR 1 bands amplified from DLKPA-derived cDNA. This suggests that the PCR range of exponential product accumulation is between 16 - 21 cycles, after which the plateau phase is reached. (Experiment 1, Table 3.4.1).

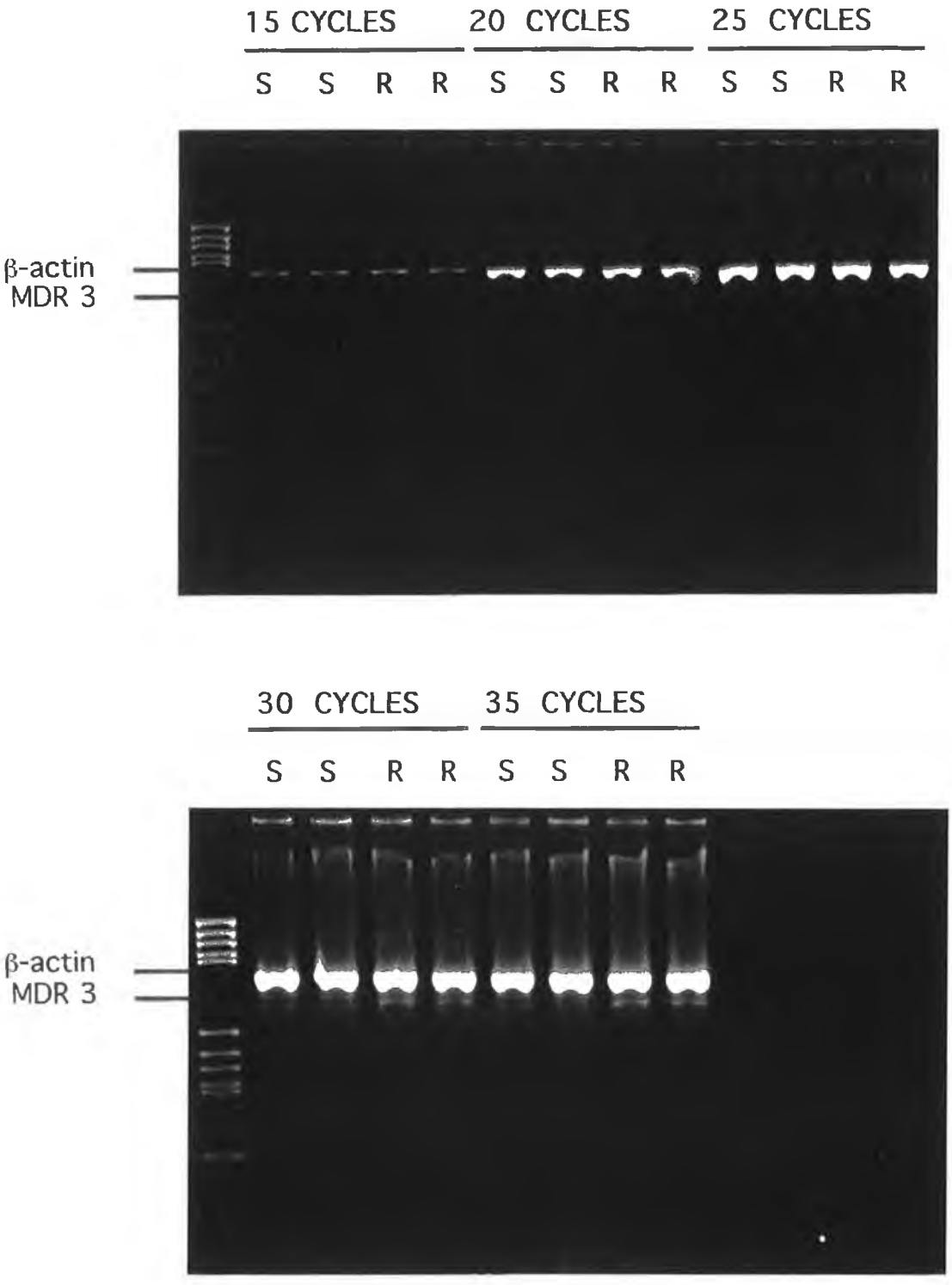


Fig. 3.4.2 Amplification of the MDR 3 target region of DLKP (S) and DLKPA (R) cDNA resulted in a visible band (321 bp) being produced after 25, 30 and 35 PCR cycles (but not at earlier cycle numbers) for DLKPA. The intensity of this band apparently increased with increasing PCR cycles. No corresponding bands were produced for DLKP.

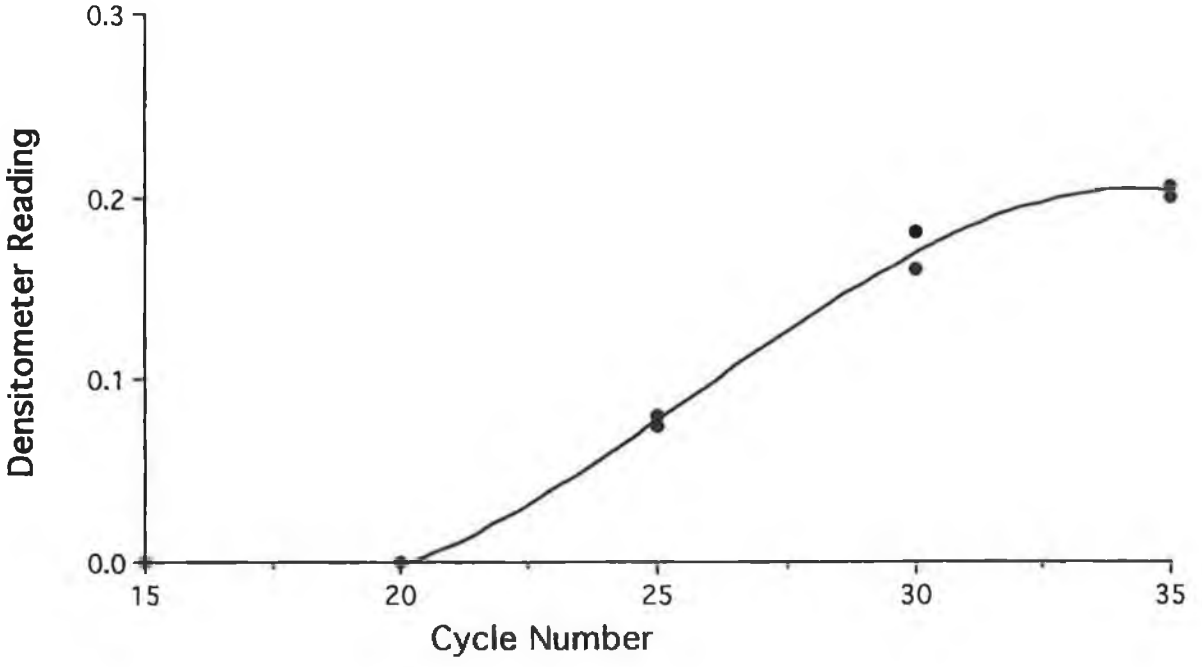


Fig. 3.4.2.1 Relationship between PCR cycle numbers and corresponding MDR 3 product accumulation with DLKPA cDNA. From this plot, it appears that the range of exponential product accumulation is between 23/24 - 32/33 cycles. (Experiment 1, Table 3.4.1).

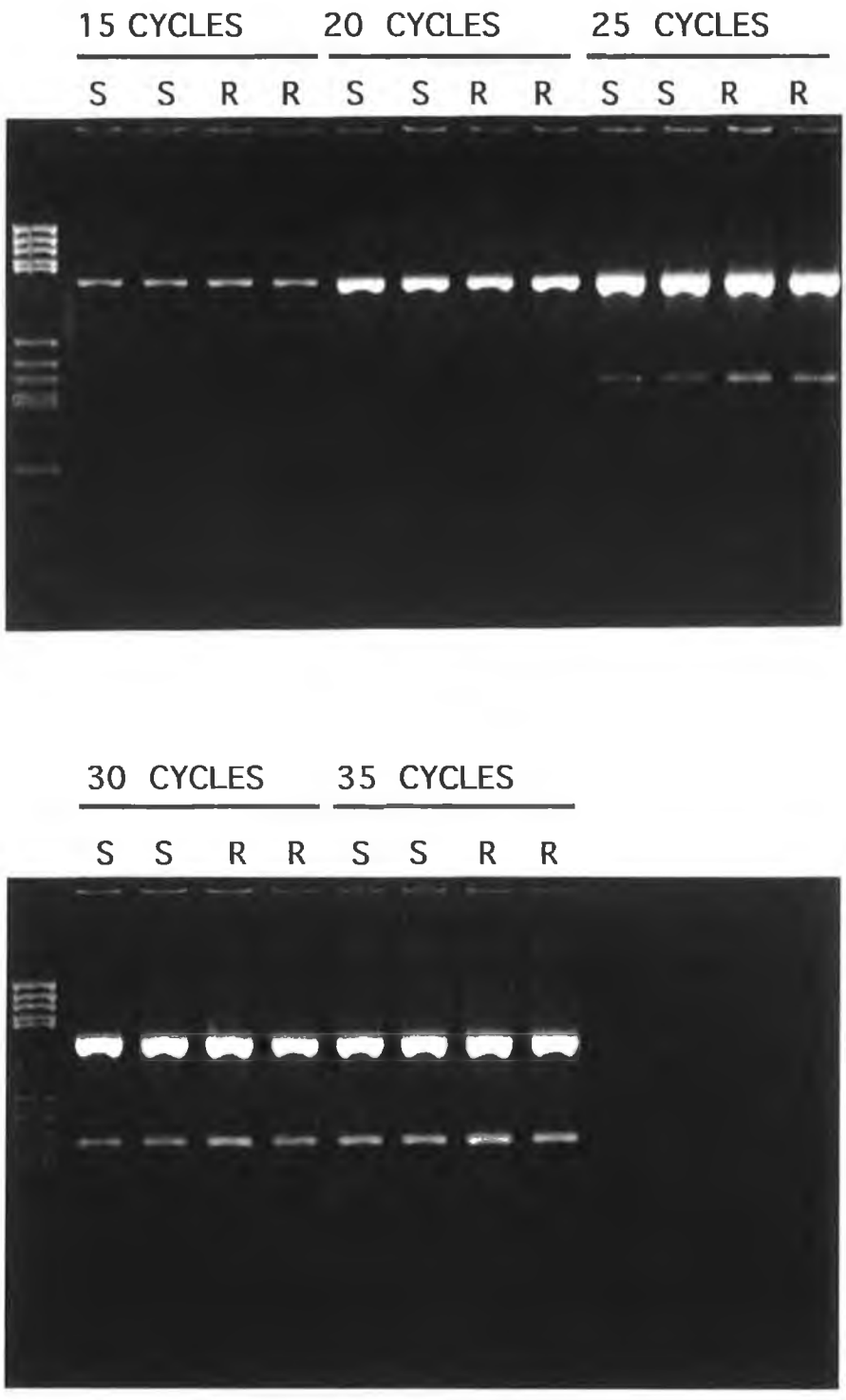
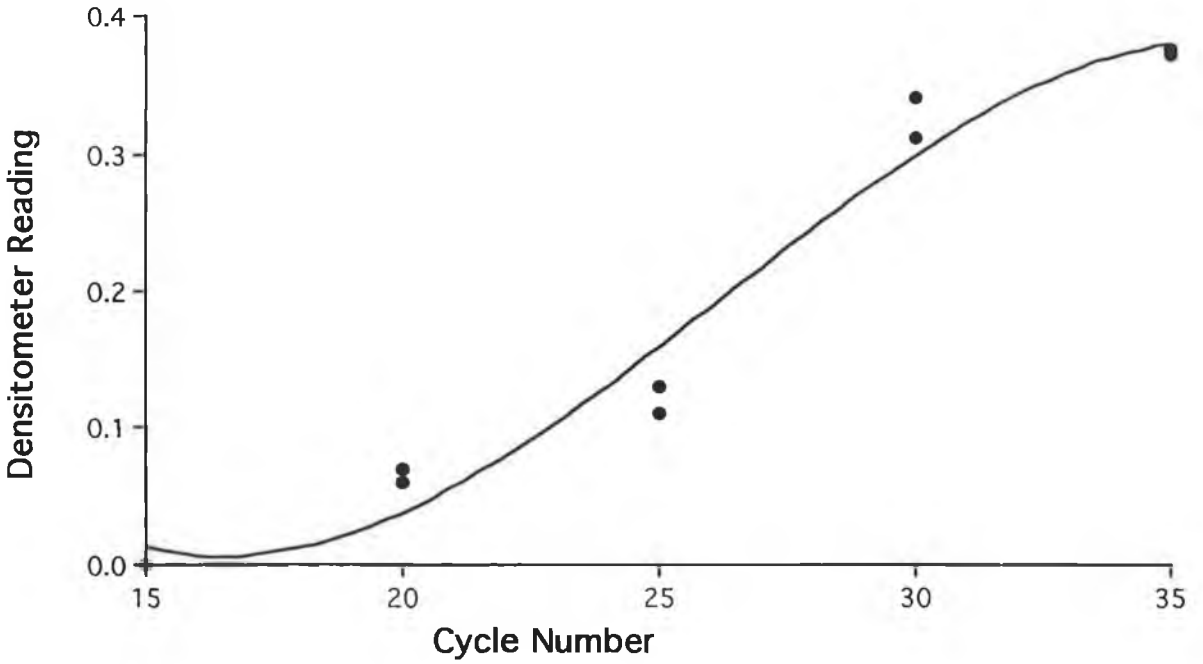
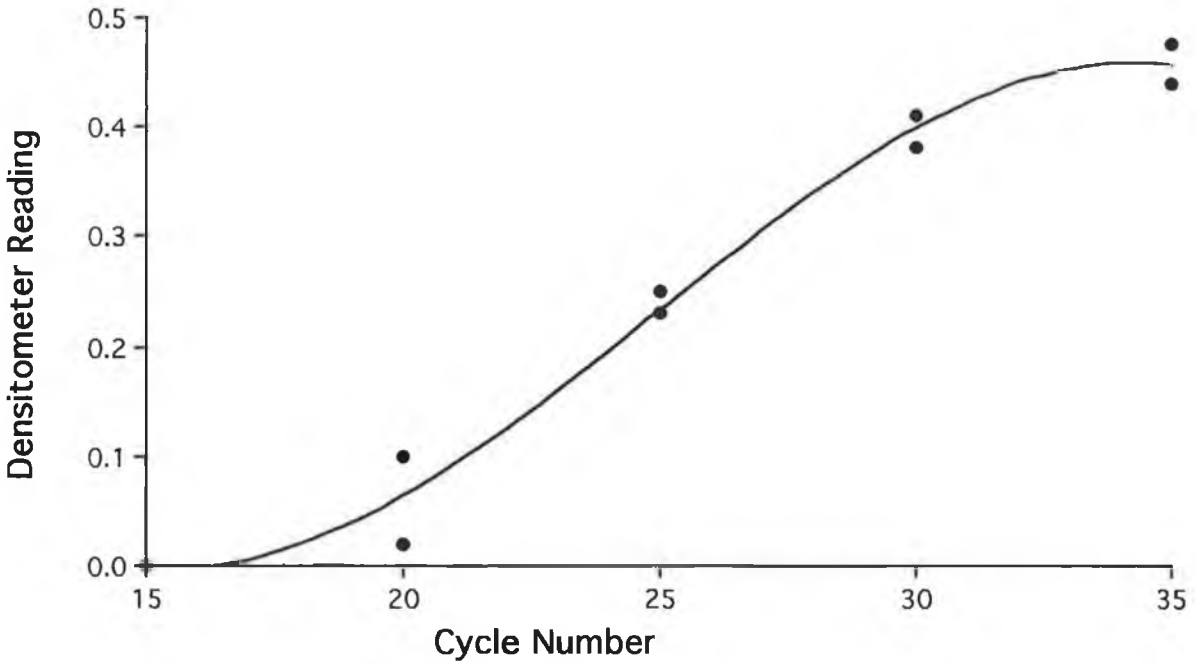
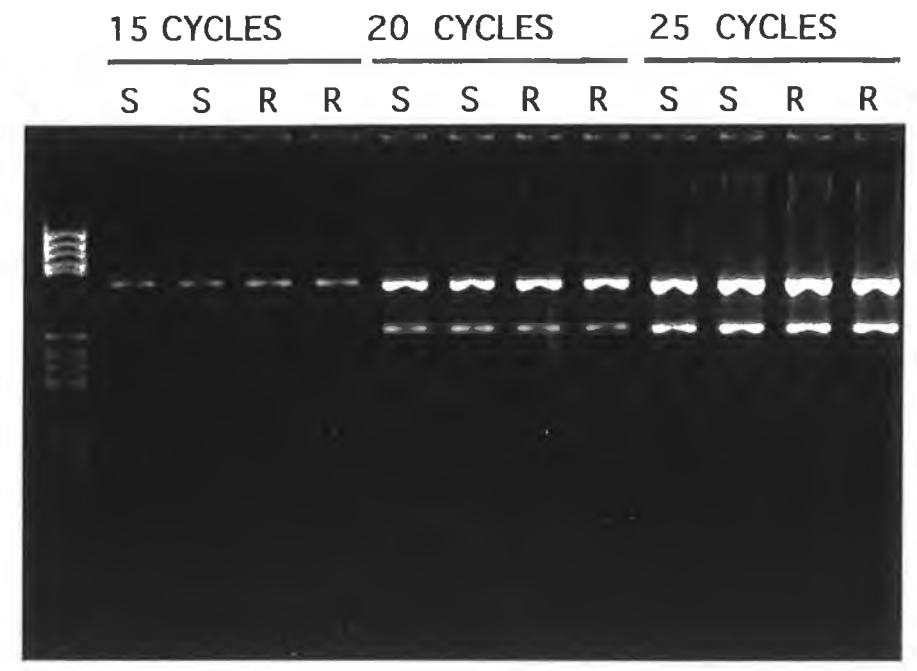


Fig. 3.4.3 Amplified MRP mRNA (203 bp) was first noted after 20 cycles of PCR amplification with both DLKP (S) and DLKPA (R) cDNA. The most significant increases in transcript levels were between 20 - 25 cycles and, again, between 25 - 30 cycles. No great differences in MRP band intensities between 30 and 35 cycles were apparent. MRP band intensities were greater for DLKPA (R) than for DLKP (S). This difference was most visible after 20 and 25 cycles of amplification, and was apparent, although less obviously, after 30 and 35 cycles.





Figs. 3.4.3.1 and 3.4.3.2 Results from MRP analysis in DLKP and DLKPA cell lines, respectively, when presented as PCR cycle numbers versus corresponding densitometry reading of MRP band intensities. From these plots, product accumulation seems to be exponential between approximately cycle numbers 20/21 - 32, when studying cDNA from both the sensitive and MDR cell lines. (Experiment 1, Table 3.4.1).



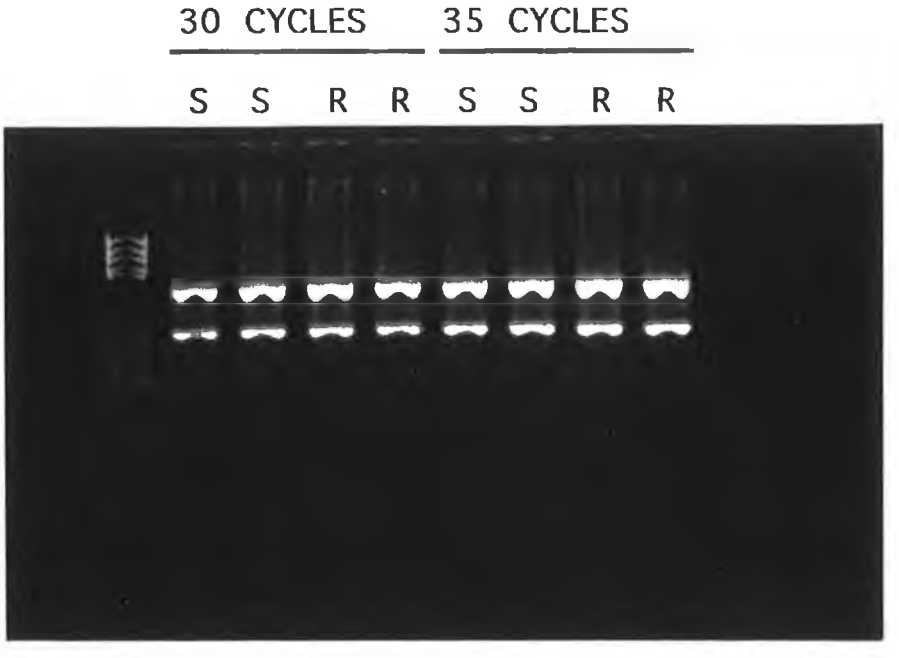
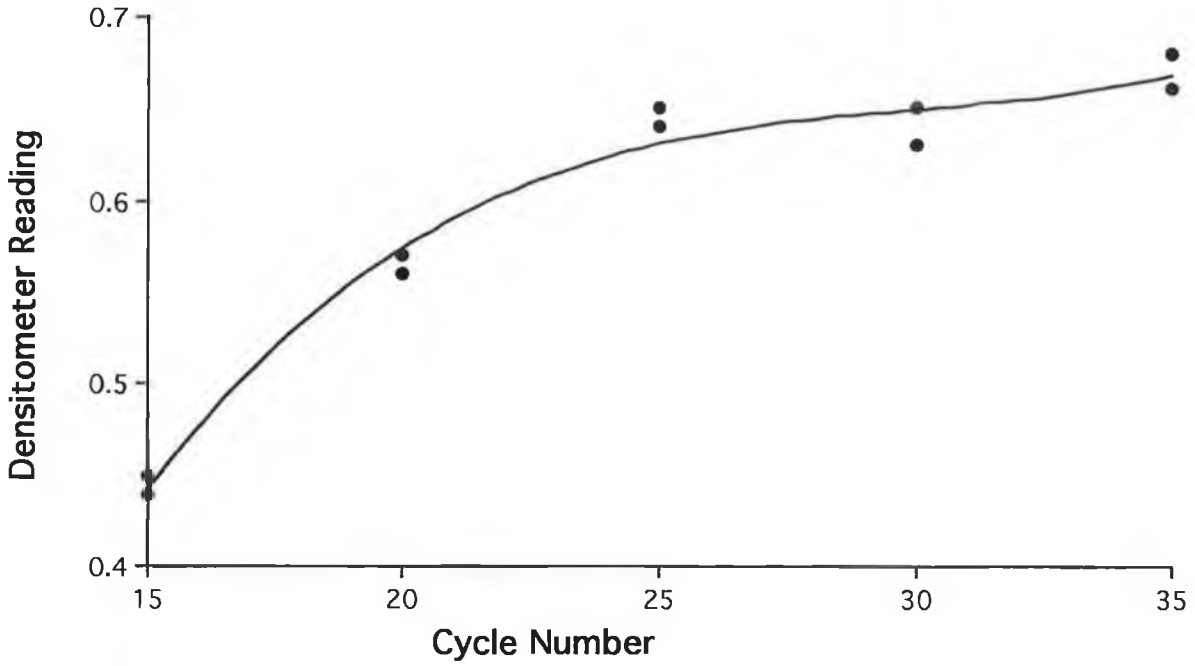
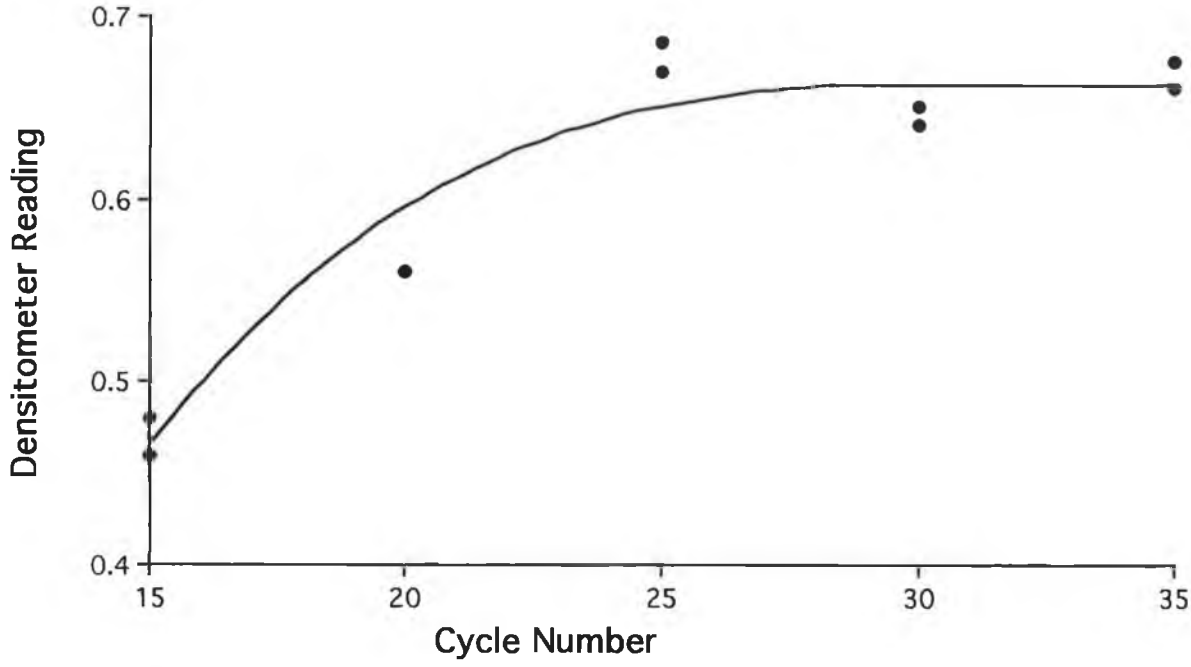


Fig. 3.4.4 Bands representing amplified GST π mRNA (270 bp) were visible after 15 cycles of PCR amplification when analysis of cDNA from both DLKP (S) and DLKPA (R) cells lines was performed. The intensities of these bands, which did not seem to differ significantly between the sensitive and MDR cell lines, seemed to increase with cycle number between 15 - 20 cycles and 20 - 25 cycles. No changes in band intensities were noted for GST π beyond 25 cycles of amplification.





Figs. 3.4.4.1 and 3.4.4.2 The range of exponential accumulation of GST π product seems to be between cycle numbers 16 - 22 for DLKP (Fig. 3.4.4.1) and 16 - 23, when analysing DLKPA cDNA (Fig. 3.4.4.2). After this a plateau phase occured, suggesting no further product formation with extra PCR cycles. (Experiment 1, Table 3.4.1).

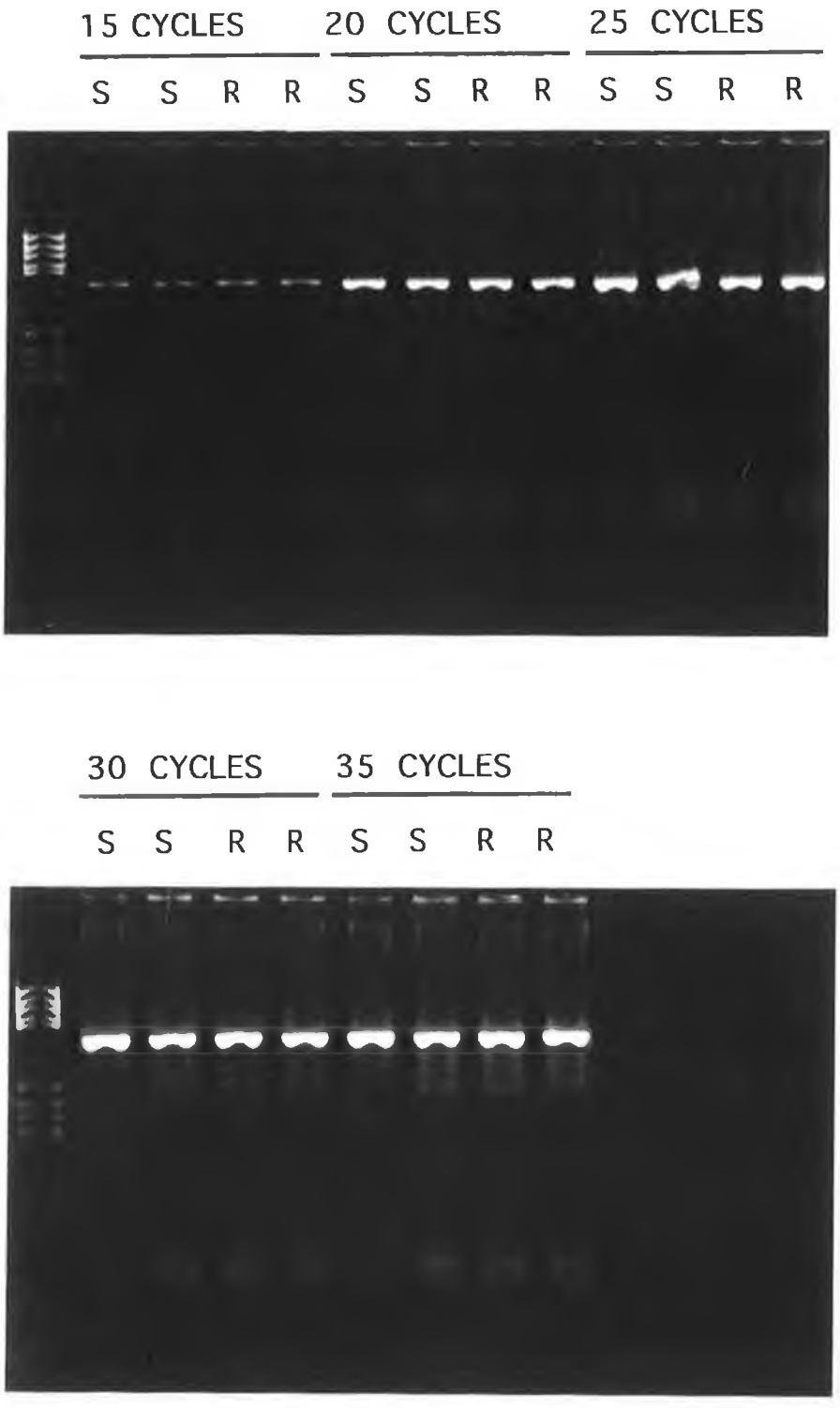


Fig. 3.4.5 No detectable levels of CYPIA1 mRNA (327 bp) were produced by RT-PCR analysis of DLKP (S) or DLKPA (R) cDNA. At higher PCR cycle numbers, a "smear" consisting of faint bands occurred below the β-actin band, in and below the region where the CYPIA1 amplified product should occur. Successful amplification of β-actin cDNA was observed (as when amplifying with all the other primer pairs included in this study), resulting in bands of similar intensities for both the sensitive and MDR cell lines.

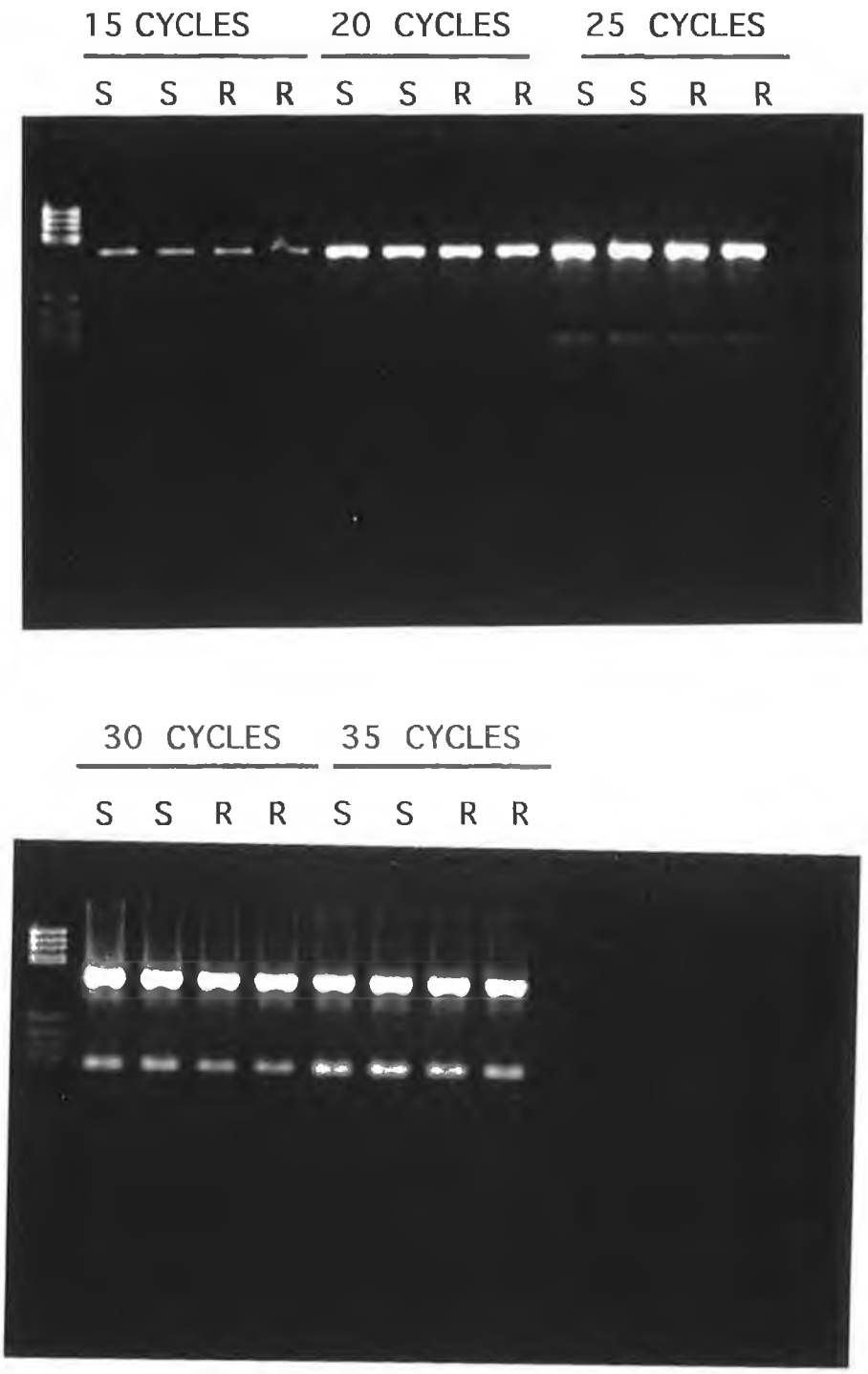
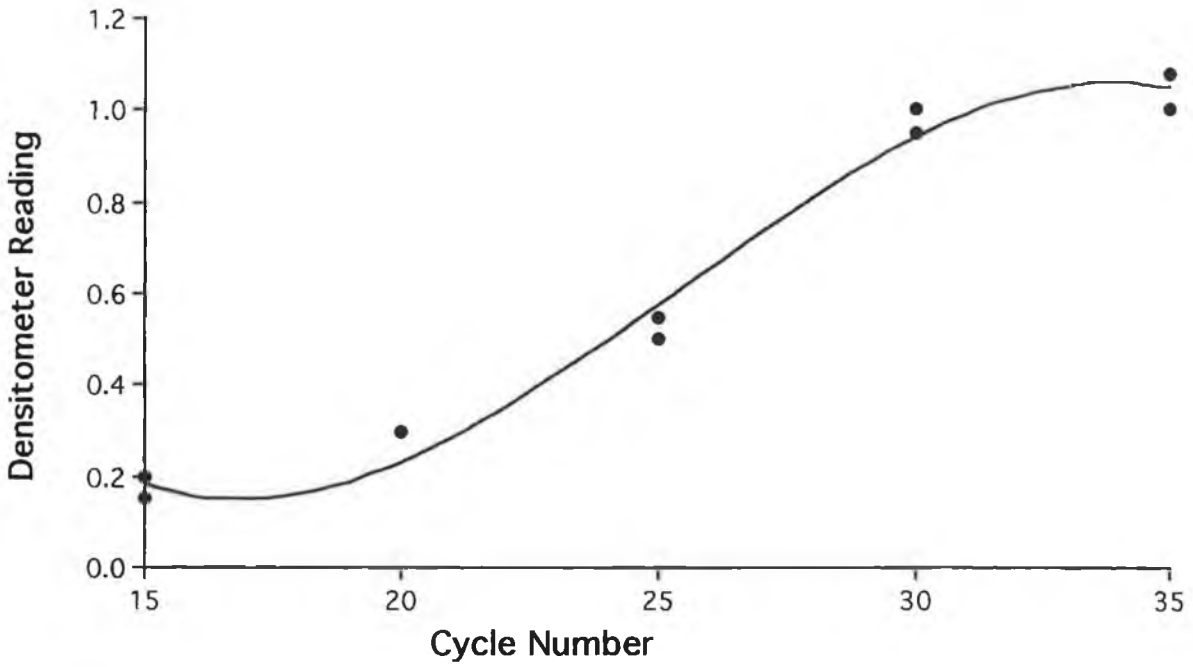
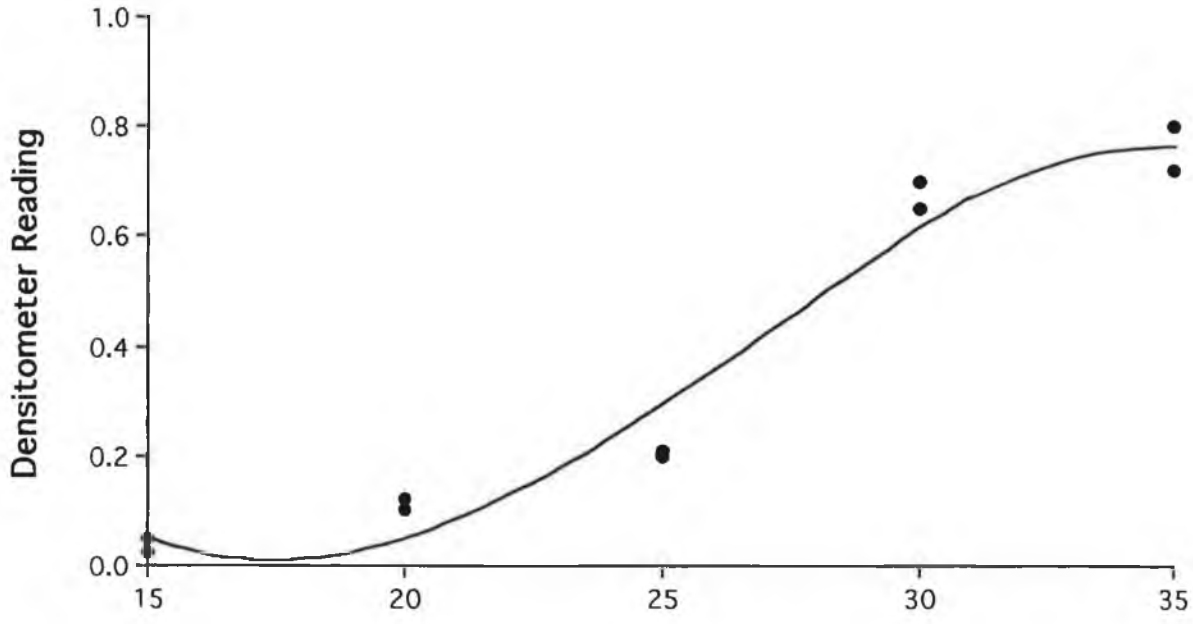


Fig. 3.4.6 Products of Topoisomerase I amplification (180 bp) with cDNA derived from DLKP (S) and DLKPA (R) cell lines were not visible after 15 cycles of amplification, but were detected (although quite weak) after 20 PCR cycles. The intensities of these bands increased significantly between 20 - 25 cycles and, again, between 25 - 30 cycles. A slight increase between 30 - 35 cycles was apparent.





Figs. 3.4.6.1 and 3.4.6.2 The plots of PCR cycle numbers versus densitometry readings of the resulting Topoisomerase I bands amplified from DLKP (Fig. 3.4.6.1) and DLKPA (Fig. 3.4.6.2) cDNA suggest the range of exponential product accumulation to be between 21 - 31 PCR cycles, for both cell lines. After this, a plateau phase was reached, indicative of no further product formation. (Experiment 1, Table 3.4.1).

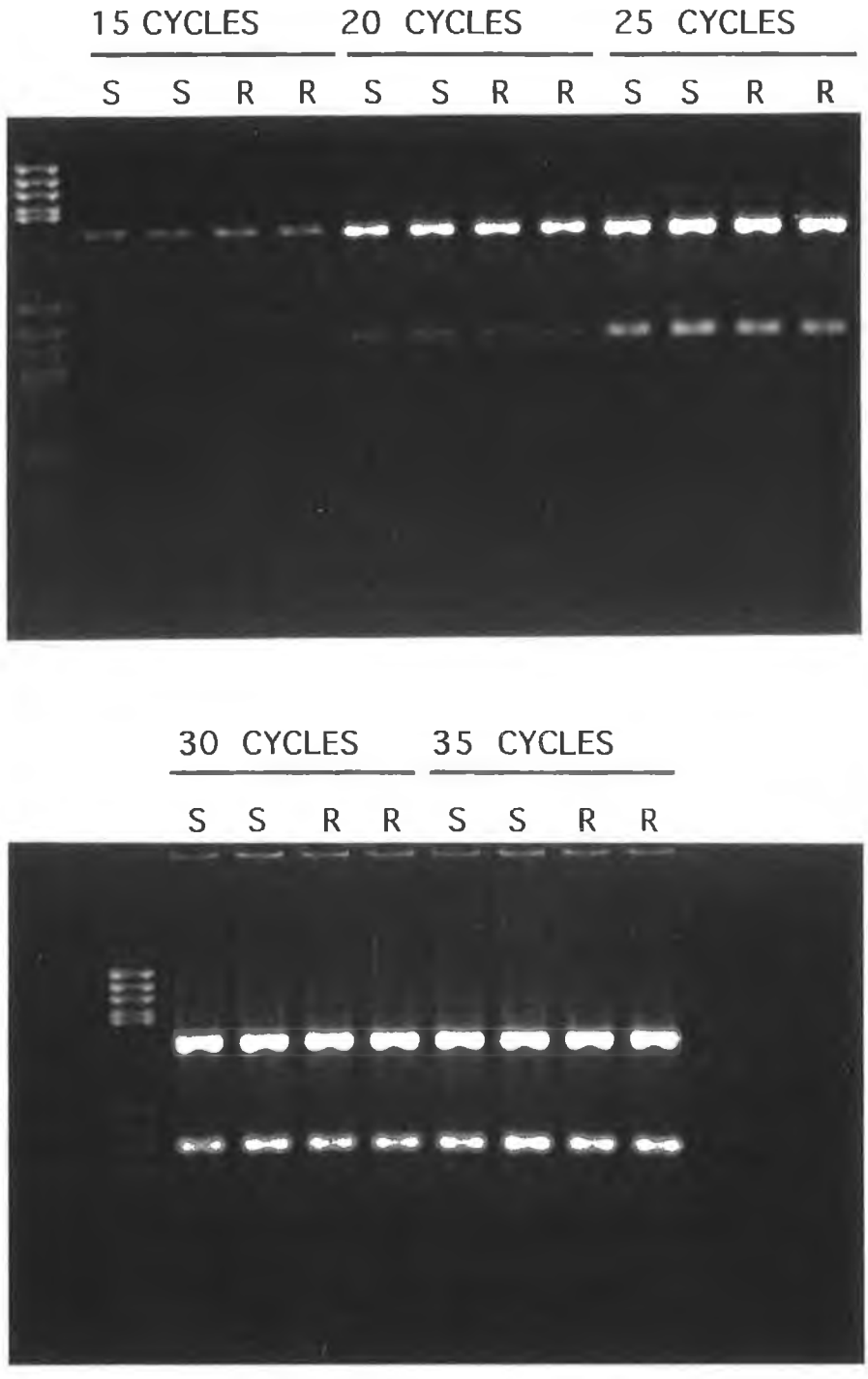
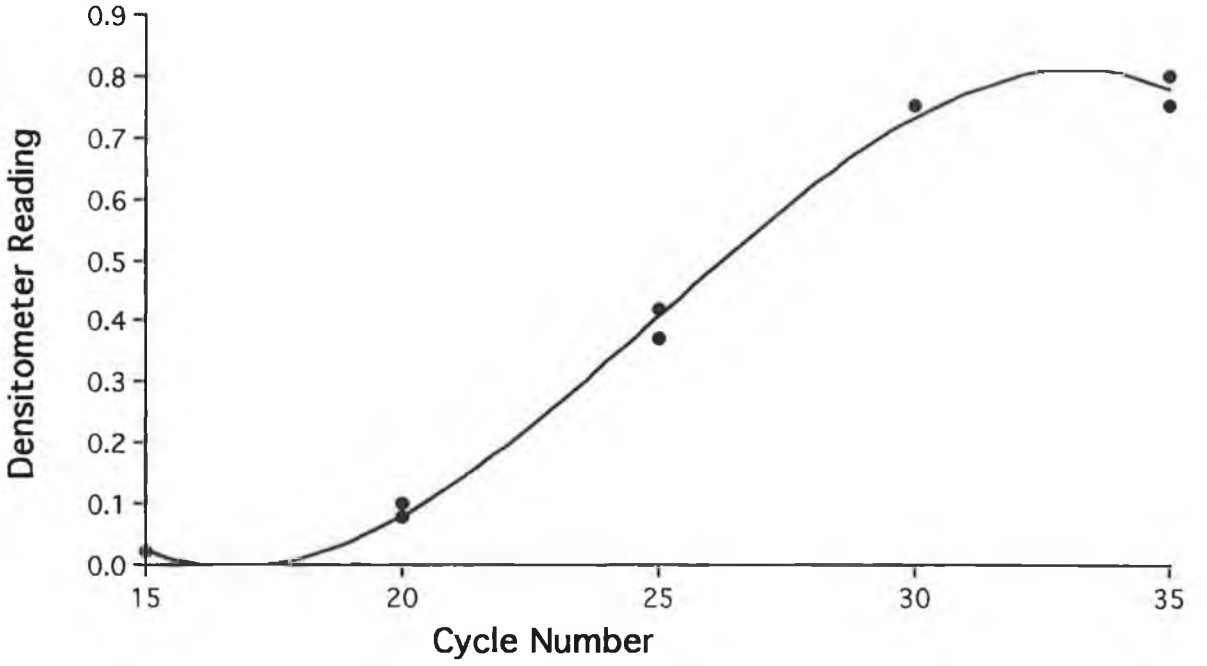
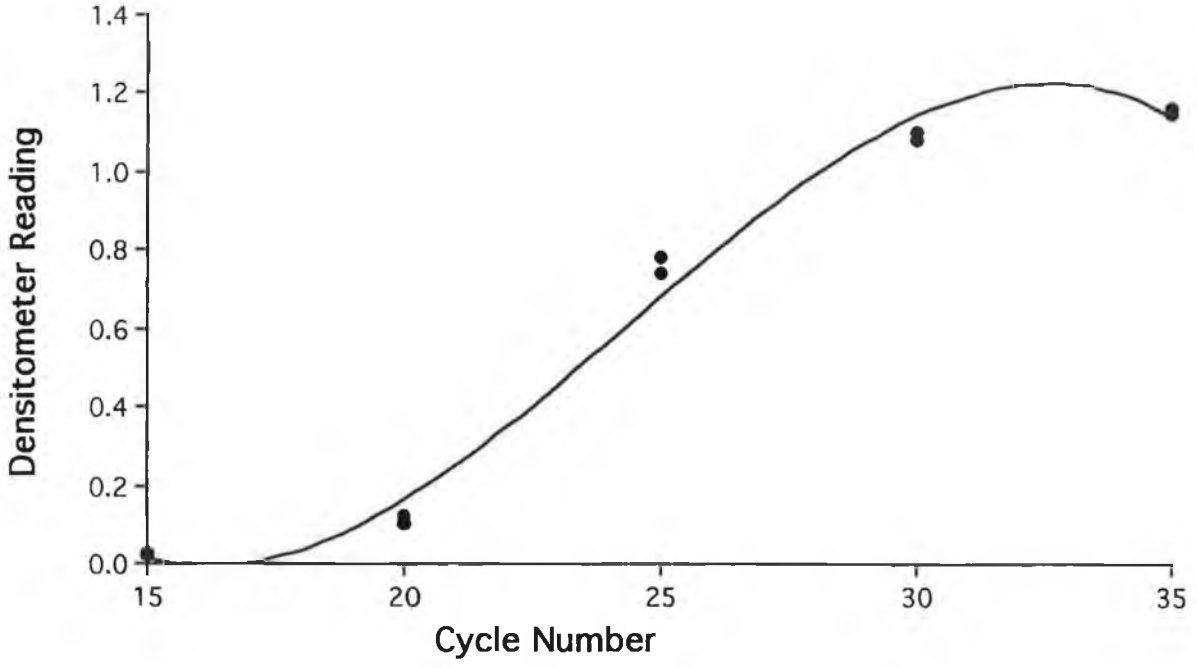


Fig. 3.4.7 No detectable levels of Topoisomerase II mRNA were produced with either DLKP (S) or DLKPA (R) cDNA after 15 cycles of amplification. Bands were visible, however, after 20 cycles of PCR. The band intensities seemed to increase significantly between 20 - 30 cycles and, to a lesser extent, between 30 - 35 cycles. Topoisomerase II mRNA transcript levels were apparently greater in the sensitive cell line, DLKP, compared to the MDR cell line, DLKPA. This difference was observed after 20 and 25 cycles of amplification, but was not so obvious after 30 and 35 PCR cycles.





Figs. 3.4.7.1 and 3.4.7.2 Results from Topoisomerase II analysis in DLKP (Fig. 3.4.7.1) and DLKPA (Fig. 3.4.7.2) cell lines presented as numbers of cycles of PCR amplification versus corresponding densitometry readings of Topoisomerase II band intensities. These plots suggest the exponential range of Topoisomerase II product accumulation to be between 20 - 31 cycles, for both the drug sensitive and resistant cell lines. (Experiment 1, Table 3.4.1).

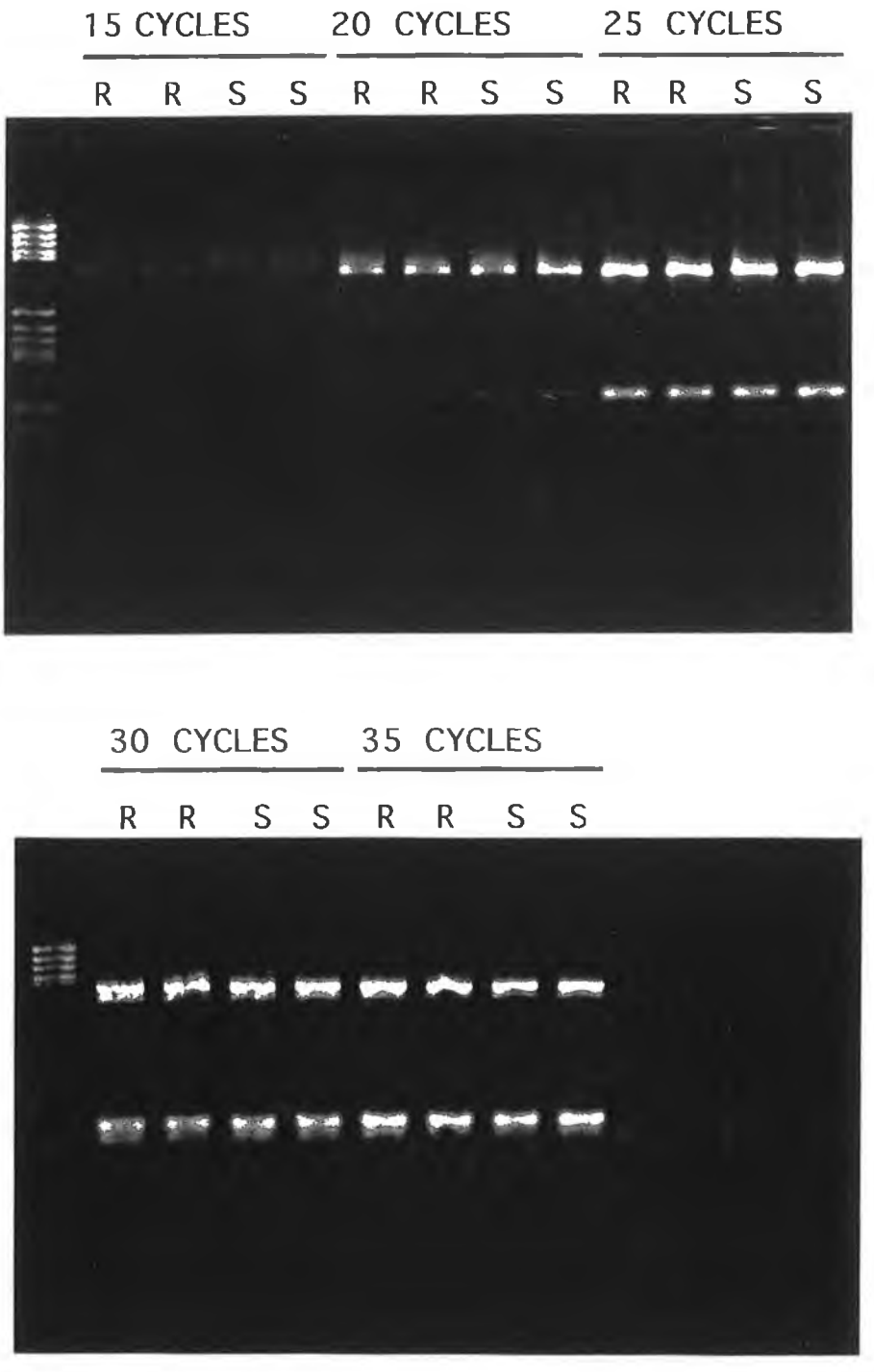
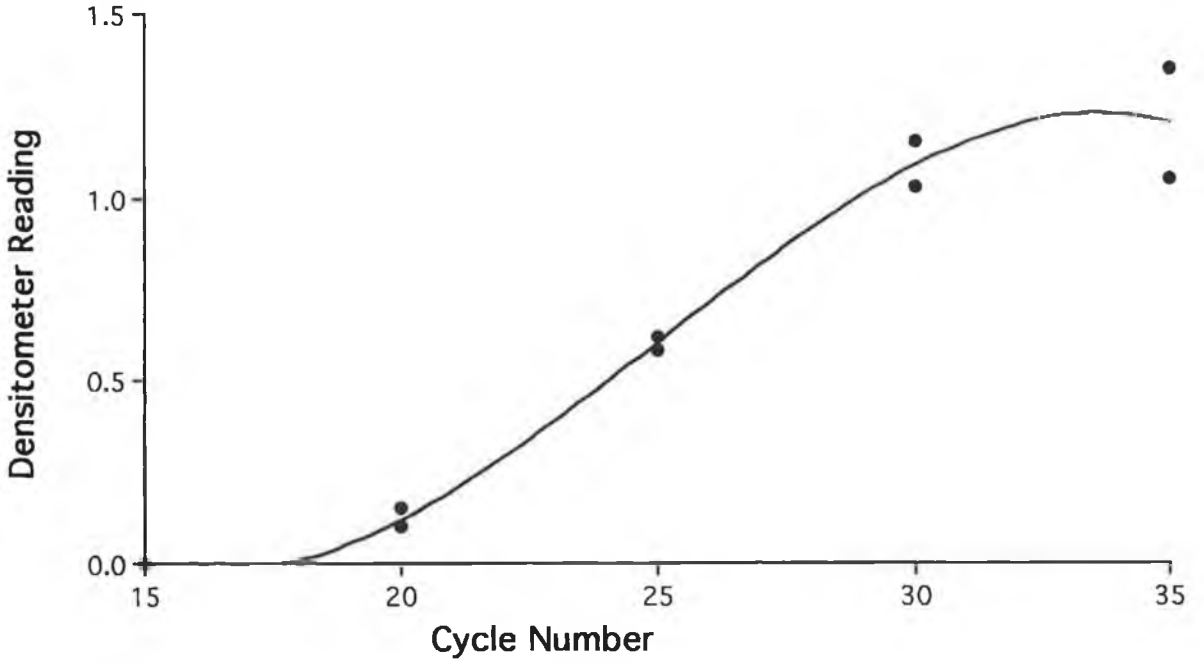
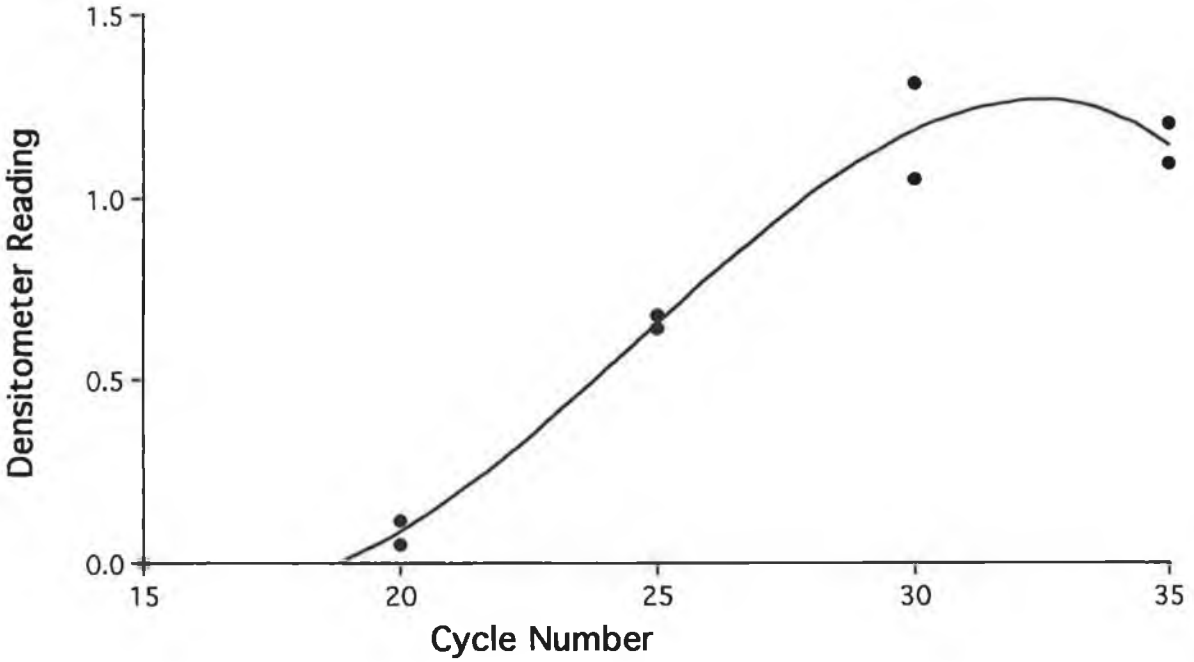


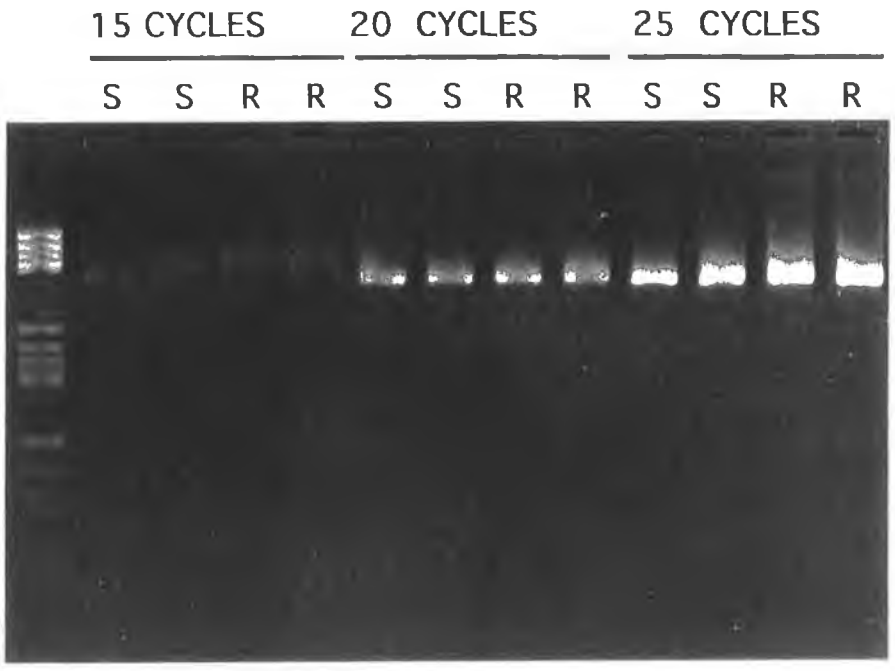
Fig. 3.4.8 Topoisomerase IIα transcript products were detected after 20 - 35 cycles of amplification, the levels of which seemed to increase significantly up to 30 cycles, but not beyond this. The intensities of the bands resulting from Topoisomerase IIα amplification were similar, although slightly reduced in the MDR cell line, DLKPA (R), compared to the sensitive cell line, DLKP (S). This was observed after lower PCR cycle numbers (20 and 25), but was not apparent after 30 and 35 cycles of amplification.

Note: The order in which the "R" and "S" samples were loaded on the gel was not the same as for all of the other gels described in section 3.4.





Figs. 3.4.8.1 and 3.4.8.2 The range of exponential product accumulation when studying Topoisomerase IIα gene transcripts was estimated, from the plots of cycle numbers *versus* corresponding densitometry readings for both DLKP (Fig. 3.4.8.1) and DLKPA (Fig. 3.4.8.2), to be between 21 - 30 PCR cycles of amplification. (Experiment 1, Table 3.4.1).



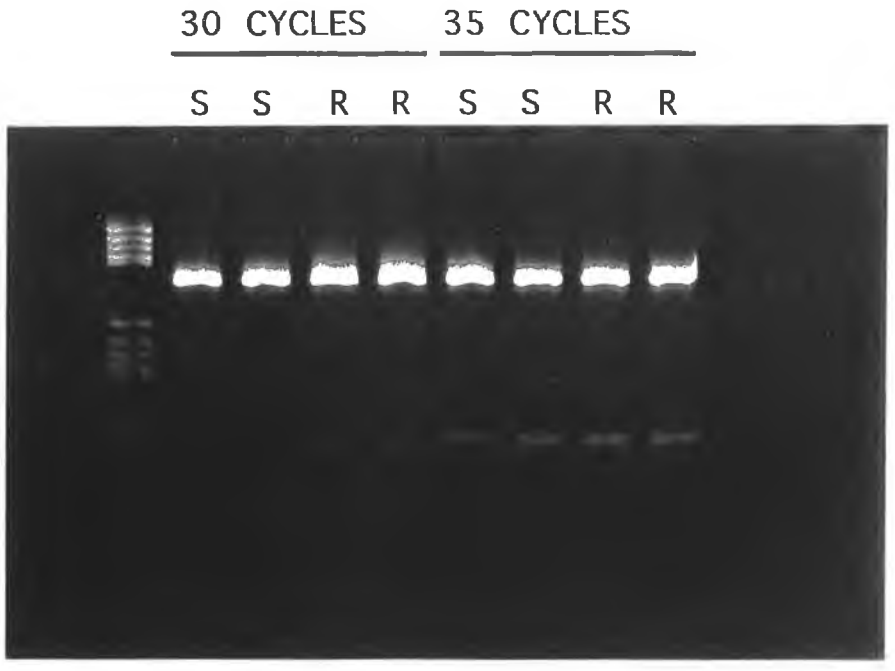
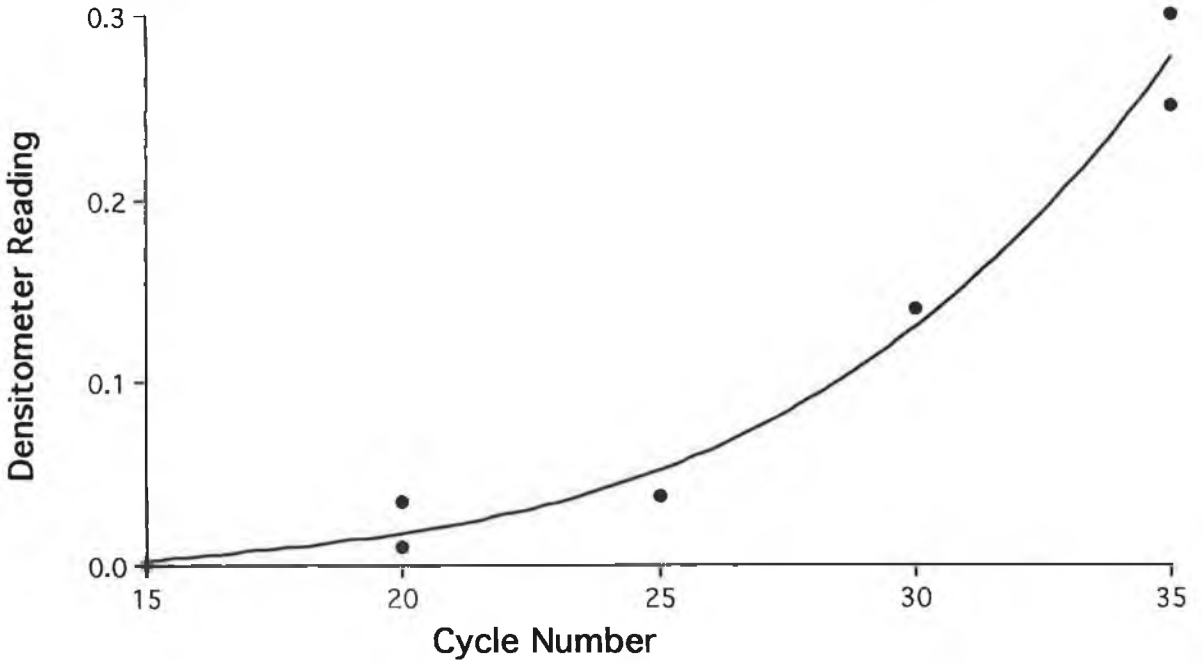
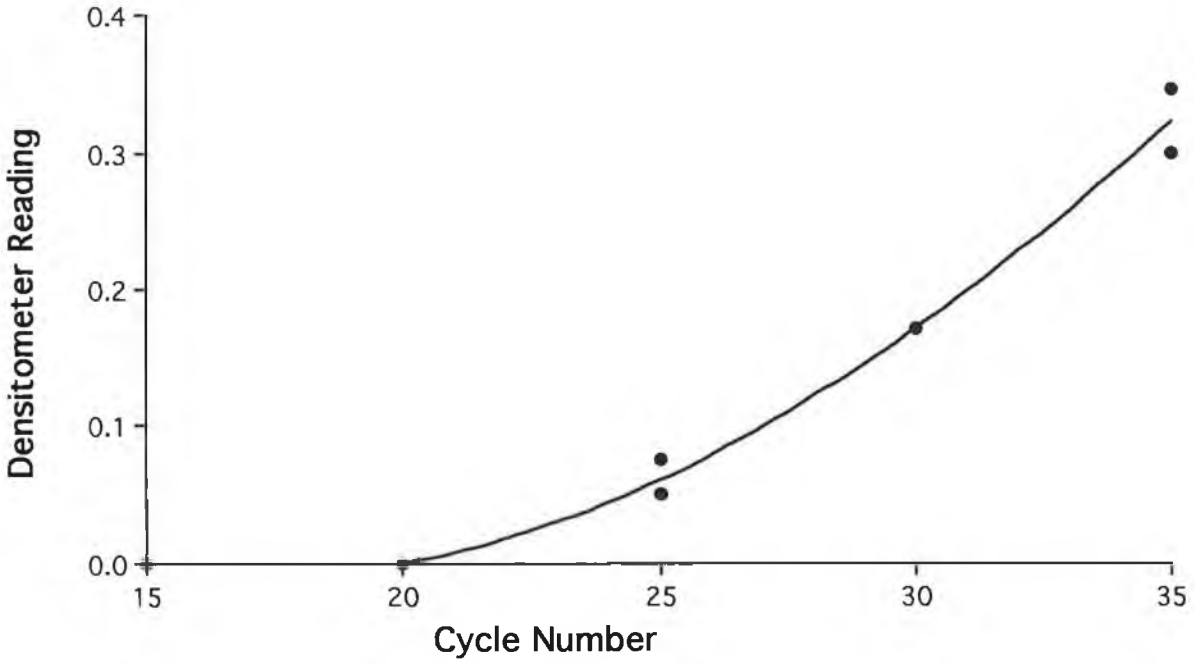


Fig. 3.4.9 Detectable levels of Topoisomerase IIβ mRNA were not produced after 15 or 20 cycles of PCR amplification with either DLKP or DLKPA-derived cDNA. Faint bands representing Topoisomerase IIβ mRNA in DLKPA cDNA were realised after 25 cycles and more intense bands resulted from amplification of both DLKP (S) and DLKPA (R) cDNA, after 30 and 35 PCR cycles. The band intensities were apparently greater in the MDR cell line in comparison to the sensitive cell line.





Figs. 3.4.9.1 and 3.4.9.2 Plots of the relationship between PCR cycle numbers and densitometry readings of the corresponding Topoisomerase IIβ amplified product intensities suggest that the exponential range of product accumulation is between 26 - 35 cycles for DLKP and 25 - 35 cycles of PCR for DLKPA. Product accumulation apparently had not reached a plateau within the PCR cycle range studied. (Experiment 1, Table 3.4.1).

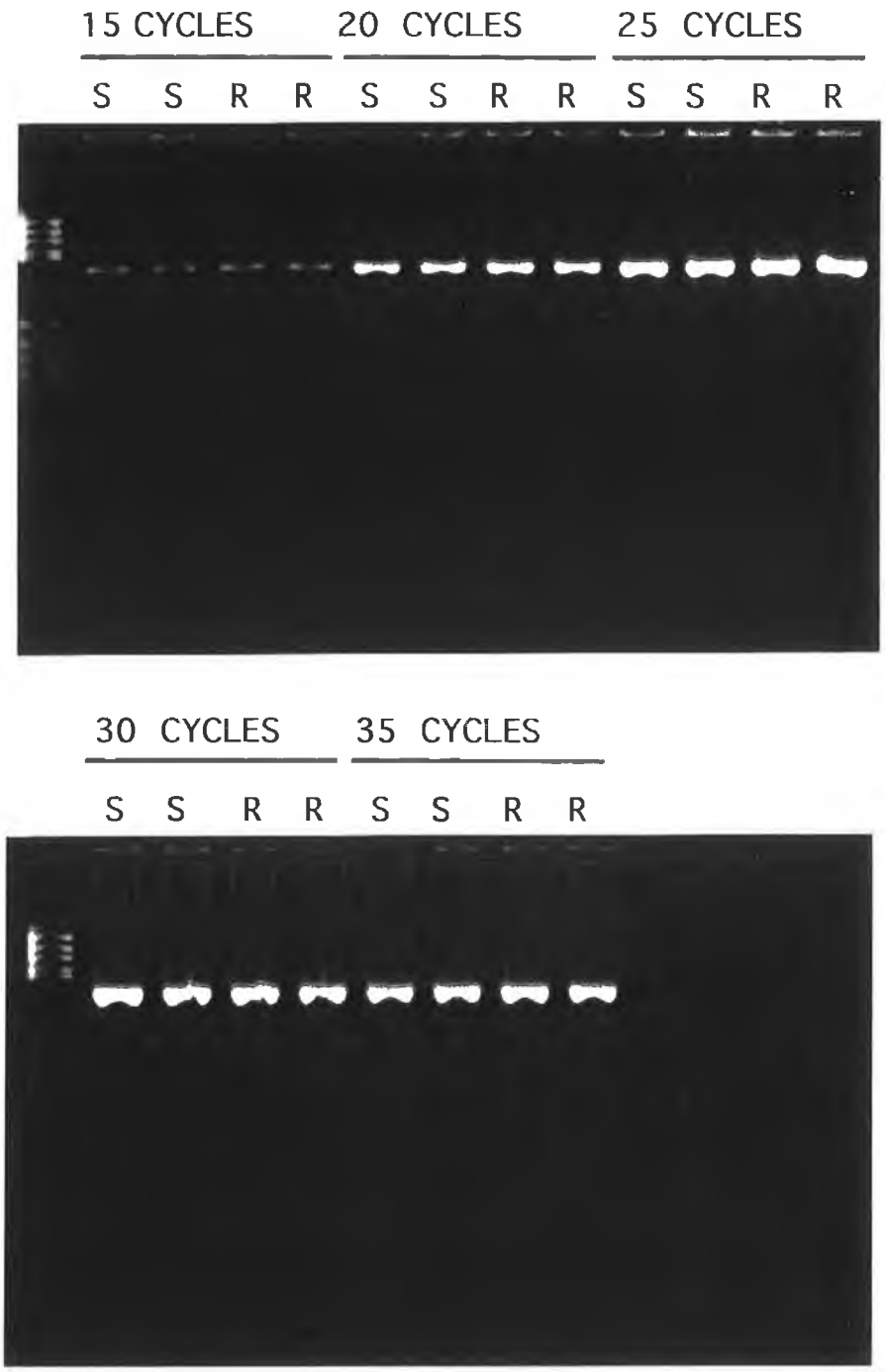
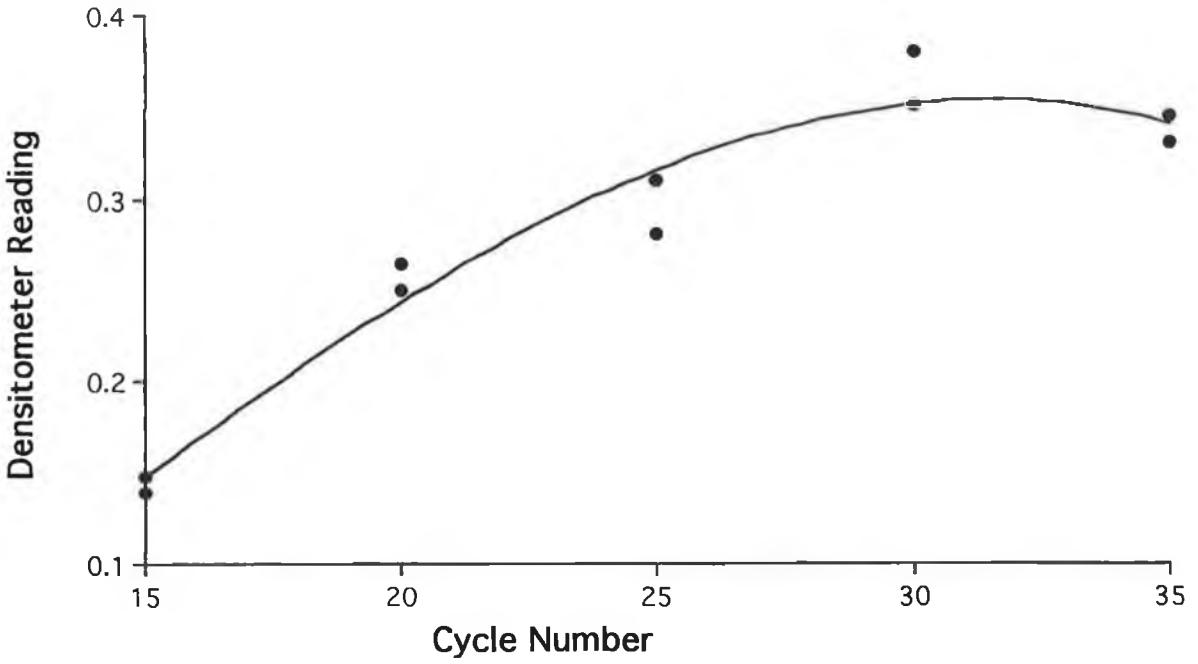
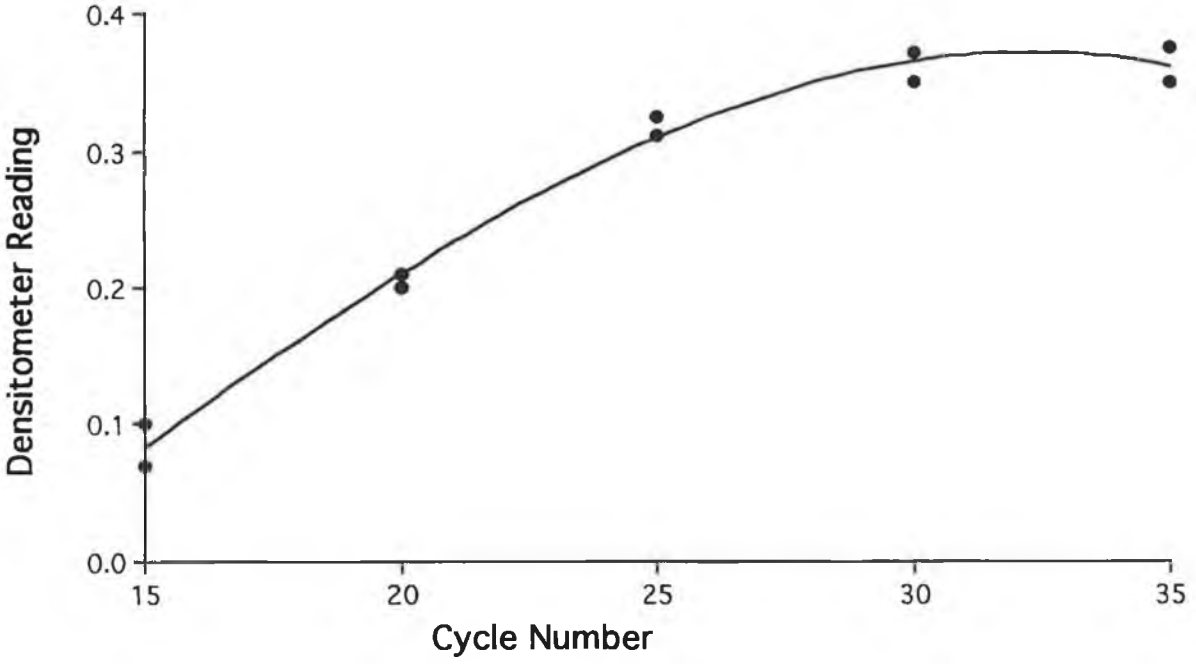


Fig. 3.4.10 When amplified independently, β-actin band intensities increased, with PCR cycle number, from 15 - 30 cycles of PCR amplification. No changes in band intensities were observed with further PCR cycles. After any given cycle number, the band intensities were similar in the drug sensitive, DLKP (S), and resistant, DLKPA (R) cell lines.





Figs. 3.4.10.1 and 3.4.10.2 From the plots of PCR number *versus* densitometry readings of the resulting β-actin bands, it can be estimated that the PCR range of exponential product accumulation is between 16 - 27 cycles, when amplifying both DLKP (Fig. 3.4.10.1) and DLKPA (Fig. 3.4.10.2) derived cDNA. After this, product accumulation reached a plateau, followed by an apparent slight reduction in product levels. (Experiment 1, Table 3.4.1).

Table 3.4.1: Exponential phase estimated for each mRNA region of study from DLKP and DLKPA cells.

Amplified Region	Cell Type	Method *	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
	DLKP	-----	-----	-----	-----	-----	-----	-----
MDR 1	DLKPA	(a)	16 - 21	16 - 21	16 - 23	16 - 24	16 - 22	16 - 22
		(b)	16 - 21	16 - 21	16 - 23	16 - 23	16 - 22	16 - 21
		(c)	16 - 21	16 - 21	16 - 24	16 - 23	16 - 23	16 - 22
MDR 3	DLKP	-----	-----	-----	-----	-----	-----	-----
	DLKPA	(a)	24 - 33	24 - 33	26 - 35	26 - 35	26 - 34	26 - 35
		(b)	24 - 34	25 - 30	26 - 34	25 - 34	26 - 35	25 - 35
		(c)	25 - 36	25 - 36	25 - 34	25 - 36	25 - 35	25 - 36
MRP	DLKP	(a)	21 - 31	21 - 31	20 - 29	20 - 32	22 - 30	21 - 30
		(b)	20 - 29	20 - 32	21 - 35	19 - 30	21 - 29	20 - 30
		(c)	20 - 32	20 - 30	20 - 30	20 - 29	21 - 28	22 - 30
	DLKPA	(a)	20 - 30	20 - 30	20 - 30	19 - 32	20 - 30	20 - 29
		(b)	20 - 29	20 - 30	20 - 29	22 - 29	20 - 29	19 - 31
		(c)	19 - 30	20 - 32	19 - 29	21 - 29	20 - 32	19 - 31
GST π	DLKP	(a)	16 - 22	16 - 22	16 - 24	16 - 24	16 - 22	16 - 23
		(b)	16 - 22	16 - 22	16 - 24	16 - 24	16 - 24	16 - 23
		(c)	16 - 21	16 - 24	16 - 24	17 - 24	16 - 24	16 - 24
	DLKPA	(a)	16 - 23	16 - 23	18 - 26	19 - 26	16 - 24	16 - 23
		(b)	16 - 20	16 - 26	16 - 26	16 - 26	16 - 26	16 - 26
		(c)	16 - 22	16 - 26	17 - 24	17 - 24	16 - 25	16 - 26
CYPIA1	DLKP		-----	-----	-----	-----	-----	-----
	DLKPA		-----	-----	-----	-----	-----	-----
TOPO I	DLKP	(a)	23 - 30	23 - 30	22 - 29	22 - 28	24 - 28	23 - 27
		(b)	23 - 30	23 - 27	23 - 29	23 - 28	24 - 28	24 - 29
		(c)	21 - 29	24 - 28	23 - 28	22 - 29	23 - 27	23 - 28
	DLKPA	(a)	24 - 31	24 - 31	24 - 28	23 - 27	22 - 27	22 - 27
		(b)	25 - 30	24 - 27	23 - 28	22 - 27	23 - 29	22 - 29
		(c)	21 - 30	23 - 29	22 - 28	22 - 28	23 - 29	22 - 28
TOPO II	DLKP	(a)	20 - 29	20 - 31	21 - 31	21 - 30	20 - 29	20 - 28
		(b)	20 - 31	20 - 31	22 - 31	22 - 31	23 - 31	22 - 30
		(c)	20 - 29	21 - 31	22 - 31	22 - 31	24 - 31	22 - 30
	DLKPA	(a)	20 - 29	21 - 30	22 - 30	22 - 29	22 - 31	21 - 29
		(b)	21 - 29	21 - 31	21 - 29	21 - 28	24 - 30	24 - 29
		(c)	21 - 29	20 - 30	22 - 30	22 - 29	24 - 30	23 - 31
TOPO IIα	DLKP	(a)	21 - 29	21 - 30	22 - 31	22 - 31	23 - 30	22 - 29
		(b)	23 - 29	22 - 29	22 - 29	23 - 29	23 - 29	22 - 29
		(c)	21 - 29	23 - 29	20 - 28	21 - 30	22 - 29	22 - 30
	DLKPA	(a)	21 - 29	21 - 29	22 - 31	21 - 29	21 - 29	21 - 29
		(b)	24 - 33	23 - 29	23 - 30	23 - 29	22 - 29	23 - 30
		(c)	22 - 29	21 - 30	21 - 29	22 - 29	21 - 29	22 - 29
TOPO IIβ	DLKP	(a)	26 - 35	26 - 35	25 - 34	24 - 35	27 - 32	27 - 31
		(b)	26 - 35	26 - 33	24 - 34	24 - 35	27 - 33	26 - 32
		(c)	26 - 32	26 - 32	25 - 35	24 - 37	26 - 33	26 - 31
	DLKPA	(a)	25 - 35	25 - 35	24 - 33	23 - 35	26 - 32	26 - 33
		(b)	25 - 32	25 - 37	23 - 33	23 - 35	26 - 32	25 - 33
		(c)	25 - 32	25 - 36	23 - 34	22 - 35	24 - 34	24 - 35
β-Actin	DLKP	(b)	16 - 26	16 - 27	16 - 26	16 - 27	16 - 26	16 - 26
		(c)	16 - 26	16 - 26	16 - 26	16 - 26	16 - 27	16 - 25
	DLKPA	(b)	16 - 26	16 - 27	16 - 27	16 - 26	16 - 26	16 - 26
		(c)	16 - 27	16 - 27	16 - 26	16 - 26	16 - 26	16 - 26

Note:
----- = no bands detected.
Method * = Method of Calculation (see 3.4).

Table 3.4.2: Exponential phase estimated for each mRNA region of study from OAW42 and OAW42A cells.

Amplified Region	Cell Type	Method *	Expt. 1	Expt. 2	Expt. 3	Expt. 4
MDR 1	OAW42	(a)	21 - 31	21 - 31	21 - 31	21 - 31
		(b)	21 - 30	20 - 30	21 - 30	21 - 30
		(c)	20 - 33	20 - 32	20 - 33	20 - 33
	OAW42A	(a)	19 - 29	20 - 29	20 - 29	20 - 29
		(b)	19 - 29	21 - 29	20 - 29	20 - 29
		(c)	19 - 29	21 - 29	20 - 29	20 - 29
MDR 3	OAW42		-----	-----	-----	-----
	OAW42A		-----	-----	-----	-----
MRP	OAW42	(a)	21 - 30	21 - 30	21 - 30	21 - 30
		(b)	22 - 29	22 - 30	22 - 30	21 - 29
	OAW42A	(c)	22 - 31	22 - 29	22 - 31	22 - 31
		(a)	21 - 31	21 - 31	21 - 31	21 - 31
		(b)	21 - 29	21 - 29	21 - 29	22 - 30
		(c)	21 - 30	22 - 31	21 - 29	22 - 30
GST π	OAW42	(a)	16 - 22	16 - 22	16 - 22	16 - 22
		(b)	16 - 20	16 - 22	16 - 24	16 - 23
		(c)	16 - 21	16 - 20	16 - 23	16 - 23
	OAW42A	(a)	16 - 22	16 - 22	16 - 24	16 - 24
		(b)	16 - 22	16 - 22	16 - 23	16 - 22
		(c)	16 - 24	16 - 21	16 - 23	16 - 21
CYPIA1	OAW42		-----	-----	-----	-----
	OAW42A		-----	-----	-----	-----
TOPO I	OAW42	(a)	22 - 27	22 - 27	22 - 27	22 - 27
		(b)	22 - 27	22 - 28	21 - 28	21 - 27
		(c)	22 - 28	21 - 27	21 - 28	21 - 27
	OAW42A	(a)	22 - 28	22 - 28	22 - 28	22 - 28
		(b)	23 - 26	23 - 26	22 - 28	22 - 27
		(c)	22 - 28	22 - 28	22 - 28	22 - 28
TOPO II	OAW42	(a)	16 - 25	16 - 25	16 - 25	16 - 25
		(b)	16 - 24	16 - 25	16 - 25	17 - 25
		(c)	16 - 23	17 - 23	16 - 24	17 - 23
	OAW42A	(a)	18 - 26	18 - 26	18 - 26	18 - 26
		(b)	18 - 26	18 - 25	17 - 25	18 - 26
		(c)	18 - 25	17 - 25	17 - 25	17 - 25
TOPO IIα	OAW42	(a)	19 - 28	19 - 28	19 - 28	19 - 28
		(b)	16 - 32	18 - 31	19 - 30	19 - 30
		(c)	21 - 32	20 - 31	20 - 30	19 - 30
	OAW42A	(a)	16 - 29	19 - 29	19 - 29	19 - 29
		(b)	21 - 29	20 - 29	20 - 30	20 - 30
		(c)	21 - 29	19 - 29	20 - 30	20 - 30
TOPO IIβ	OAW42	(a)	26 - 35	26 - 35	26 - 35	26 - 35
		(b)	26 - 35	26 - 35	26 - 35	26 - 35
		(c)	26 - 34	26 - 35	26 - 34	26 - 35
	OAW42A	(a)	26 - 35	26 - 35	26 - 34	26 - 34
		(b)	26 - 35	26 - 36	26 - 34	26 - 35
		(c)	26 - 36	26 - 35	26 - 35	26 - 35
β-Actin	OAW42	(b)	16 - 27	16 - 27	16 - 27	16 - 27
		(c)	16 - 27	16 - 26	16 - 27	16 - 26
	OAW42A	(b)	16 - 26	16 - 26	16 - 27	16 - 26
		(c)	16 - 27	16 - 26	16 - 26	16 - 27

Note:
----- = no bands detected;
Method * = Method of Calculation (see 3.4).

As these results were fairly reproducible, they can be summed up as in Table 3.4.3. Although the ranges when amplification of product remains exponential varies to some degree from cell line to cell line, and to a lesser extent from sensitive to MDR variant; with the exception of MDR 1, the "optimum" cycle number can be considered to be the same for each of the cell lines studied. (Of course, many other cycle numbers within this range could possibly be performed with similar success).

With the MDR 1 primers, although 20 and 25 cycles may be the time points of choice when amplifying the lung and ovarian cell lines, respectively, 20 cycles could, in fact, be performed quite successfully for each of the cell types. These results, therefore, suggest that the proposed "optimum" numbers of cycles may be suitable for studying the relevant transcript levels in a range of other cell types. Furthermore, these results support the choice of β-actin as an internal control. The exponential range of PCR amplification for β-actin cDNA spans the proposed "optimum" cycle numbers for all of the other parameters under study. As required (1.4.1), the internal control should not have reached a plateau before the target cDNA of interest so that the analysis is completed within the PCR exponential phase of product accumulation.

Table 3.4.3

	DLKP	DLKPA	Optimum Cycle No.	OAW42	OAW42A	Optimum Cycle No.
MDR1	-------	16 - 23	20	19 - 33	19 - 29	25
MDR3	-------	24 - 35	27	-------	-------	-------
MRP	20 - 32	20 - 32	25	20 - 32	20 - 32	25
GST π	16 - 24	16 - 26	20	16 - 24	16 - 24	20
CYPIA1	-------	-------	-------	-------	-------	-------
Topo I	21 - 30	21 - 30	25	22 - 38	22 - 38	25
Topo II	20 - 31	20 - 31	25	16 - 26	16 - 26	25
Topo IIα	21 - 30	21 - 30	25	21 - 30	21 - 30	25
Topo IIβ	26 - 35	25 - 35	27	26 - 35	26 - 35	27
β-Actin	16 - 27	16 - 27		16 - 27	16 - 27	

Results from this phase of the experiment emphasise the importance of working within the exponential range of amplification for each particular pair of primers and RNA type. This is supported *e.g.* when studying Topoisomerase I transcript levels in DLKP and DLKPA cell lines. At 25 cycles, differences in Topoisomerase I mRNA levels were apparent in the DLKP and DLKPA cell lines, in two of the three independent RNA transcripts (*i.e.* four of six experiments), but only in one of three (*i.e.* two of six experiments) at 30 cycles. This also emphasises the importance of doing many repeat experiments (as with many techniques), to prove the reliability of these findings.

When plotted as cycle number *versus* intensity of band, in most cases the resulting graph was indicative of an exponential and plateau phase of product accumulation (Figs. 3.4.1.1. - 3.4.10.2). However, in some cases, the plateau did not seem to be maintained, but an apparent decreases in product amounts were apparent *e.g.* when studying β-actin levels (Fig. 3.4.10.1). This was possibly due to product degradation. Similar results were reported by Douglas and Atchison (1993) who, when amplifing a fragment of the apo AI gene set up a number of replicate

samples, subjecting them to different lengths of the denaturation temperature (94 ^{o}C).

The most significant overall trend from these results (when working within the exponential phase) is a very significant increase in MDR 1 mRNA levels in DLKPA, by comparison to DLKP. This was seen, also, for OAW42A *versus* OAW42, but to a lesser extent. The results also imply that the levels of Topoisomerase II mRNA are reduced in both resistant variants in comparison to their sensitive parent cell line. This seems to be due to a reduced Topoisomerase IIα transcript level and may be partially counter-balanced by a slight increase in Topoisomerase IIβ levels. MRP mRNA levels seem to be slightly increased in the resistant variant of the lung cell line, but unchanged in the ovarian cell line; whereas GST π is apparently slightly increased in OAW42A compared to OAW42, but not obviously affected in the multiple drug resistant lung cell line in comparison to its parent line. The results from the Topoisomerase I study are inconclusive for DLKP and DLKPA. A slight decrease in Topoisomerase I transcript level with multiple drug resistance was suggested from studies on two RNA extracts. This observation was not backed up by studies on a third. In all studies of the ovarian cell lines, however, Topoisomerase I mRNA levels were reduced with multiple drug resistance.

3.5 Semi-Quantitative Analysis of MDR 1 and Topoisomerase II in DLKP and its Resistant Variants.

3.5.1(i) Semi-Quantitative Analysis - within and between experiments.

As detailed in 3.4, preliminary studies of gene transcript levels of MDR-related factors suggest that the most obvious differences between the lung carcinoma cell line, DLKP, and its resistant variant, DLKPA, involve MDR 1 and Topoisomerase II gene products. Although other differences were noted, they were not as consistent or as obvious and so possibly not as fundamentally significant. A semi-quantitative study was, therefore, attempted to establish the fold-differences of MDR 1, Topoisomerase II and the Topoisomerase IIα subunit, independently, between a number of multiple drug resistant variants of the lung carcinoma cell line, DLKP.

DLKPA, an adriamycin selected MDR variant of DLKP, consists of a heterogenous population of cells from which a number of independent MDR cell lines, representing individual homogenous populations with differing toxicity profiles have been cloned. The resulting cell lines included in this phase of the study - DLKPA-2B, DLKPA-6B and DLKPA-5F were cloned from the heterogenous population (by Dr. Mary Heenan). DLKP/VP-3 and DLKP/VP-8, two other resistant variants of DLKP selected to different concentrations of VP-16 (by Dr. Heenan), which also have different MDR profiles, were also included in this analysis.

MDR 1 gene transcript levels were studied in the following cell lines: DLKPA, DLKPA-2B, DLKPA-6B, DLKPA-5F, DLKP/VP-3 and DLKP/VP-8. The sensitive cell line (DLKP) was excluded from this analysis as previous experimental results suggested that it contains no detectable levels of MDR 1 mRNA. Similar attempts to investigate Topoisomerase II and Topoisomerase IIα mRNA levels in DLKP, DLKPA, DLKP/VP-3 and DLKP/VP-8 cells were conducted.

In all cases, analysis was performed on three concentrations of RNA extracted from the cell lines of interest *i.e.* 0.5 μg/μl, 1.0 μg/μl and 2.0 μg/μl. RT and PCR reactions were carried out as previously described (2.10.4 & 2.10.5), using equal volumes of cDNA formed from the three RNA stocks, in each PCR reaction. Samples from all MDR 1 amplifying tubes were removed at 15, 20, 25 and 30 cycles, and at 20, 25 and 30 cycles when amplifying Topoisomerase II or Topoisomerase IIα derived cDNA, respectively. This analysis was done for MDR 1, Topoisomerase II and Topoisomerase IIα and each of the relevant cell lines (mentioned above) a total of four times *i.e.* on duplicate stocks of cDNA formed from two RNA extracts of independent cell line stocks. All reaction tubes were set up in duplicate.

Results from this analysis are shown in Figs. 3.5.1.1 - 3.5.1.6 for MDR 1 studies of DLKPA, DLKPA-2B, DLKPA-6B, DLKPA-5F, DLKP-VP-3 and DLKP-VP/8, respectively. In each Fig., E1/R1 E1/R2, E2/R1 and E2/R2 represent the two RNA extracts and the duplicate analysis of both extracts *e.g.* E1/R1 (RNA extract no. 1; 1st RT-PCR analysis), *etc.* The results from the densitometric analysis are presented after subtraction of background and normalising with β-actin, in tabulated form. In all cases, densitometry analysis was done on negatives, to prevent the inclusion of bias resulting from differential printing. The results for each of the four analyses (*i.e.* two independent studies on two independent extracts) were plotted as RNA concentration *versus* densitometrical reading at 20 cycles (as this has previously been proposed as a suitable cycle number at which to study MDR 1 mRNA levels in DLKP variants (see 3.4)). Similarly, the results from the Topoisomerase II and Topoisomerase IIα analysis are presented in Figs. 3.5.1.7 - 3.5.1.10 and 3.5.1.11 - 3.5.1.14, respectively. In these cases, however, the plotted graphs of RNA concentration *versus* densitometry reading represent the product formed for each of the three RNA concentrations at 25 cycles. This cycle number was chosen as this has been shown (see 3.4) to be within the exponential range of PCR product accumulation when amplifying

cDNA derived from DLKP variants, with either the Topoisomerase II or Topoisomerase IIα primers listed.

With both increasing cycle number (*i.e.* 15, 20, 25 and 30 cycles) and increasing cDNA concentration (based on increasing concentrations of RNA - 0.5 μg/μl, 1.0 μg/μl and 2.0 μg/μl - from which cDNA was formed), increasing intensities of bands were seen for all of the cell lines studied, with few exceptions. This indicates that different RNA concentrations are reflected in the resulting bands. However, according to the tabulated densitometry readings, in all cases, the intensities of the bands produced, although linearly related, were not proportional to the initial quantities of RNA. Although it is difficult to accurately judge this relationship by eye, as each band must be normalised with β-actin, following subtraction of background, it seemed that in some situations this relationship was apparent from the gel photographs. This, however, was not reflected in the densitometry readings. Examples of this include DLKPA (Fig. 3.5.1.1 (E2/R1)), DLKPA-2B ((Fig. 2.5.1.2 (E1/R1) and (E1/R2)), DLKPA-6B (Fig. 3.5.1.3 (E1/R1) and (E2/R1)), DLKPA-5F (Fig. 3.5.1.4 (E2/R1) and (E2/R2)), DLKP/VP-3 (Fig. 3.5.1.5 (E1/R2) and (E2/R1)) and DLKP/VP-8 (Fig. 3.5.1.6 (E1/R1) and (E2/R2), when visualised after 20 cyles of PCR. (The bands produced on analysis of DLKPA, DLKP/VP-3 and DLKPA-5F are relatively strong at 20 cycles and so this can be estimated more easily (and possibly more accurately) after 25 cycles of PCR amplification. This point is illustrated in Figs. 3.5.1.1, 3.5.1.4 and 3.5.1.5). Results from the densitometry analysis suggest that for DLKPA, DLKPA-5F and DLKP/VP-3, the band intensities increase from 15 - 25 cycles (as normal), but then decrease from 25 - 30 cycles. This is not obvious from the gels represented by this data. The fact that this trend was not seen with other cell lines studied *e.g.* DLKPA-2B and DLKPA-6B, may be because their product accumulations have not reached a plateau to the same extent as DLKPA, DLKPA-5F and DLKP/VP-3, which seem to have greater levels of MDR 1 mRNA present. The apparent decrease in intensity may be due to product degradation.

Results from Topoisomerase II (Figs. 3.5.1.7 - 3.5.1.10) and Topoisomerase IIα mRNA transcripts (Figs. 3.5.1.11 - 3.5.1.14) similarly suggest a correlation between RNA concentrations and resulting densitometry readings. As with the MDR 1 studies, however, the densitometry analysis of band intensity did not correlate proportionally with the initial RNA concentrations, although in many cases, by visual analysis, such a correlation could possibly be made. An example of this is Fig. 3.5.1.7 where increasing band intensities were performed in proportion to the starting RNA concentrations. This increase was most obvious between the three RNA concentrations after 20 cycles, when fold-differences were apparent. Differences in relative band intensities were also observed at 25 cycles, whereas at 30 cycles, the differences were very slight.

These results of MDR 1, Topoisomerase II and Topoisomerase IIα mRNA studies, in all cell lines included, suggest a limitation at some stage(s) of the procedure of cDNA formation, product amplification by PCR, or analysis of resulting products by densitometry. Before proceeding to establish the efficiencies of the RT and PCR reactions using a range of concentrations of RNA and resulting cDNA, respectively, a series of studies was performed to detect any possible limitations of the densitometry analysis.

3.5.1(ii) Semi-Quantitative Analysis within an Experiment

The products of MDR 1, Topoisomerase II and Topoisomerase IIα mRNA analysis from each cell line studied were ran on individual agarose gels (3.5(i)) so that attempts to correlate differences in band intensities with the initial RNA concentrations, after specific PCR cycle numbers, could be made. It seemed, however, that although the densitometry readings may be relatively accurate within a given gel, data from different gels could not be accurately compared. For this reason, it was necessary to study further samples from each extract of the cell line for comparisons between cell lines be made. 1.0 $\mu g/\mu l$ RNA

from DLKPA, DLKPA-2B, DLKPA-6B, DLKPA-5F, DLKP/VP-3 and DLKP/VP-8 were used as template in this analysis and 20 cycles of PCR amplification of MDR 1 cDNA were performed. The results are shown in Fig. 3.5.1.15 and Table 3.5.1.15.1. Similarly, 1.0 $\mu g/\mu l$ RNA from DLKP, DLKPA, DLKP/VP-3 and DLKP/VP-8 cDNAs were analysed together to establish if significant differences existed between their Topoisomerase IIα levels (Fig. 3.5.1.16 and Table 3.5.1.16.1). These studies were performed a total of four times for MDR 1 and for Topoisomerase IIα *i.e.* two repeat studies on two RNA extracts.

From the results of MDR 1 mRNA studies in adriamycin-selected DLKP variants after analysis by densitometry, it seems that the levels of this gene transcript in rank order are as follows: DLKPA > DLKPA-5F > DLKP/VP-3 > DLKP/VP-8 > DLKPA-6B > DLKPA-2B. The differences in densitometry readings between these bands, however, were small. By visual analysis, the MDR 1 transcript levels were very similar, if not equal, in DLKPA, DLKPA-5F, DLKP/VP-3 and DLKP/VP-8. The rank order appeared to be: DLKPA = DLKPA-5F = DLKP/VP-3 = DLKP/VP-8 > DLKPA-6B > DLKPA-2B. In one study (E1/R2), observed both by densitometry and visual analysis, the levels in DLKPA-2B were apparently greater than in DLKPA-6B. This may possibly be an artifact, resulting from inconsistent printing. Differences in band intensities were more visible when the bands were not printed very strong. With the exception of the gels produced as E1/R2, the band intensities seemed to be increased approximately (visually) 1.5-fold in DLKPA-6B cells, compared to DLKPA-2B, and approximately 1.5-fold from DLKPA-6B to DLKPA, DLKPA-5F, DLKP/VP-3 and DLKP/VP-8. Differences of at least 2 - 3 fold were therefore visible between the least resistant and most resistant cell lines.

Analysis, by densitometry, of mRNA levels which encode the α subunit of Topoisomerase II enzyme suggested that the Topoisomerase IIα transcript levels were reduced in each of the MDR variants, by comparison to DLKP (Fig. 3.5.1.16). The densitometry readings, however, did not differ greatly (Table 3.5.1.16.1). No real differences in Topoisomerase IIα levels between these cell lines were observed by visual analysis.

Figs. 3.5.1.1 - 3.5.1.6 MDR 1 mRNA levels in DLKPA, DLKPA-2B, DLKPA-6B, DLKPA-5F, DLKP/VP-3 and DLKP/VP-8 were studied by semi-quantitative PCR analysis using a range of three RNA concentrations (0.5 μg/μl, 1.0 μg/μl and 2.0 μg/μl). This was done to establish if the resulting MDR 1 bands were indicative of the corresponding starting RNA concentrations. 100 μl PCR sample volumes were set up to amplify MDR 1 cDNA, the target gene transcript. β-actin, acting as an endogenous control and as a means to normalise the intensity of the MDR 1 band, was co-amplified with the MDR 1 cDNA. 10 μl aliquots were removed after 15, 20, 25 and 30 cycles of amplification. The resulting products were separated by gel electrophoresis through a 4 % gel and were identified with respect to molecular weight markers (left-hand lane in each gel) ranging from 587 bp to 8 bp in size.

This analysis was performed for each of the cell lines (mentioned above) a total of four times *i.e.* on duplicate stocks of cDNA formed from two RNA extracts of independent cell line stocks - designated E1/R1, E1/R2, E2/R1 and E2/R2. All reactions were performed in duplicate.

Tables 3.5.1.1.1 - 3.5.1.6.1 MDR 1 band intensities (Figs. 3.5.1.1 - 3.5.1.6, respectively) were analysed by densitometry. The resulting data was corrected by subtracting background readings and normalising with β-actin (the endogenous control).

Figs. 3.5.1.1.2 - 3.5.1.6.2 Relationship between the RNA concentrations and the corrected densitometry readings of the bands resulting after gel electrophoresis of the RT-PCR products (Figs. 3.5.1.1 - 3.5.1.6). The data plotted represents the bands produced after 20 cycles of PCR amplification, as this was found to be within the exponential phase of product accumulation with this primer pair and so was suggested as a suitable cycle number at which analysis could be performed (see 3.4).

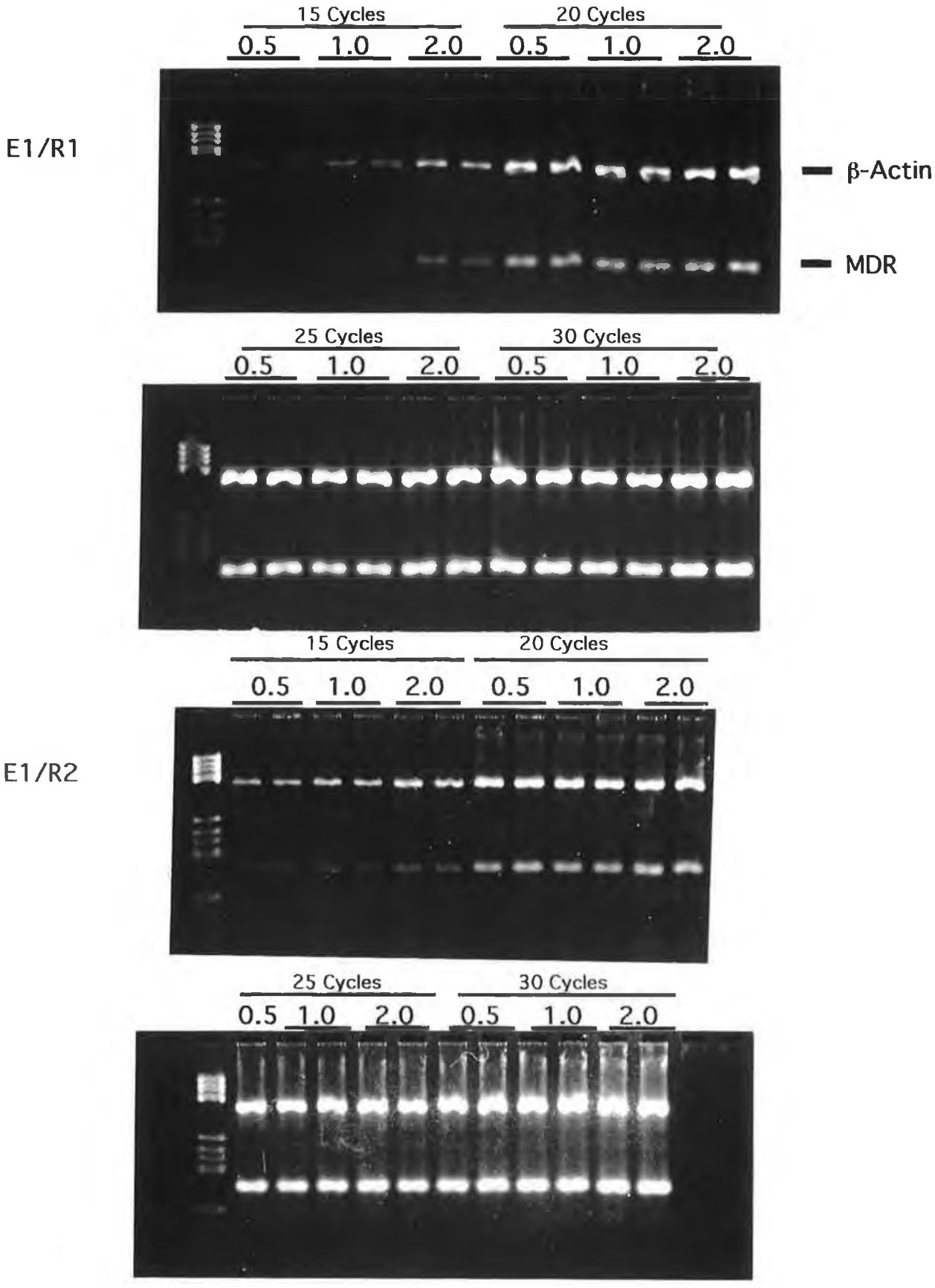
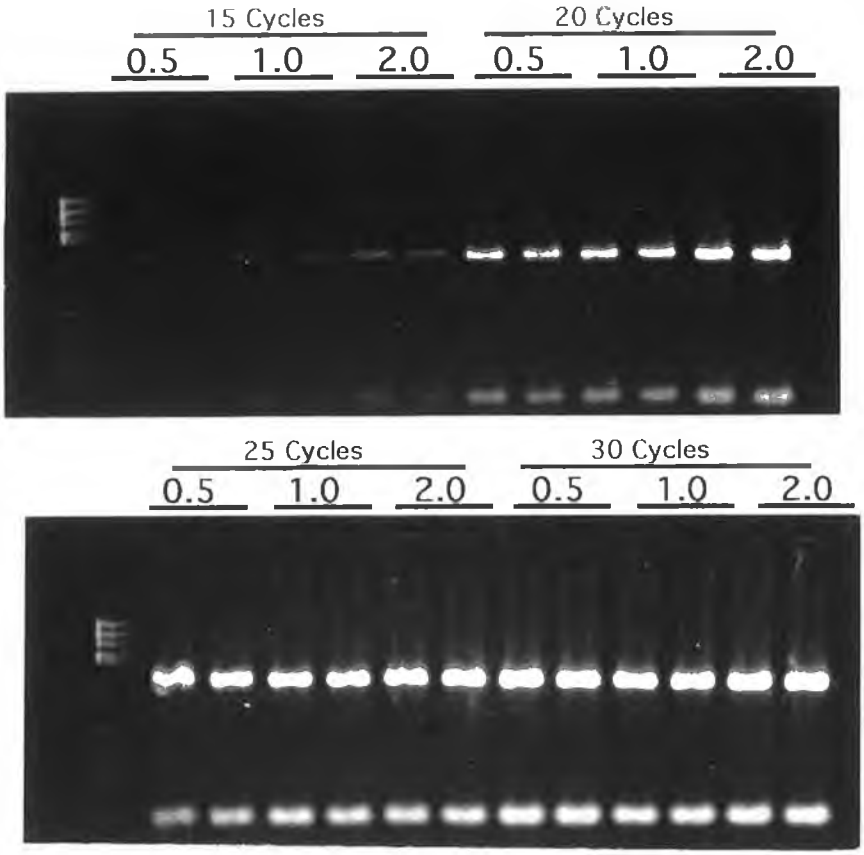


Fig. 3.5.1.1 Gel electrophoresis of the products of MDR 1 and β-actin mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKPA cultured cells and removing samples after four different PCR cycle time points. This analysis was done a total of four times.

E2/R1



E2/R2

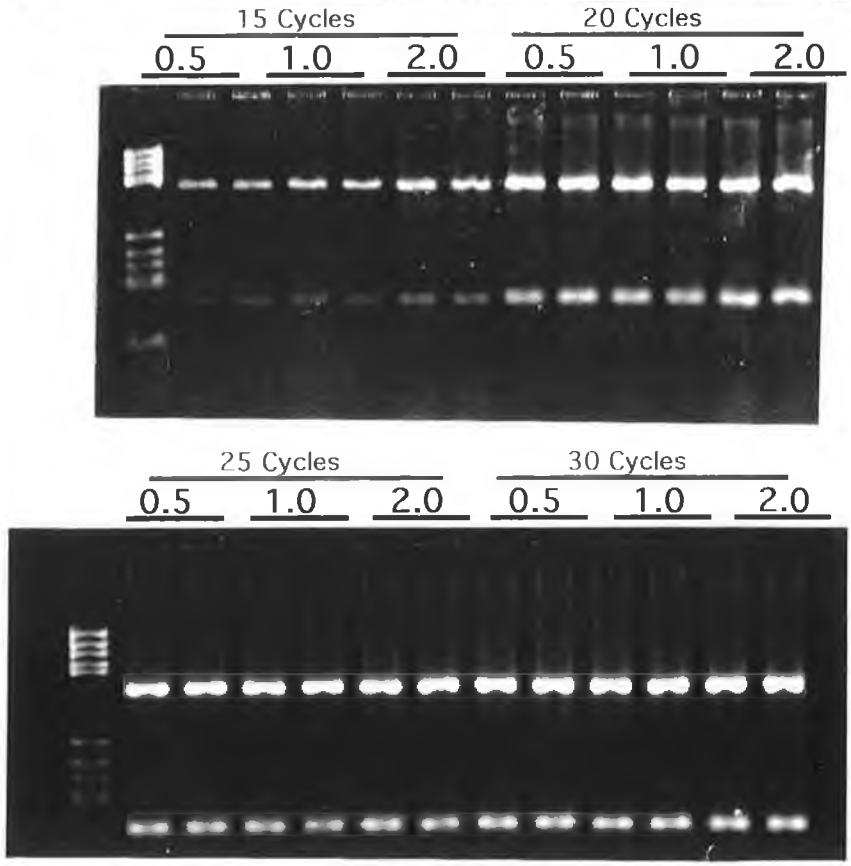


Fig. 3.5.1.1. CONTINUED

MDR 1 (DLKPA)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15	0.417	0.584	0.600
	20	0.592	0.607	0.700
	25	0.873	0.915	1.004
	30	0.873	0.876	0.946
E1 / R2	15	0.500	0.585	0.667
	20	0.584	0.654	0.727
	25	0.785	0.947	0.948
	30	0.869	0.838	0.941
E2 / R1	15	0.286	0.400	0.410
	20	0.530	0.641	0.671
	25	0.798	0.840	0.903
	30	0.733	0.786	0.846
E2 / R2	15	0.184	0.215	0.225
	20	0.500	0.560	0.631
	25	0.757	0.852	0.893
	30	0.845	0.877	0.857

Table 3.5.1.1.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA, as in Fig. 3.5.1.1

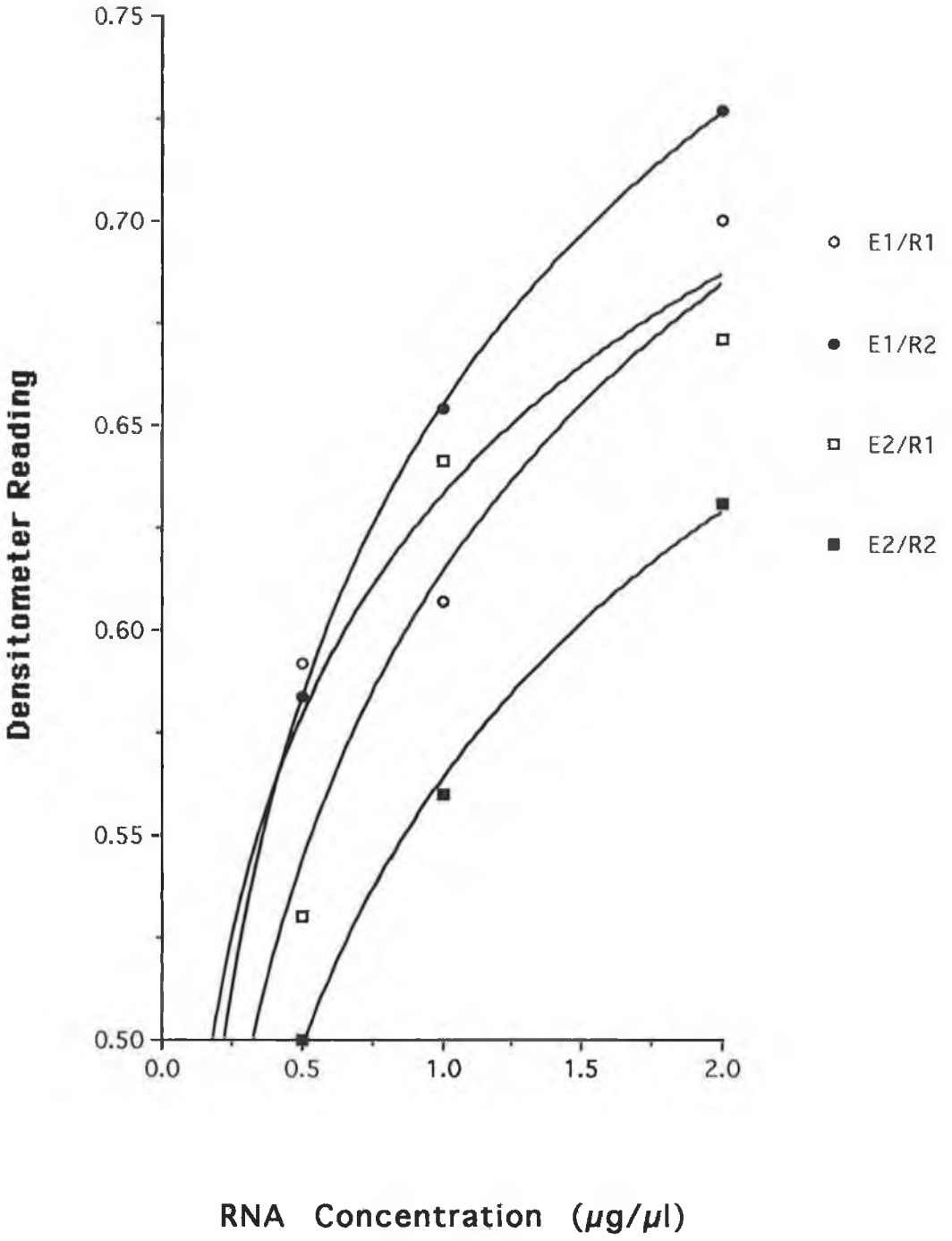


Fig. 3.5.1.1.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 20 cycles of amplification of MDR 1 cDNA derived from DLKPA cultured cells.

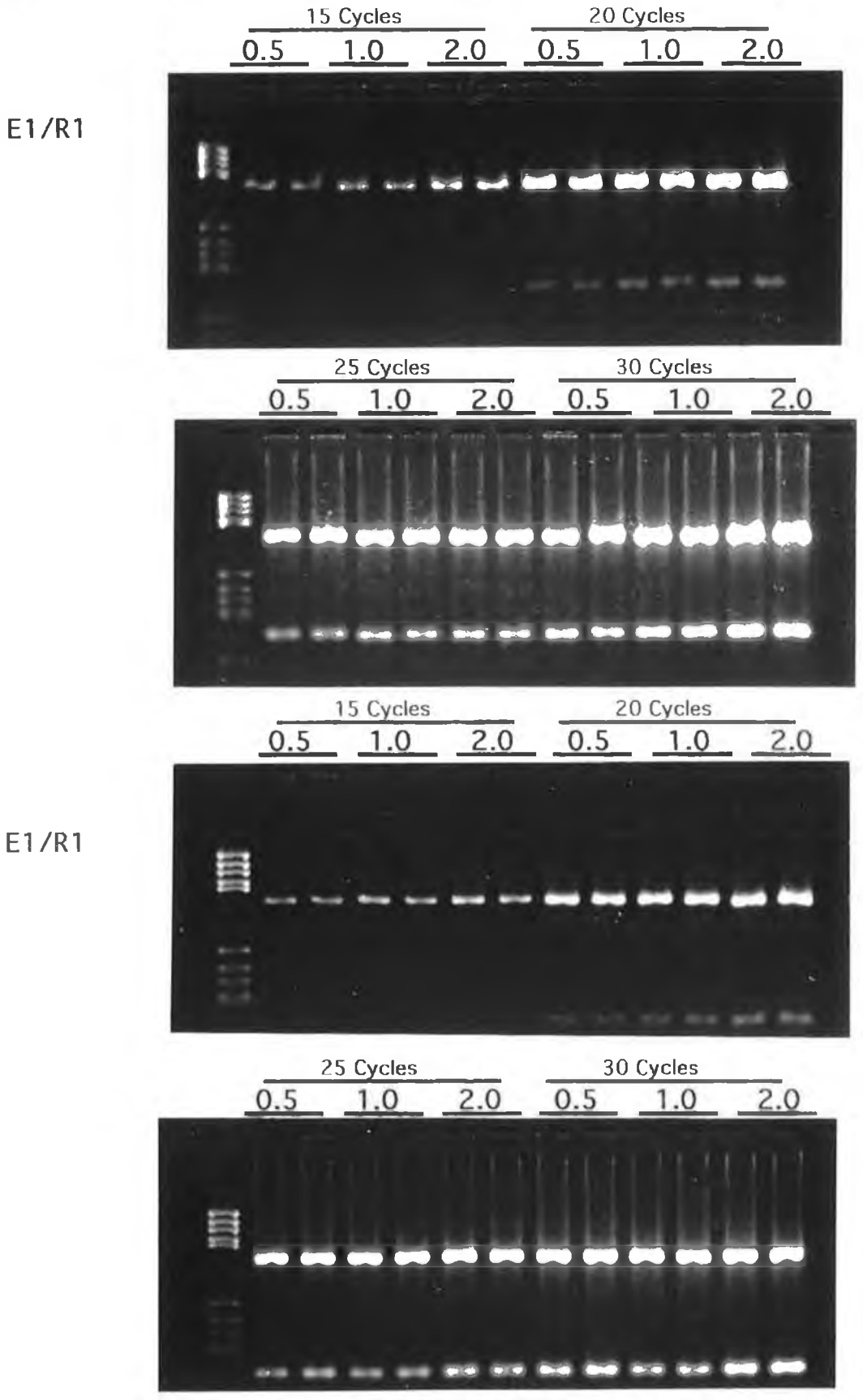


Fig. 3.5.1.2 Gel electrophoresis of the products of MDR 1 and β-actin mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKPA-2B cultured cells and removing samples after four different PCR cycle time points. This analysis was done a total of four times.

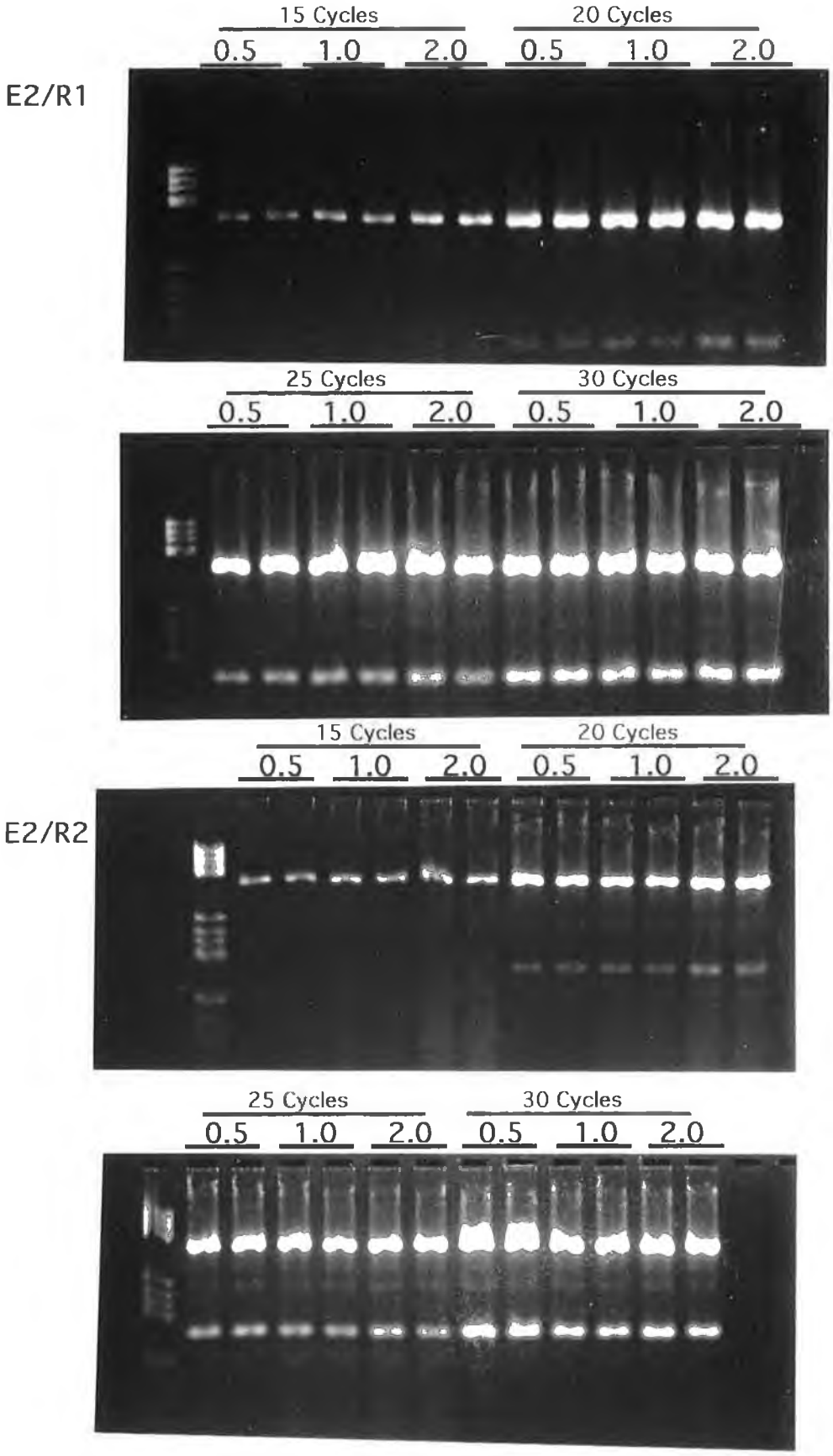


Fig. 3.5.1.2 CONTINUED

MDR 1 (DLKPA-2B)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15	0.000	0.000	0.000
	20	0.161	0.181	0.240
	25	0.488	0.534	0.549
	30	0.561	0.578	0.605
E1 / R2	15	0.000	0.000	0.000
	20	0.143	0.149	0.241
	25	0.500	0.636	0.644
	30	0.655	0.775	0.787
E2 / R1	15	0.000	0.000	0.000
	20	0.087	0.161	0.182
	25	0.379	0.456	0.536
	30	0.657	0.666	0.781
E2 / R2	15	0.000	0.000	0.000
	20	0.085	0.089	0.130
	25	0.400	0.414	0.500
	30	0.595	0.613	0.658

Table 3.5.1.2.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-2B, as in Fig. 3.5.1.2

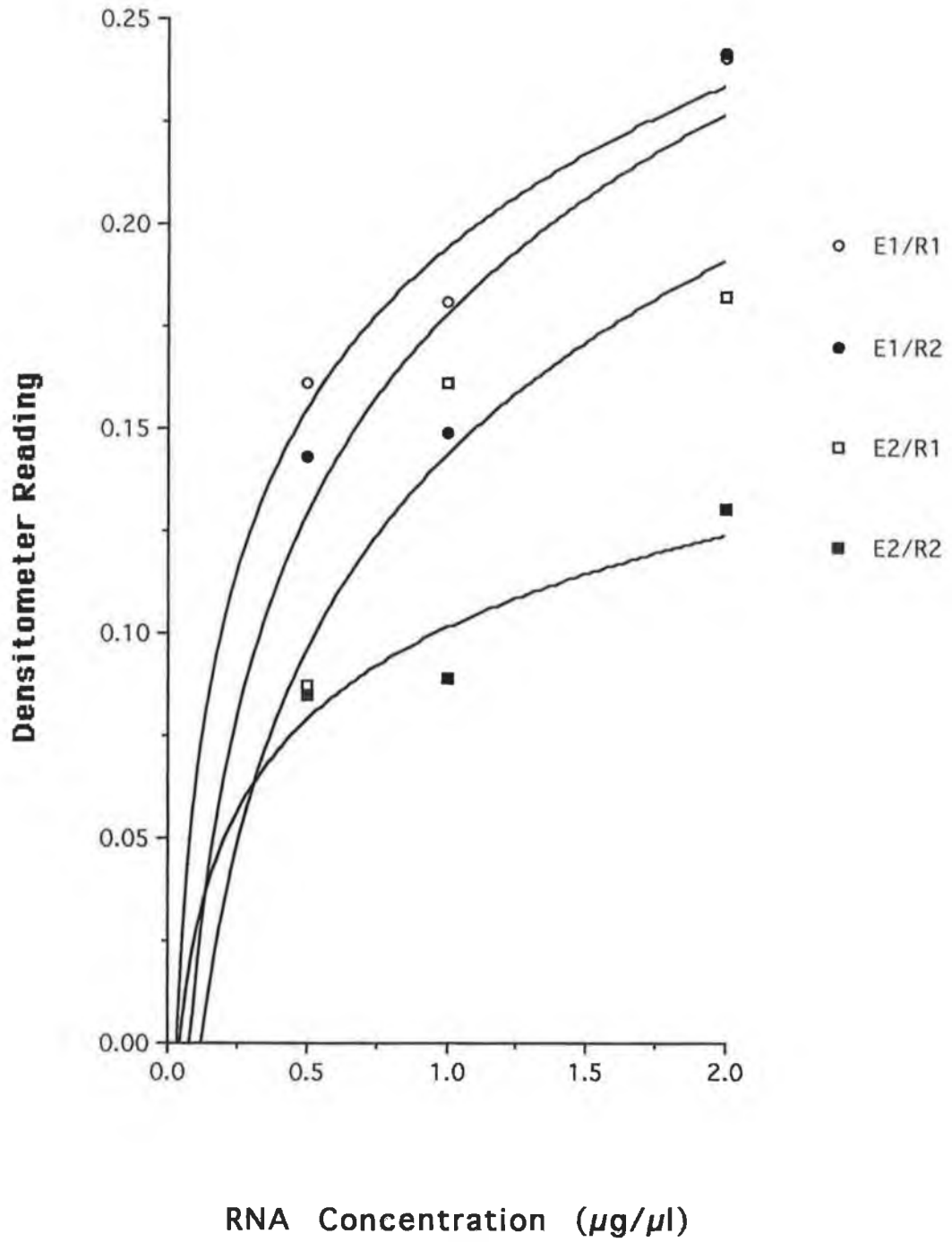


Fig. 3.5.1.2.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 20 cycles of amplification of MDR 1 cDNA derived from DLKPA-2B cultured cells.

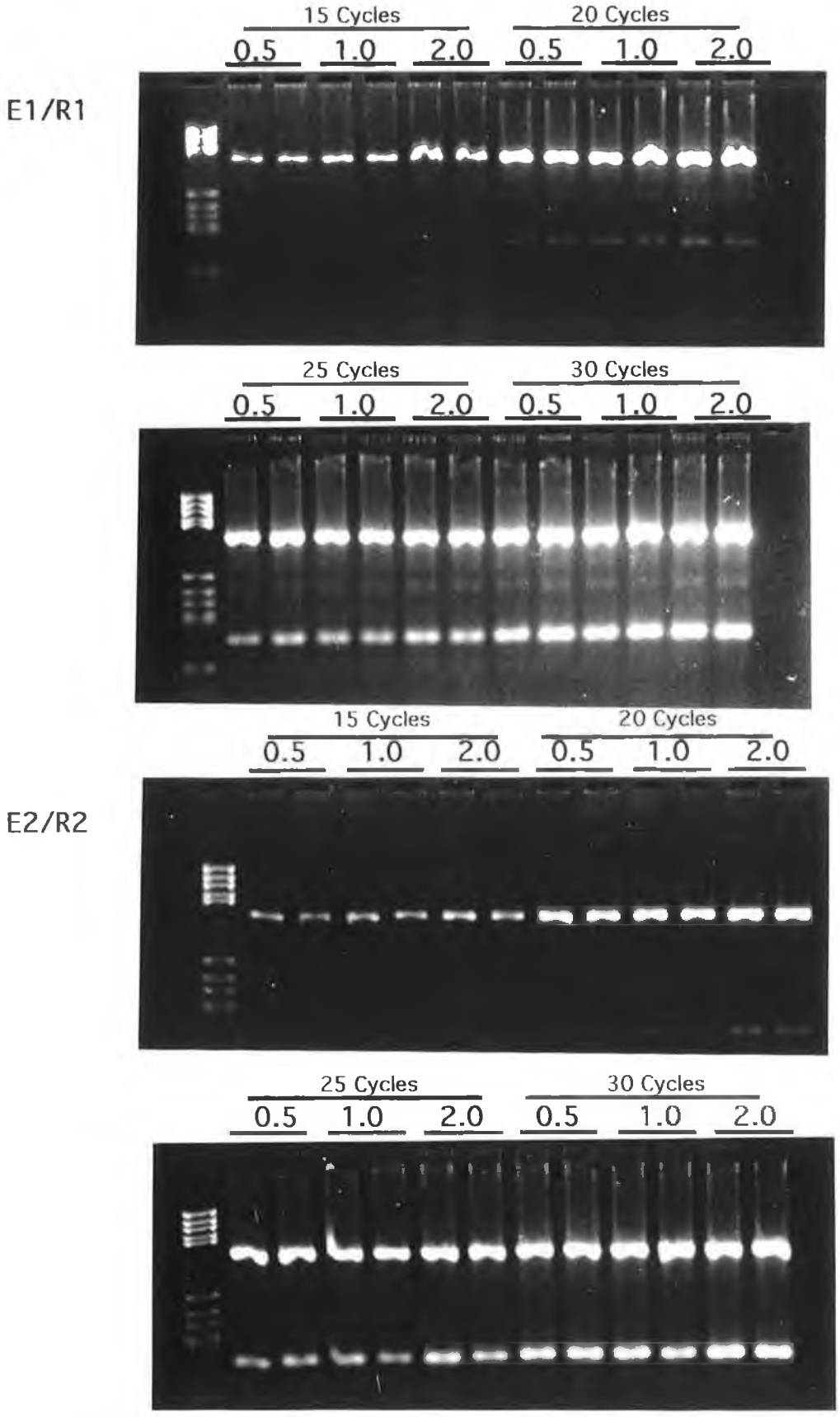
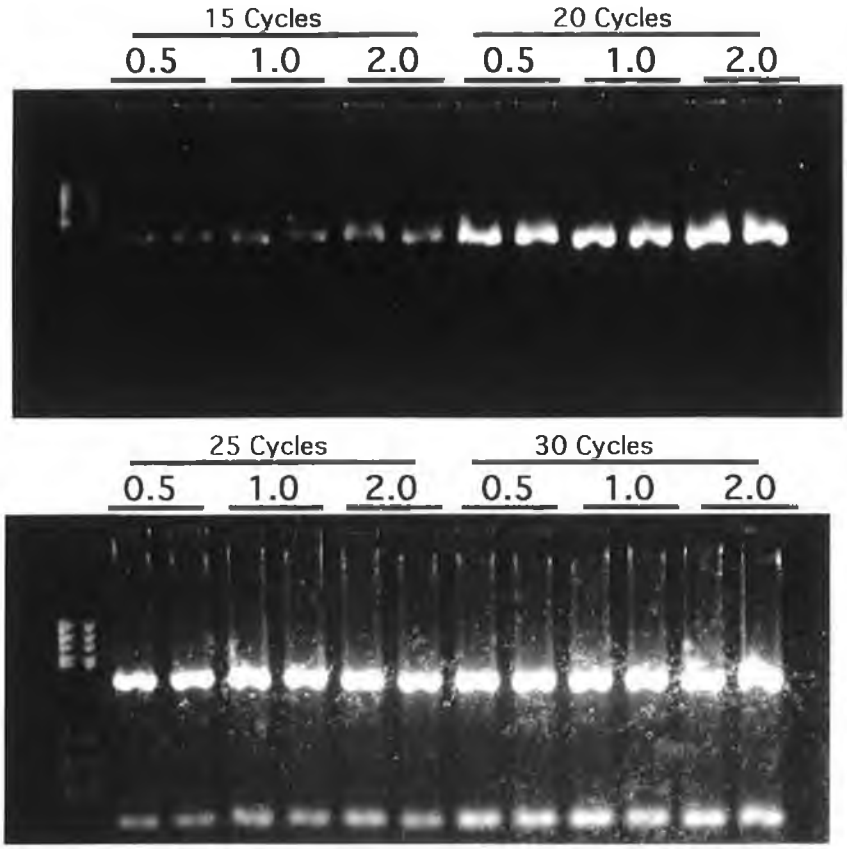


Fig. 3.5.1.3 Gel electrophoresis of the products of MDR 1 and β-actinmRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKPA-6B cultured cells and removing samples after four different PCR cycle time points. This analysis was done a total of four times.

E2/R1



E2/R2

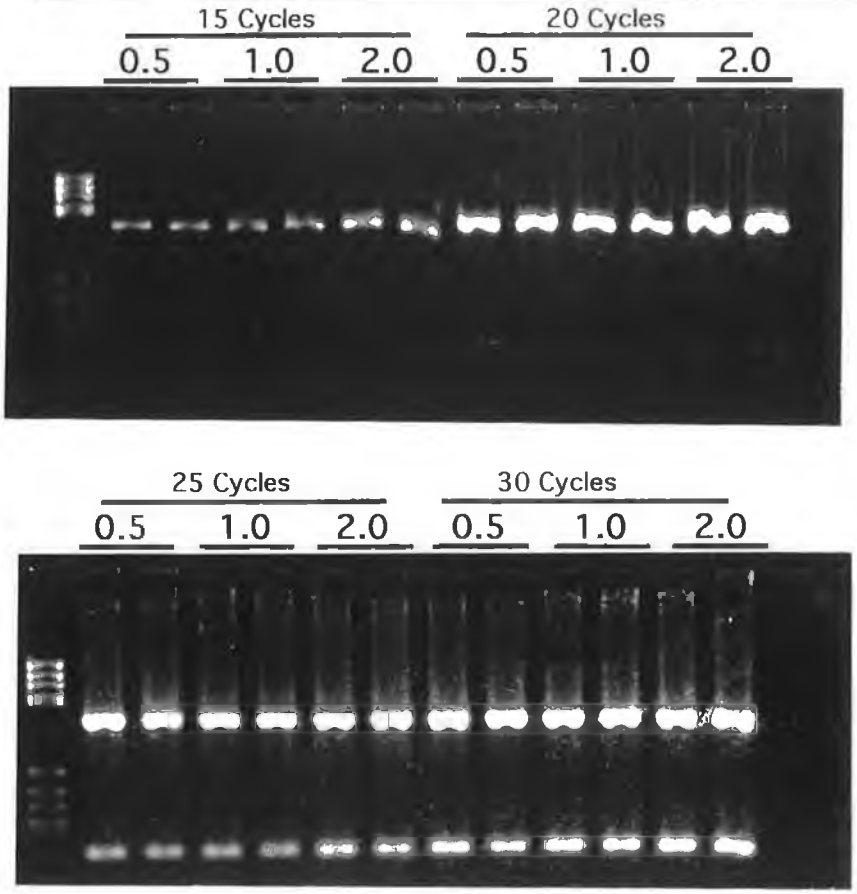


Fig. 3.5.1.3 CONTINUED

MDR 1 (DLKPA-6B)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15	0.000	0.021	0.034
	20	0.150	0.227	0.245
	25	0.486	0.487	0.499
	30	0.513	0.541	0.647
E1 / R2	15	0.000	0.024	0.052
	20	0.211	0.231	0.286
	25	0.544	0.624	0.632
	30	0.610	0.691	0.711
E2 / R1	15	0.000	0.025	0.050
	20	0.141	0.150	0.175
	25	0.481	0.542	0.545
	30	0.564	0.604	0.852
E2 / R2	15	0.000	0.021	0.039
	20	0.144	0.164	0.220
	25	0.379	0.390	0.473
	30	0.595	0.610	0.619

Table 3.5.1.3.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-6B, as in Fig. 3.5.1.3

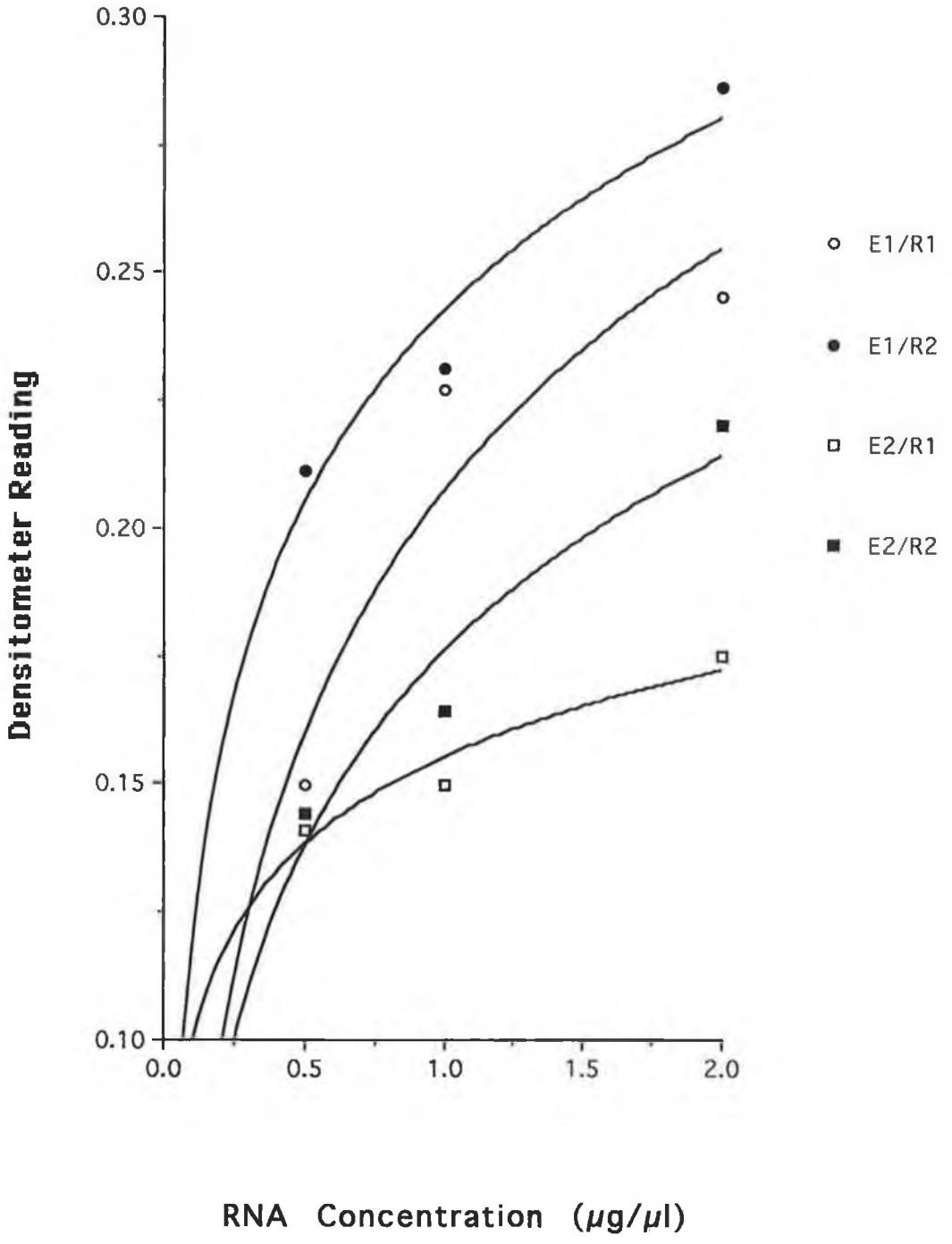


Fig. 3.5.1.3.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 20 cycles of amplification of MDR 1 cDNA derived from DLKPA-6B cultured cells.

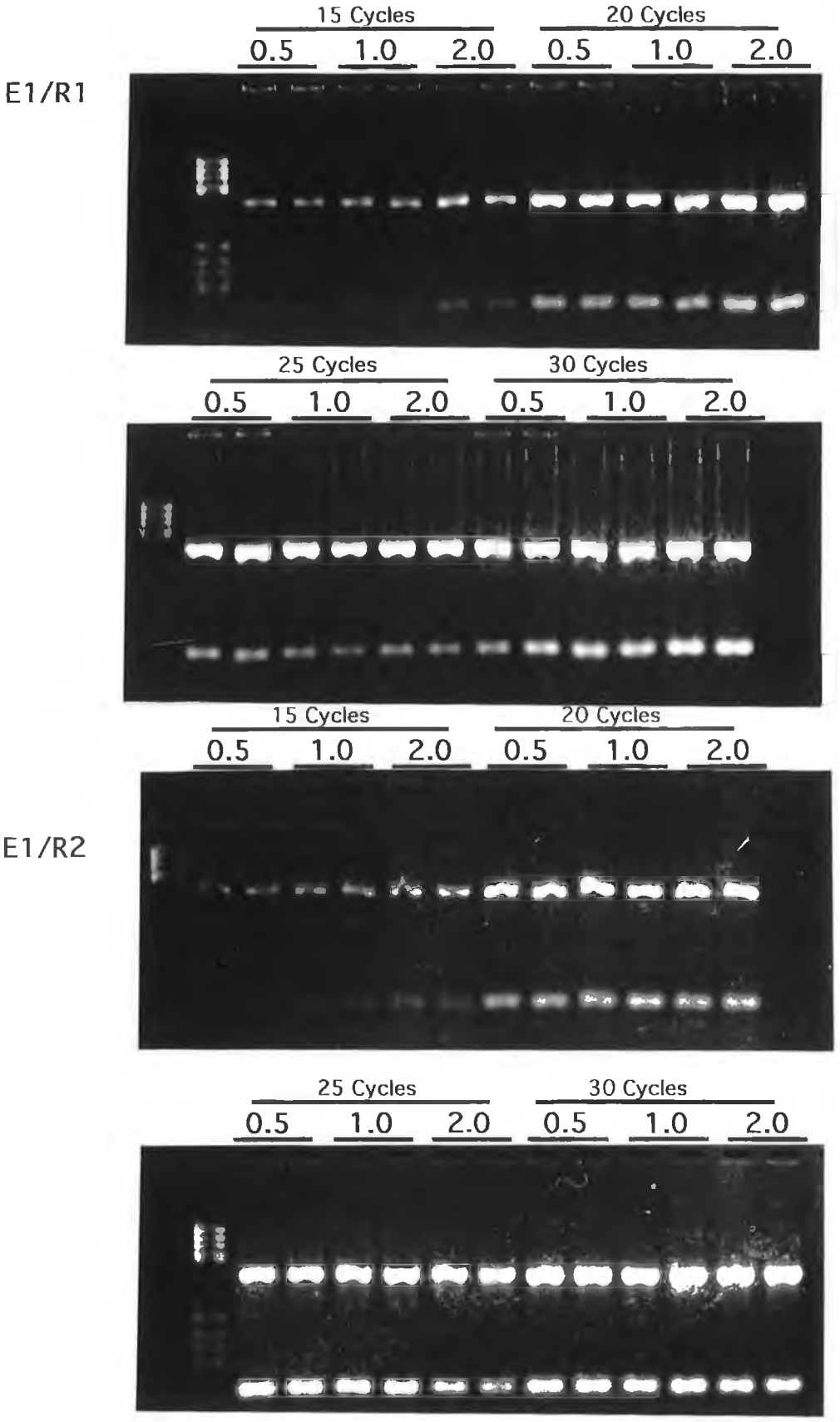


Fig. 3.5.1.4 Gel electrophoresis of the products of MDR 1 and β-actin mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKPA-5F cultured cells and removing samples after four different PCR cycle time points. This analysis was done a total of four times.

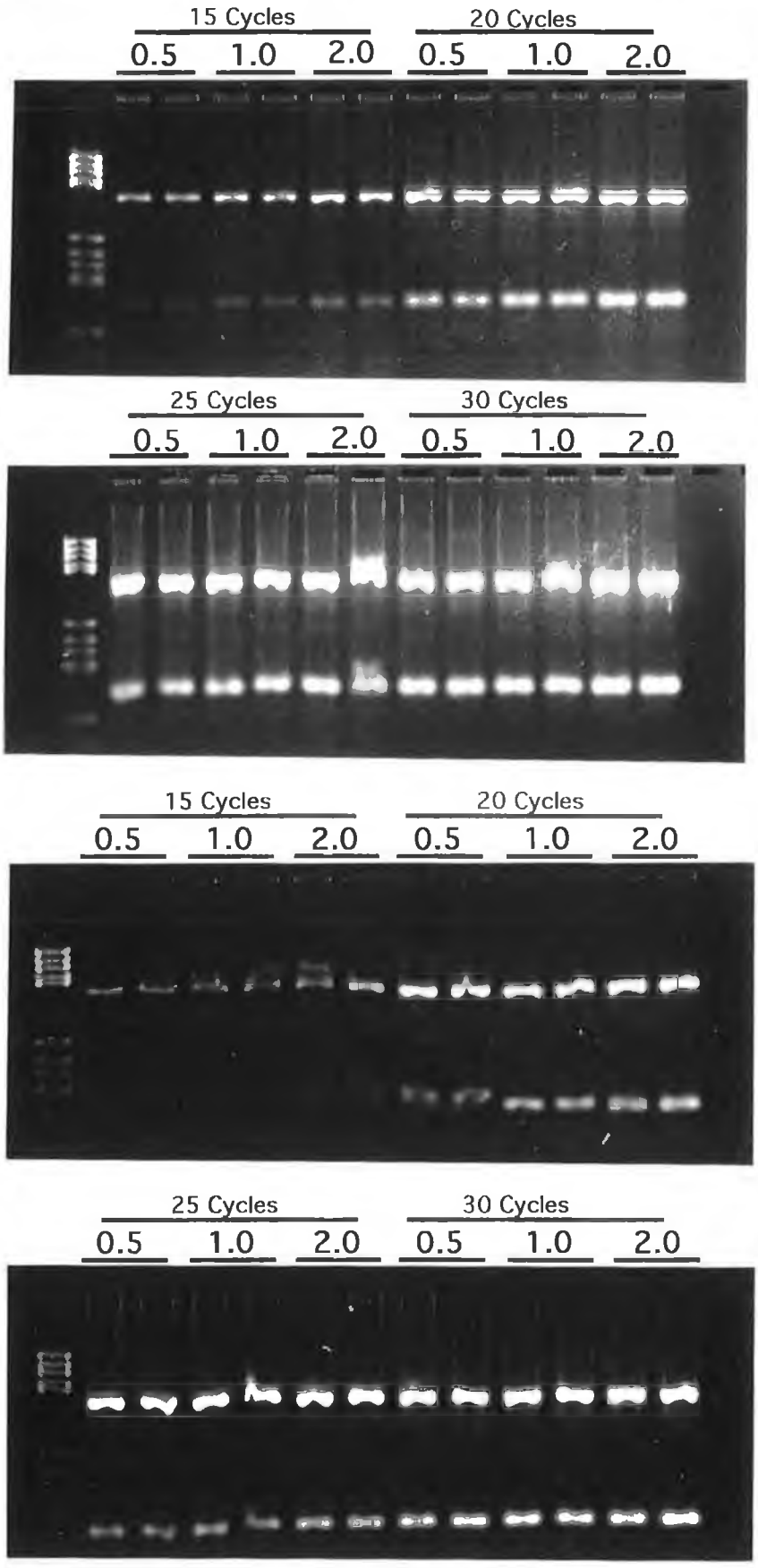


Fig. 3.5.1.4 CONTINUED

MDR 1 (DLKPA-5F)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15	0.268	0.281	0.450
	20	0.628	0.675	0.791
	25	0.741	0.869	0.892
	30	0.727	0.737	0.775
E1 / R2	15	0.631	0.646	0.775
	20	0.710	0.753	0.810
	25	0.788	0.963	1.057
	30	0.687	0.800	1.030
E2 / R1	15	0.393	0.437	0.450
	20	0.650	0.655	0.730
	25	0.885	0.907	0.960
	30	0.722	0.789	0.853
E2 / R2	15	0.094	0.123	0.293
	20	0.534	0.622	0.668
	25	0.858	0.859	0.953
	30	0.737	0.781	0.784

Table 3.5.1.4.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-5F, as in Fig. 3.5.1.4

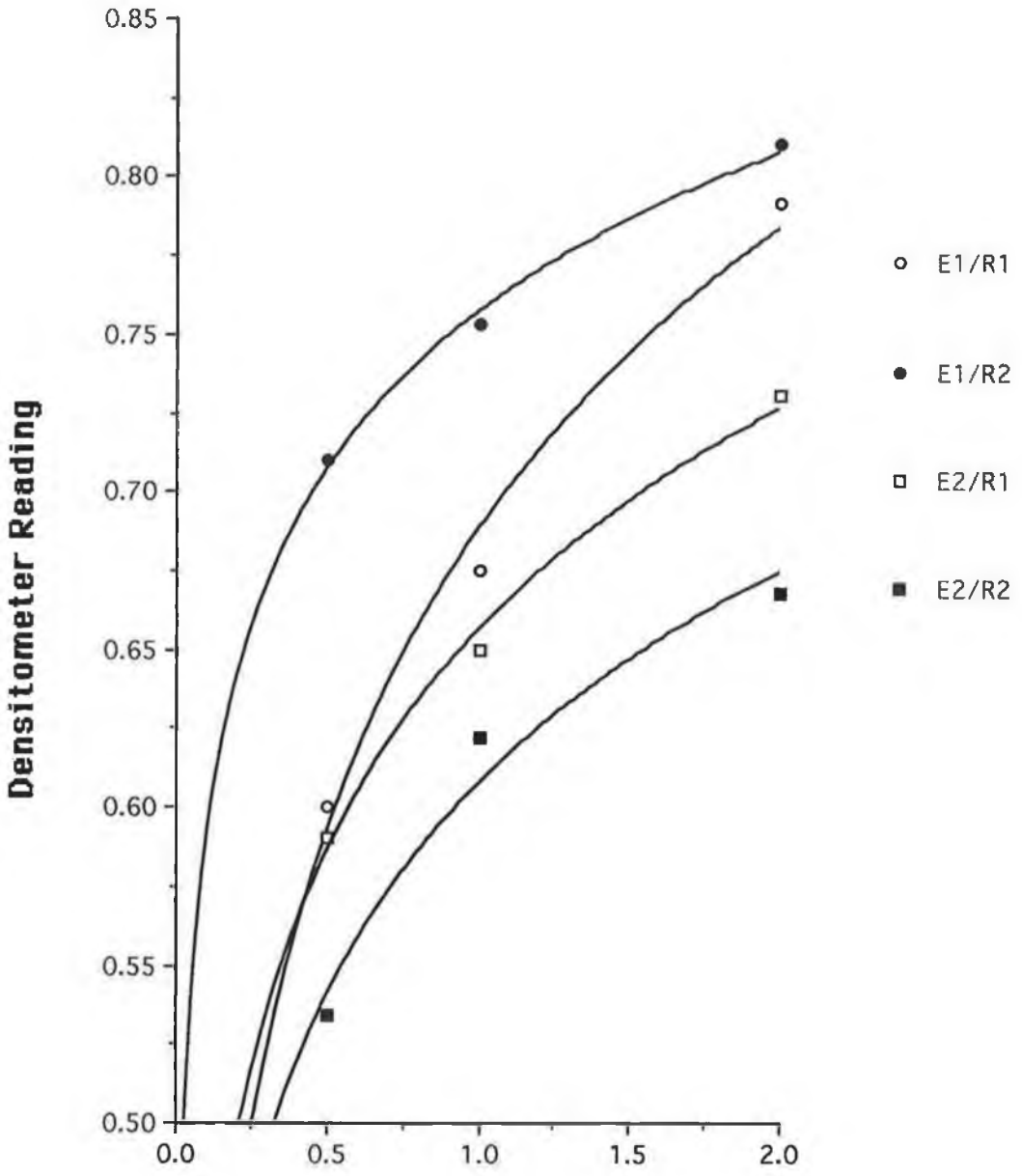


Fig. 3.5.1.4.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 20 cycles of amplification of MDR 1 cDNA derived from DLKPA-5F cultured cells.

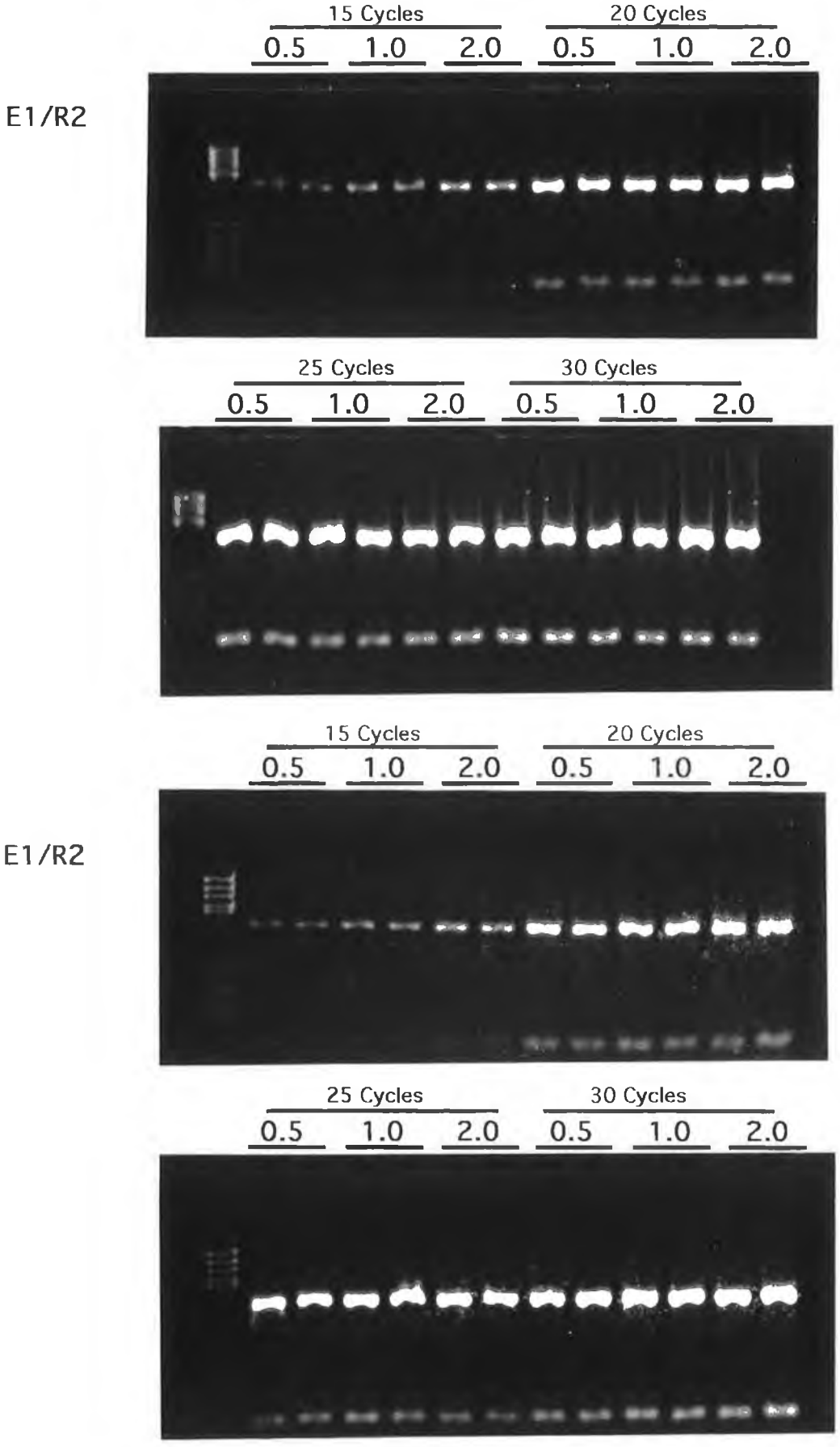
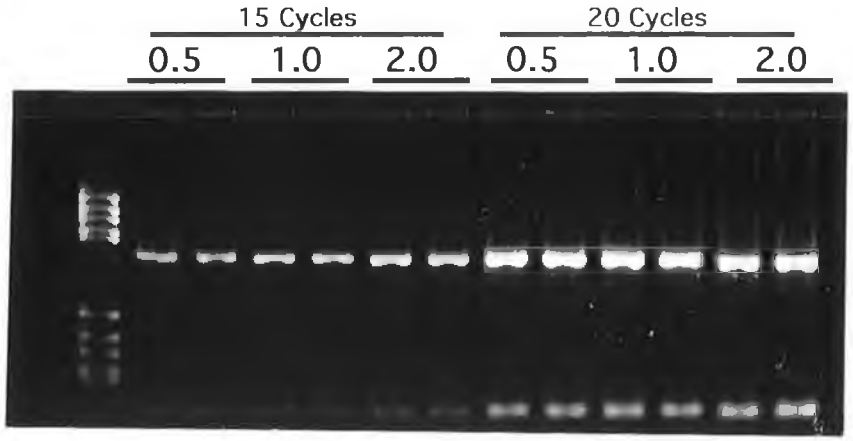
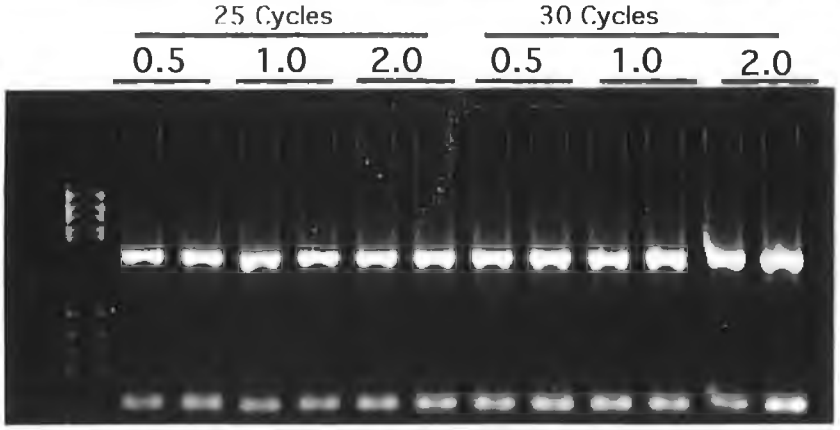


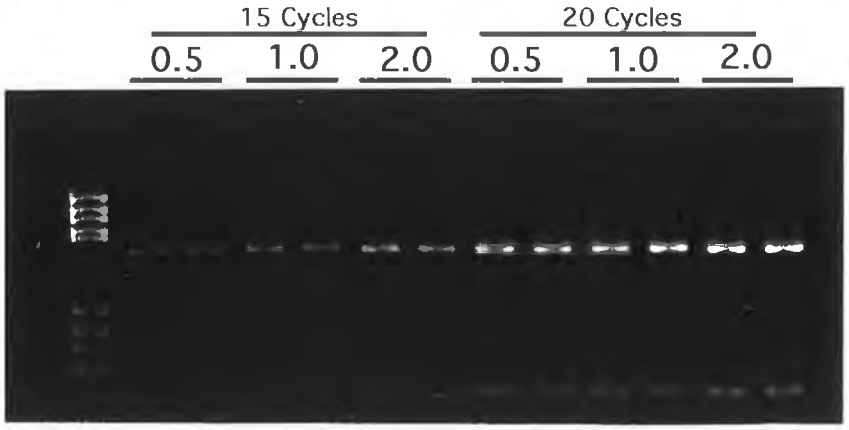
Fig. 3.5.1.5 Gel electrophoresis of the products of MDR 1 and β-actin mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKP/VP-3 cultured cells and removing samples after four different PCR cycle time points. This analysis was done a total of four times.

E2/R1





E2/R2



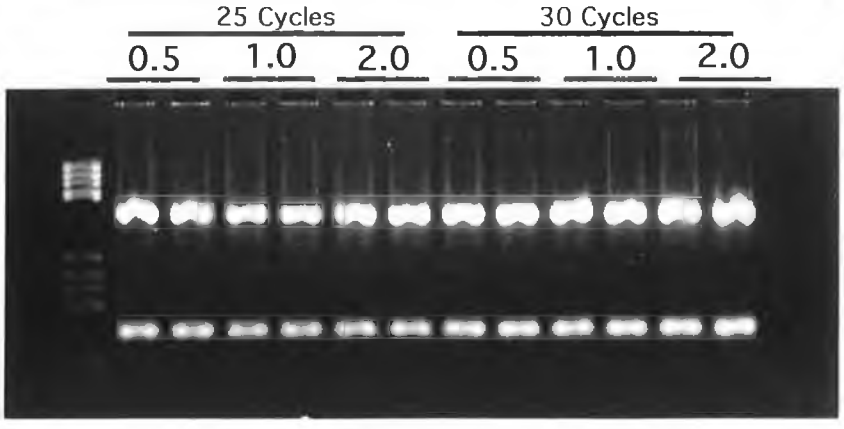


Fig. 3.5.1.5 CONTINUED

MDR 1 (DLKP/VP-3)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15	0.125	0.197	0.200
	20	0.389	0.412	0.490
	25	0.649	0.680	0.736
	30	0.537	0.564	0.666
E1 / R2	15	0.118	0.167	0.167
	20	0.282	0.346	0.420
	25	0.611	0.690	0.720
	30	0.552	0.560	0.618
E2 / R1	15	0.134	0.181	0.241
	20	0.515	0.594	0.634
	25	0.796	0.796	0.857
	30	0.781	0.781	0.793
E2 / R2	15	0.231	0.300	0.332
	20	0.553	0.579	0.650
	25	0.709	0.893	0.893
	30	0.681	0.778	0.822

Table 3.5.1.5.1 Analysis, by densitometry, of MDR 1 band intensities for DLKP/VP-3, as in Fig. 3.5.1.5

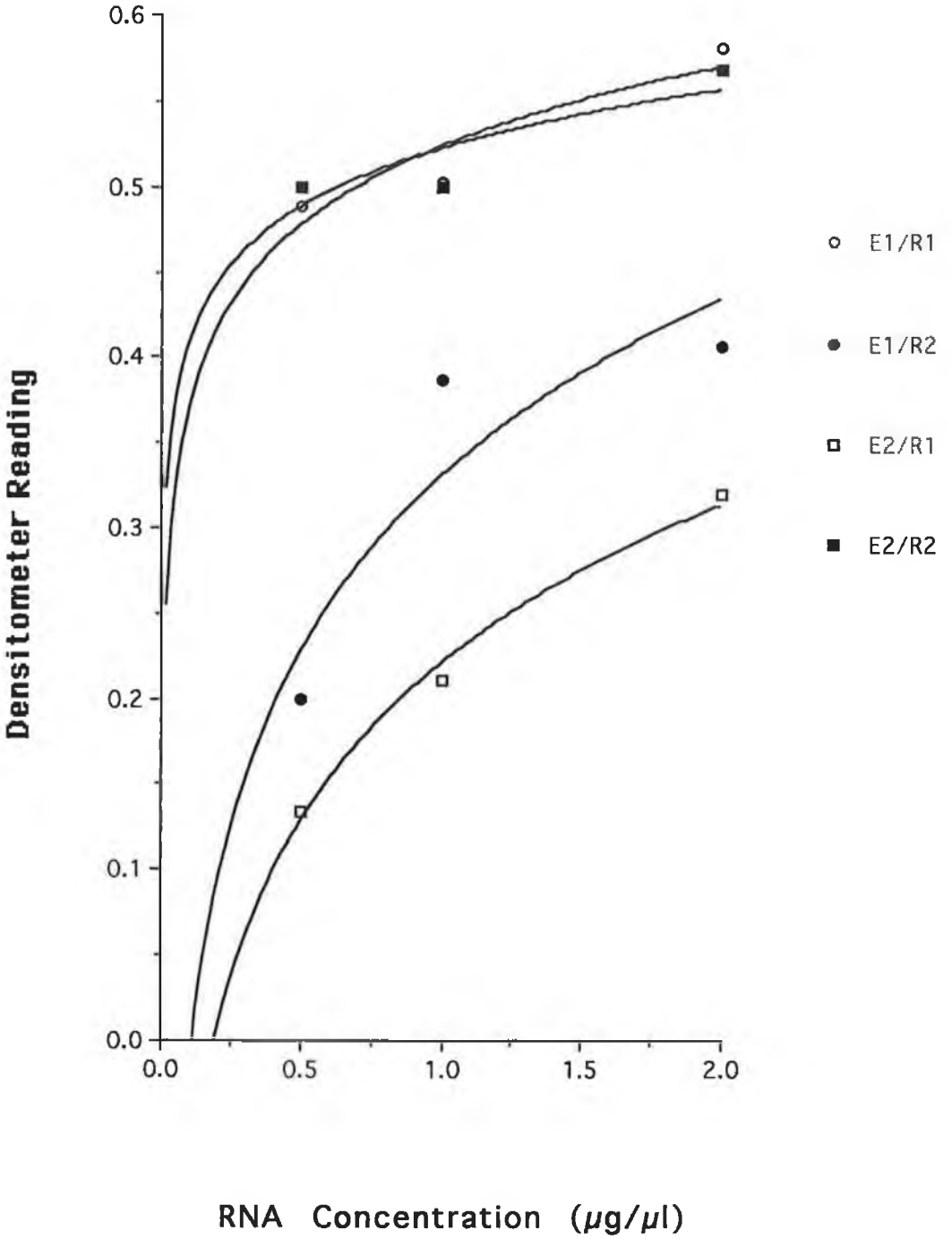


Fig. 3.5.1.5.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 20 cycles of amplification of MDR 1 cDNA derived from DLKP/VP-3 cultured cells.

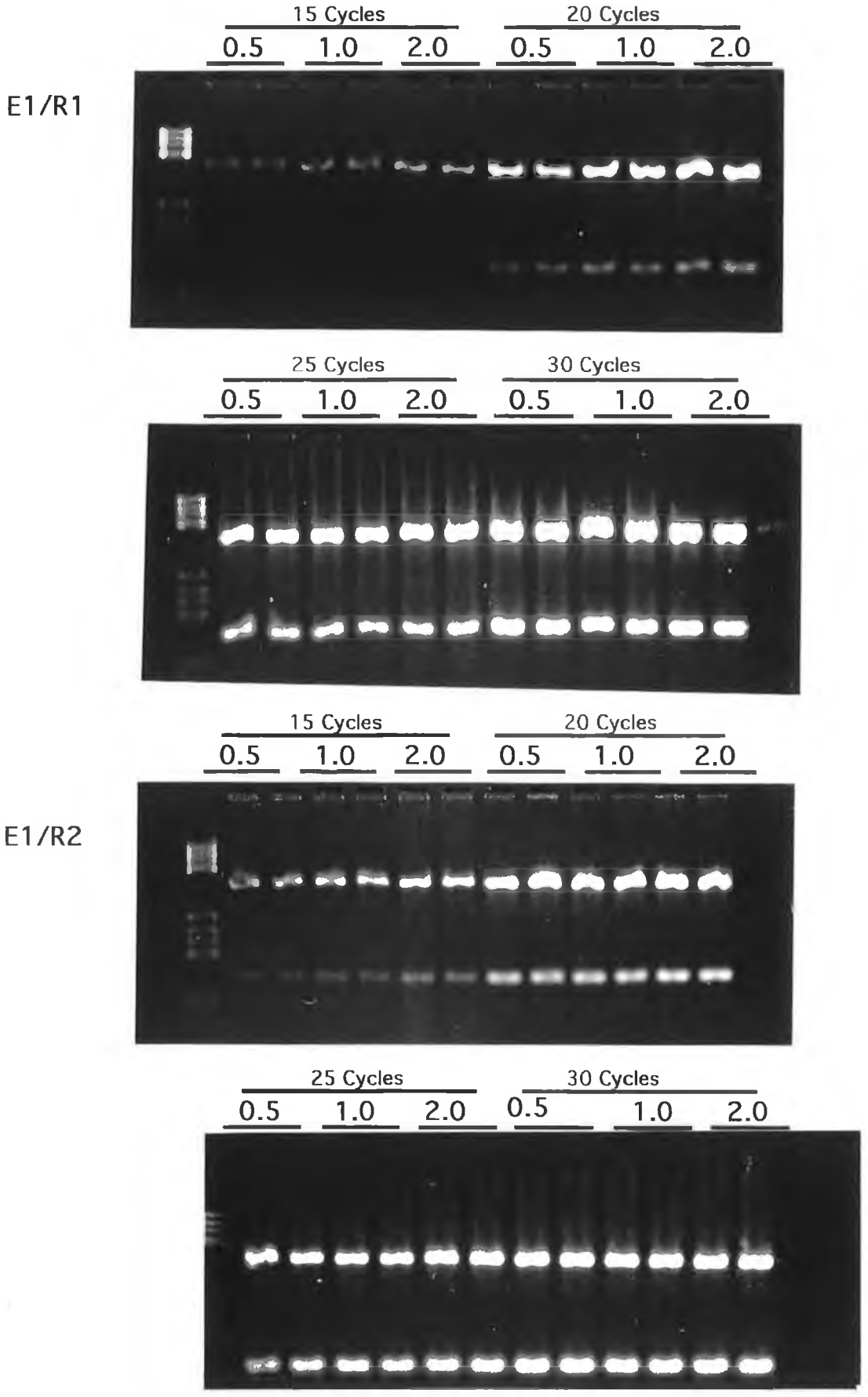


Fig. 3.5.1.6 Gel electrophoresis of the products of MDR 1 and β-actin mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKP/VP-8 cultured cells and removing samples after four different PCR cycle time points. This analysis was done a total of four times.

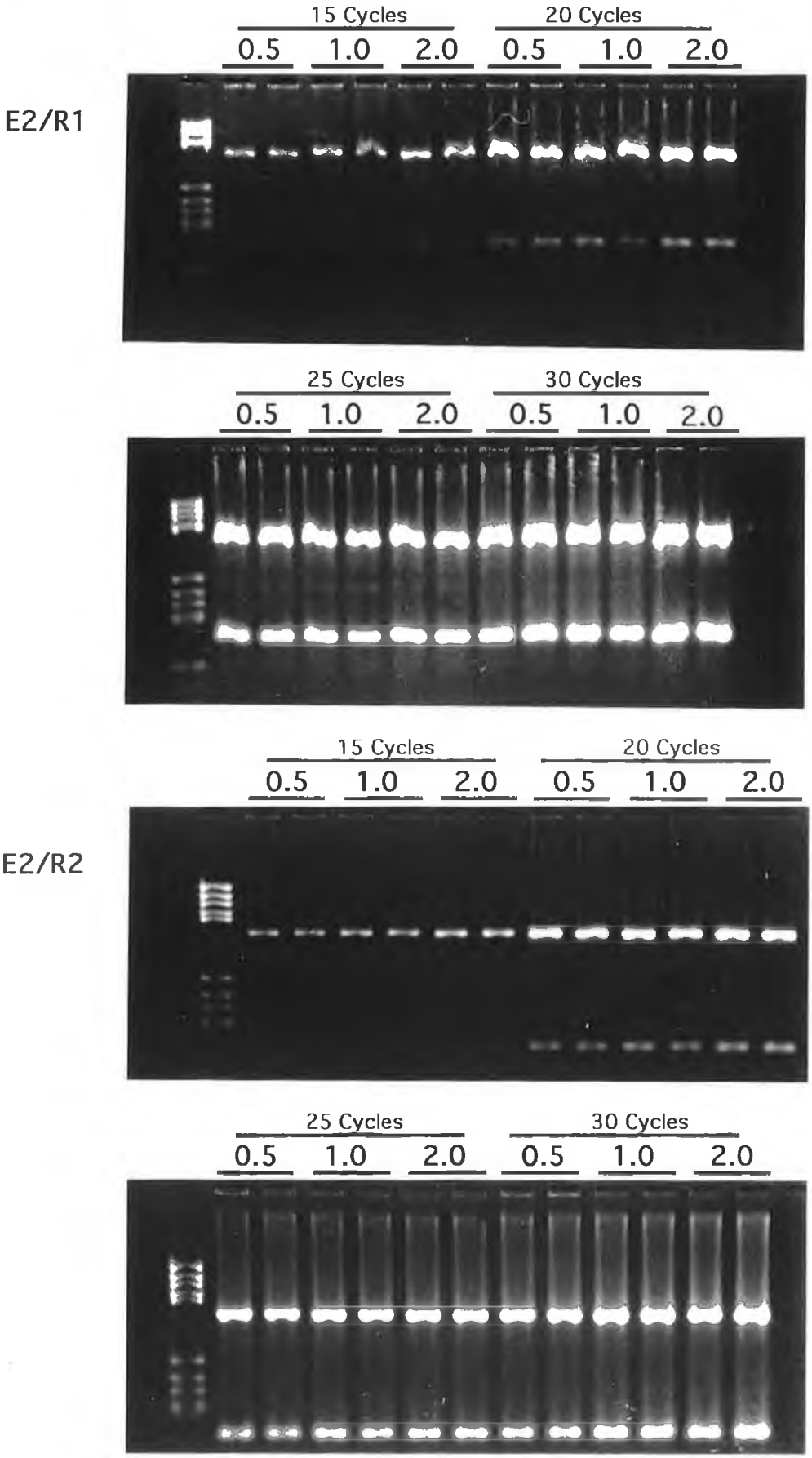


Fig. 3.5.1.6 CONTINUED

MDR 1 (DLKP/VP-8)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15	0.184	0.222	0.273
	20	0.489	0.502	0.581
	25	0.659	0.707	0.756
	30	0.813	0.837	0.870
E1 / R2	15	0.167	0.250	0.292
	20	0.200	0.387	0.406
	25	0.736	0.765	0.778
	30	0.869	0.888	0.899
E2 / R1	15	0.118	0.150	0.215
	20	0.134	0.211	0.319
	25	0.472	0.526	0.612
	30	0.534	0.579	0.711
E2 / R2	15	0.118	0.200	0.286
	20	0.500	0.500	0.568
	25	0.690	0.706	0.758
	30	0.783	0.781	0.771

Table 3.5.1.6.1 Analysis, by densitometry, of MDR 1 band intensities for DLKP/VP-8, as in Fig. 3.5.1.6

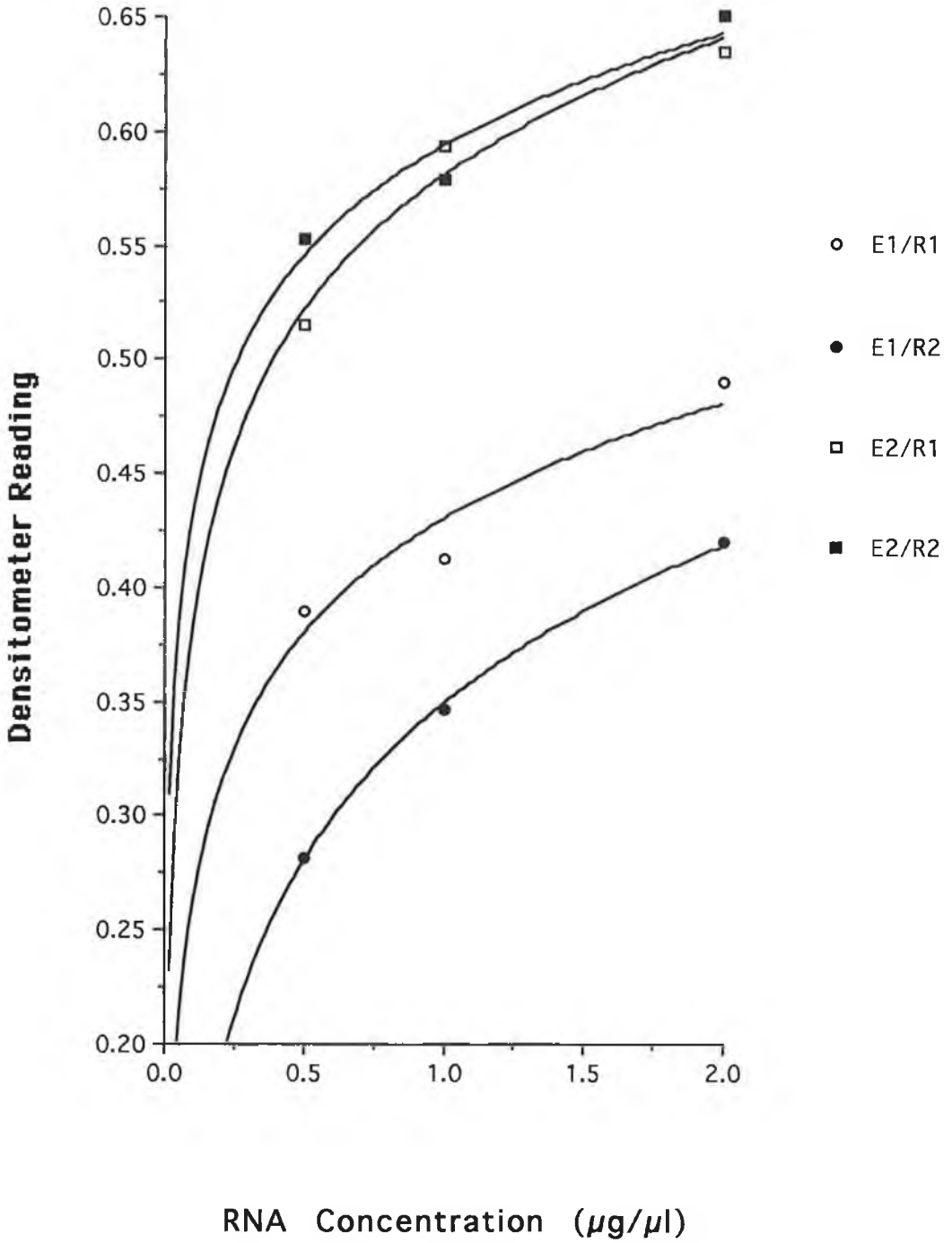


Fig. 3.5.1.6.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 20 cycles of amplification of MDR 1 cDNA derived from DLKP/VP-8 cultured cells.

Figs. 3.5.1.7 - 3.5.1.10 Topoisomerase II mRNA levels in DLKP, DLKPA, DLKP/VP-3 and DLKP/VP-8 were studied by semi-quantitative PCR analysis using a range of three RNA concentrations (0.5 μg/μl, 1.0 μg/μl and 2.0 μg/μl). This was done to establish if the resulting Topoisomerase II bands were indicative of the corresponding starting RNA concentrations. 100 μl PCR sample volumes were set up to amplify Topoisomerase II cDNA, the target gene transcript. β-actin, acting as an endogenous control and as a means to normalise the intensity of the Topoisomerase II bands, was co-amplified with the Topoisomerase II cDNA. 10 μl aliquots were removed after 20, 25 and 30 cycles of amplification. The resulting products were separated by gel electrophoresis through a 4 % gel and were identified with respect to molecular weight markers (ran on each gel) ranging from 587 bp to 8 bp in size.

This analysis was performed for each of the cell lines (mentioned above) a total of four times *i.e.* on duplicate stocks of cDNA formed from two RNA extracts of independent cell line stocks - designated E1/R1, E1/R2, E2/R1 and E2/R2. All reactions were performed in duplicate.

Tables 3.5.1.7.1 - 3.5.1.10.1 Topoisomerase II band intensities (Figs. 3.5.1.7 - 3.5.1.10, respectively) were analysed by densitometry. The resulting data was corrected by subtracting background readings and normalising with β-actin (the endogenous control).

Figs. 3.5.1.7.2 - 2.5.1.10.2 Relationship between the RNA concentrations and the corrected densitometry readings of the bands resulting after gel electrophoresis of the RT-PCR products (Figs. 3.5.1.7 - 3.5.1.10). The data plotted represents the bands produced after 25 cycles of PCR amplification, as this was found to be within the exponential phase of product accumulation with this primer pair and was suggested as a suitable cycle number at which analysis could be performed (see 3.4).

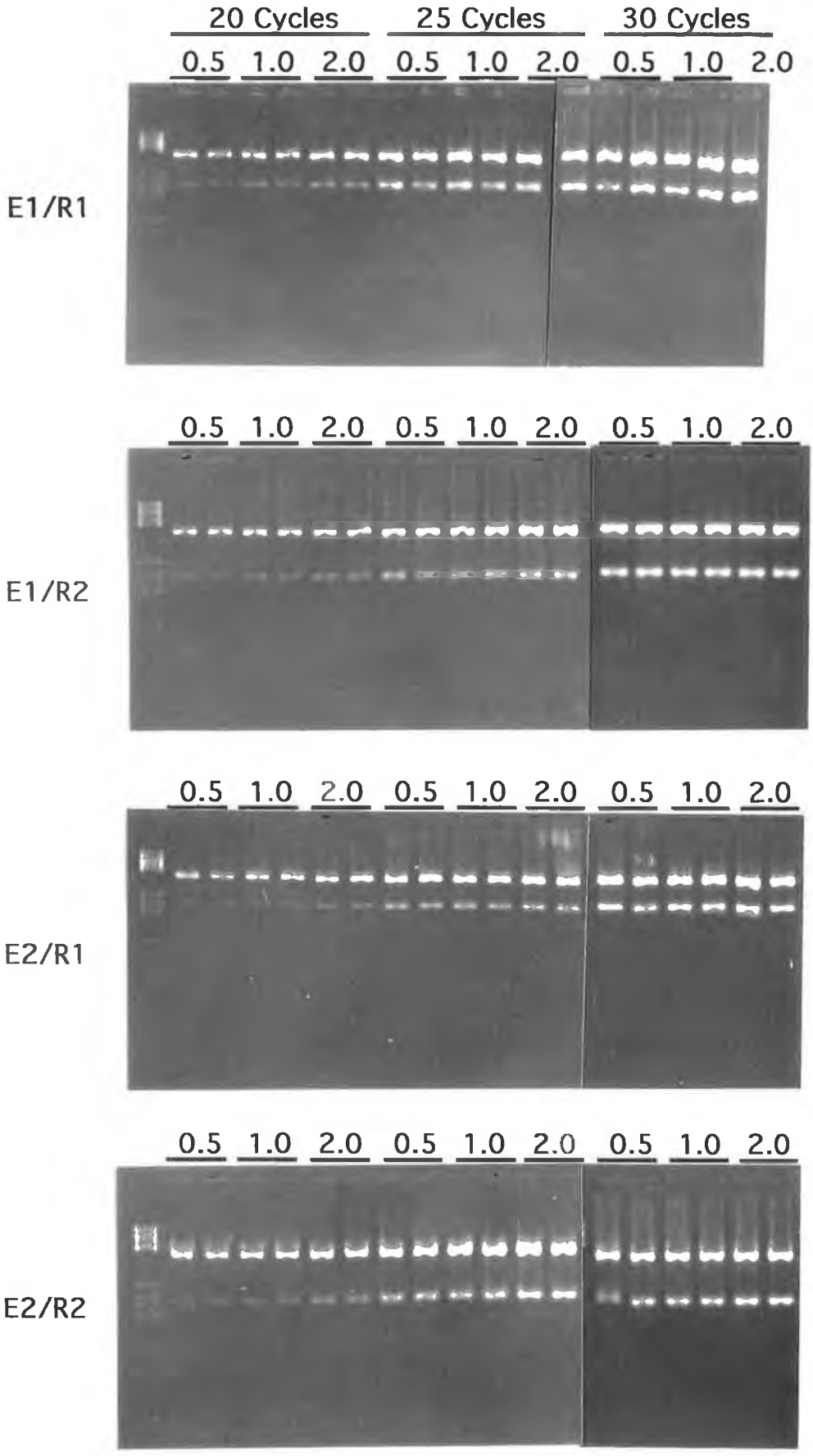


Fig. 3.5.1.7 Gel electrophoresis of the products of Topoisomerase II mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 $\mu g/\mu l$) extracted from DLKP cultured cells and removing samples after three different PCR cycle time points. This analysis was done a total of four times.

Topo II (DLKP)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15			
	20	0.209	0.268	0.313
	25	0.646	0.688	0.732
	30	0.732	0.873	1.000
E1 / R2	15			
	20	0.111	0.191	0.321
	25	0.558	0.619	0.641
	30	0.784	0.795	0.811
E2 / R1	15			
	20	0.134	0.211	0.188
	25	0.511	0.562	0.588
	30	0.700	0.770	0.795
E2 / R2	15			
	20	0.167	0.191	0.333
	25	0.532	0.586	0.594
	30	0.635	0.760	0.775

Table 3.5.1.7.1 Analysis, by densitometry, of Topoisomerase II band intensities for DLKP, as in Fig. 3.5.1.7

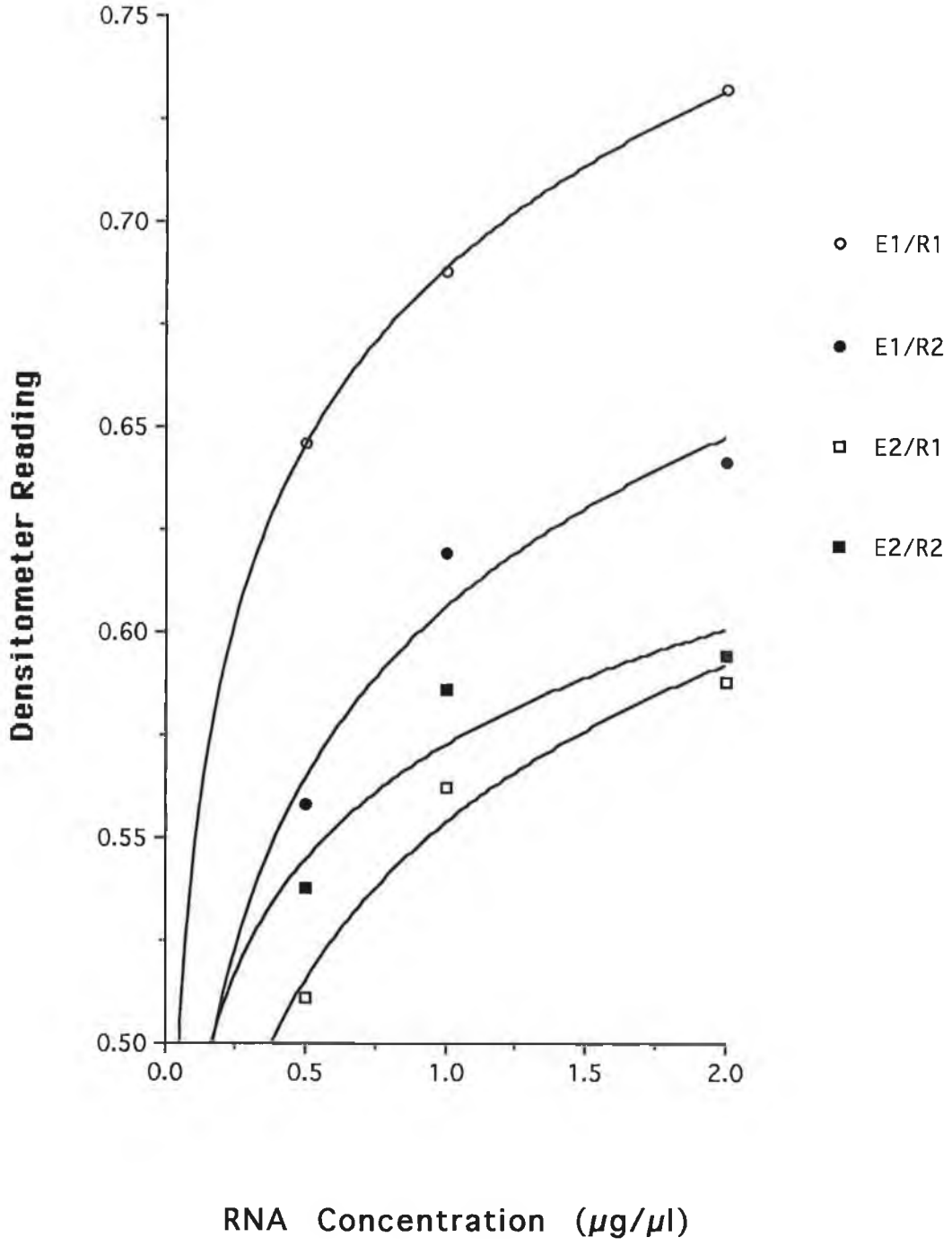


Fig. 3.5.1.7.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 25 cycles of amplification of Topoisomerase II cDNA derived from DLKP cultured cells.

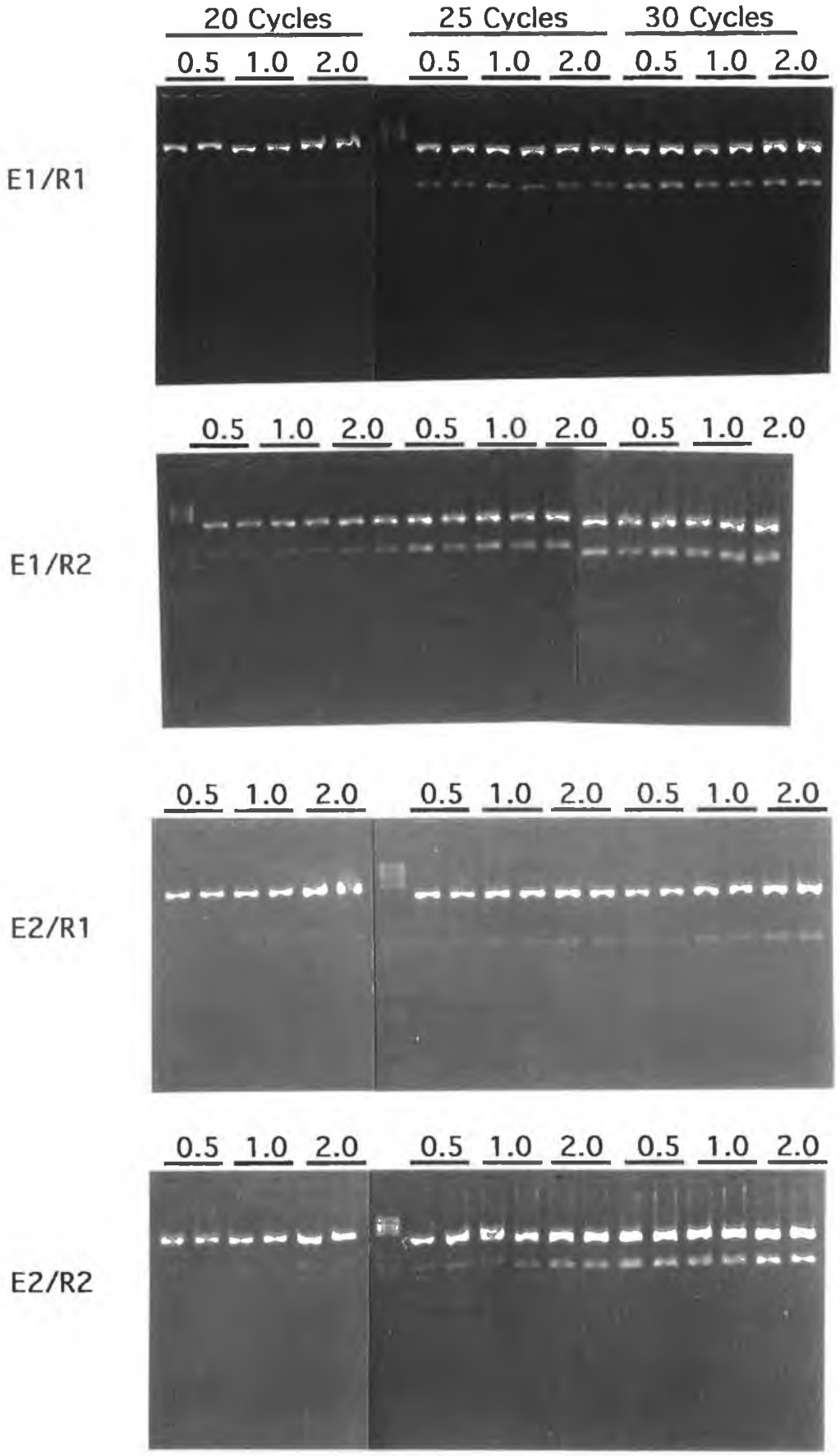


Fig. 3.5.1.8 Gel electrophoresis of the products of Topoisomerase II mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 $\mu g/\mu l$) extracted from DLKPA cultured cells and removing samples after three different PCR cycle time points. This analysis was done a total of four times.

Topo II (DLKPA)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15			
	20	0.000	0.167	0.292
	25	0.325	0.390	0.461
	30	0.508	0.534	0.591
E1 / R2	15			
	20	0.084	0.087	0.100
	25	0.091	0.172	0.278
	30	0.211	0.266	0.367
E2 / R1	15			
	20	0.092	0.113	0.118
	25	0.248	0.278	0.391
	30	0.440	0.500	0.528
E2 / R2	15			
	20	0.000	0.000	0.137
	25	0.193	0.234	0.340
	30	0.340	0.515	0.568

Table 3.5.1.8.1 Analysis, by densitometry, of Topoisomerase II band intensities for DLKPA, as in Fig. 3.5.1.8

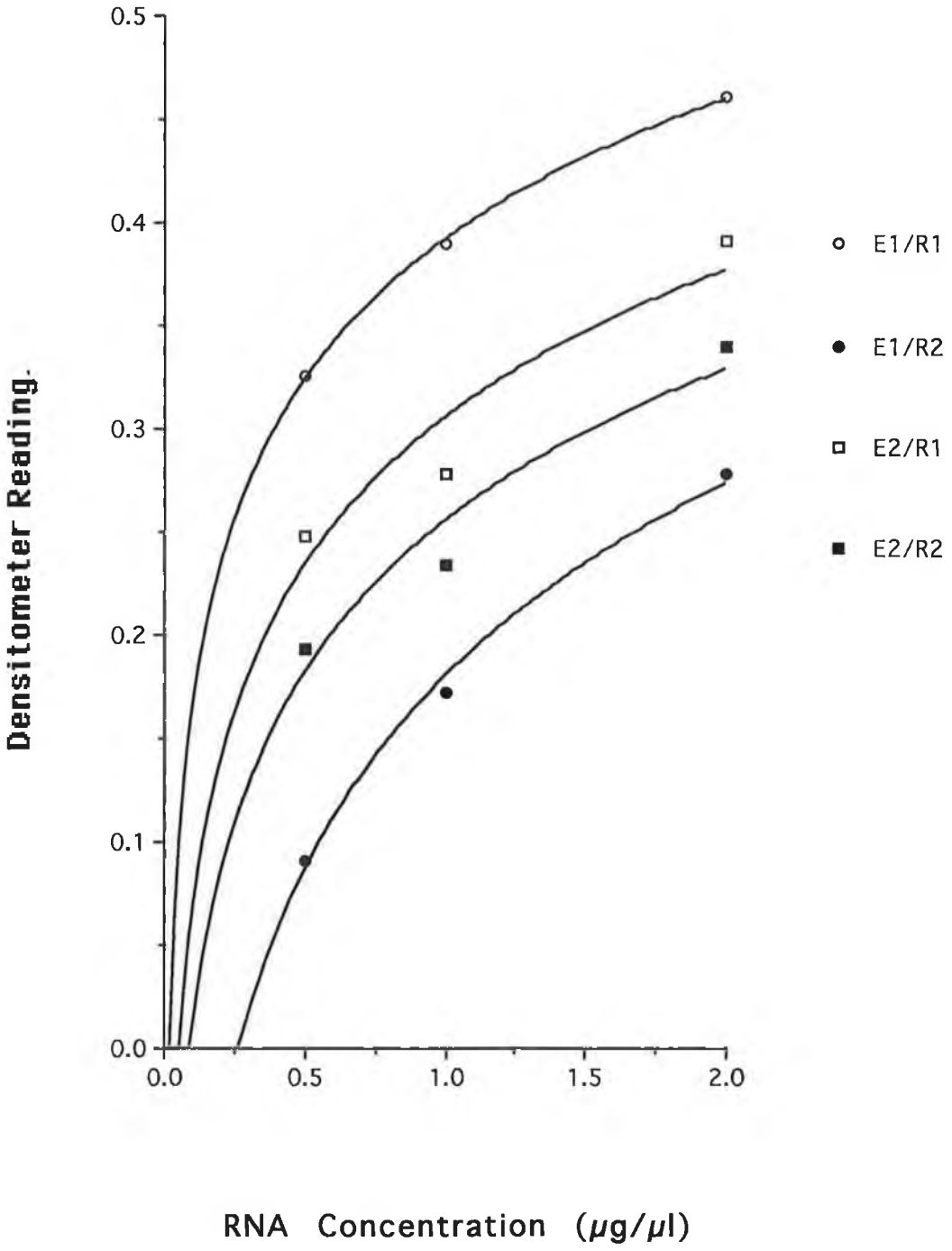


Fig. 3.5.1.8.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 25 cycles of amplification of Topoisomerase II cDNA derived from DLKPA cultured cells.

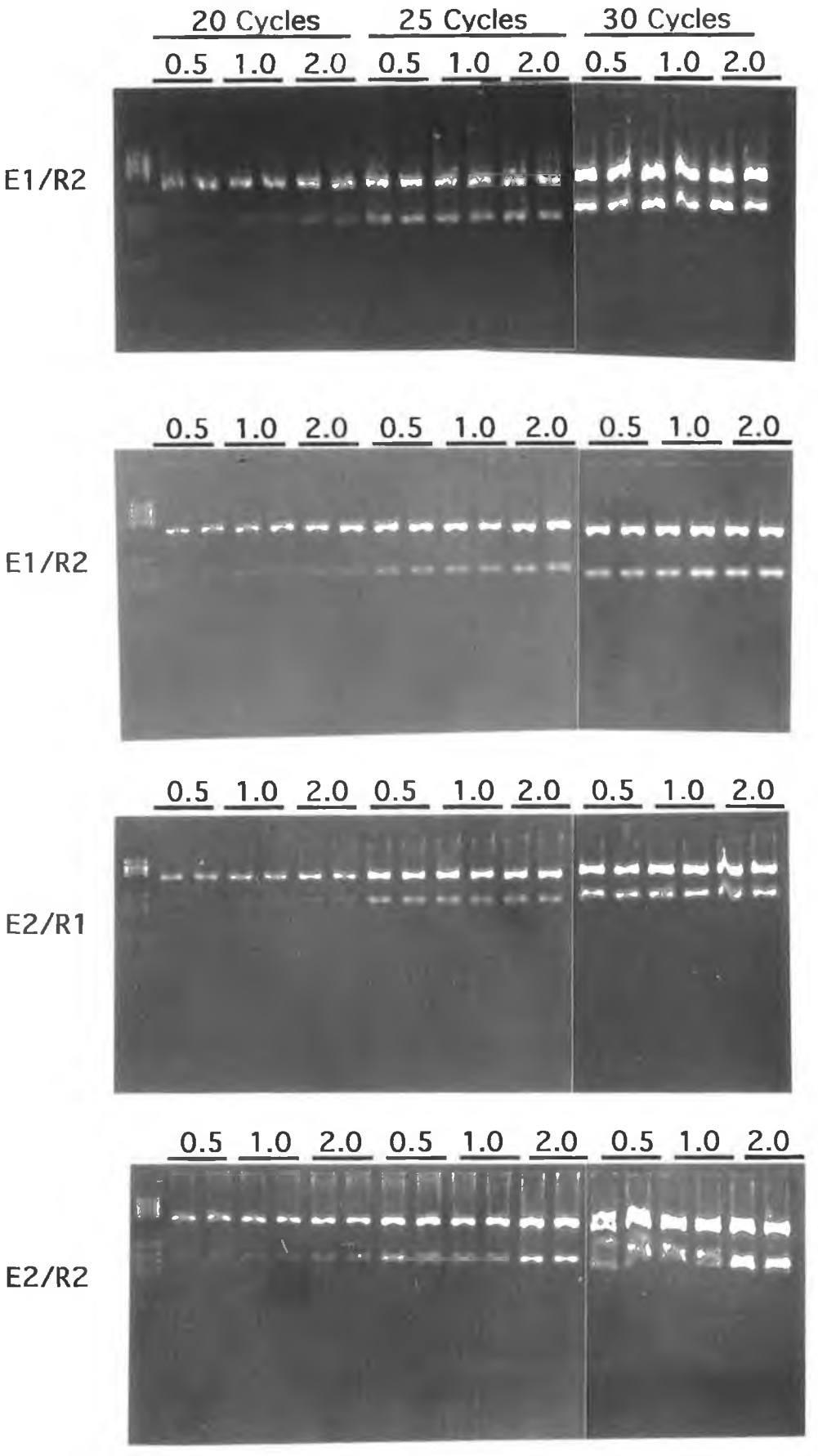


Fig. 3.5.1.9 Gel electrophoresis of the products of Topoisomerase II mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 $\mu g/\mu l$) extracted from DLKP/VP-3 cultured cells and removing samples after three different PCR cycle time points. This analysis was done a total of four times.

Topo II (DLKP/VP-3)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15			
	20	0.155	0.155	0.340
	25	0.350	0.378	0.478
	30	0.535	0.764	0.875
E1 / R2	15			
	20	0.129	0.134	0.311
	25	0.349	0.382	0.491
	30	0.567	0.656	0.720
E2 / R1	15			
	20	0.125	0.184	0.222
	25	0.364	0.393	0.489
	30	0.684	0.733	0.694
E2 / R2	15			
	20	0.125	0.134	0.267
	25	0.417	0.459	0.510
	30	0.607	0.742	0.775

Table 3.5.1.9.1 Analysis, by densitometry, of Topoisomerase II band intensities for DLKP/VP-3, as in Fig. 3.5.1.9

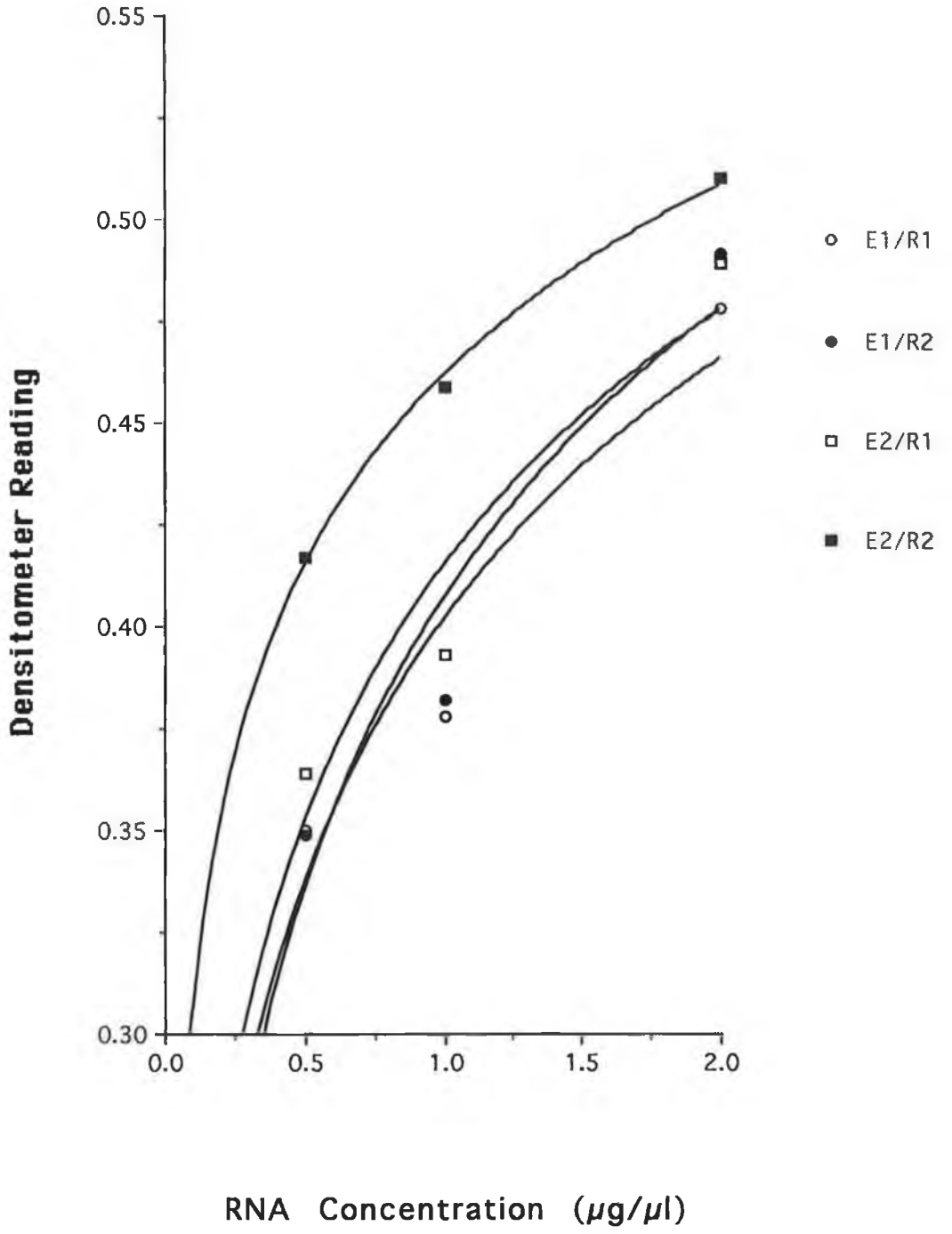


Fig. 3.5.1.9.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 25 cycles of amplification of Topoisomerase II cDNA derived from DLKP/VP-3 cultured cells.

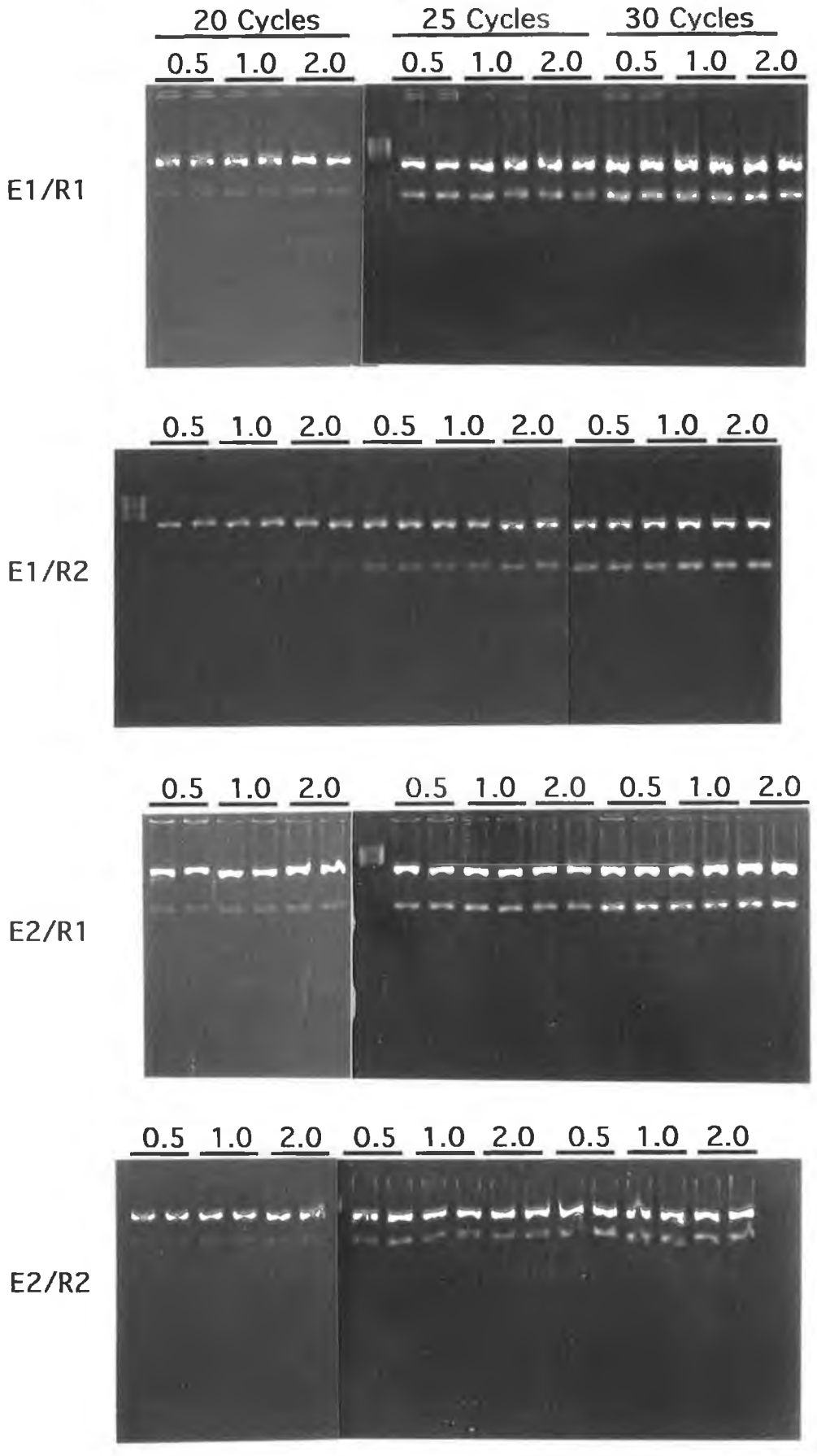


Fig. 3.5.1.10 Gel electrophoresis of the products of Topoisomerase II mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 $\mu g/\mu l$) extracted from DLKP/VP-8 cultured cells and removing samples after three different PCR cycle time points. This analysis was done a total of four times.

Topo II (DLKP/VP-8)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15			
	20	0.146	0.211	0.211
	25	0.407	0.427	0.467
	30	0.515	0.546	0.563
E1 / R2	15			
	20	0.032	0.056	0.200
	25	0.250	0.320	0.380
	30	0.217	0.343	0.459
E2 / R1	15			
	20	0.155	0.156	0.284
	25	0.343	0.417	0.437
	30	0.462	0.568	0.619
E2 / R2	15			
	20	0.156	0.167	0.211
	25	0.311	0.400	0.415
	30	0.450	0.554	0.579

Table 3.5.1.10.1 Analysis, by densitometry, of Topoisomerase II band intensities for DLKP/VP-8, as in Fig. 3.5.1.10

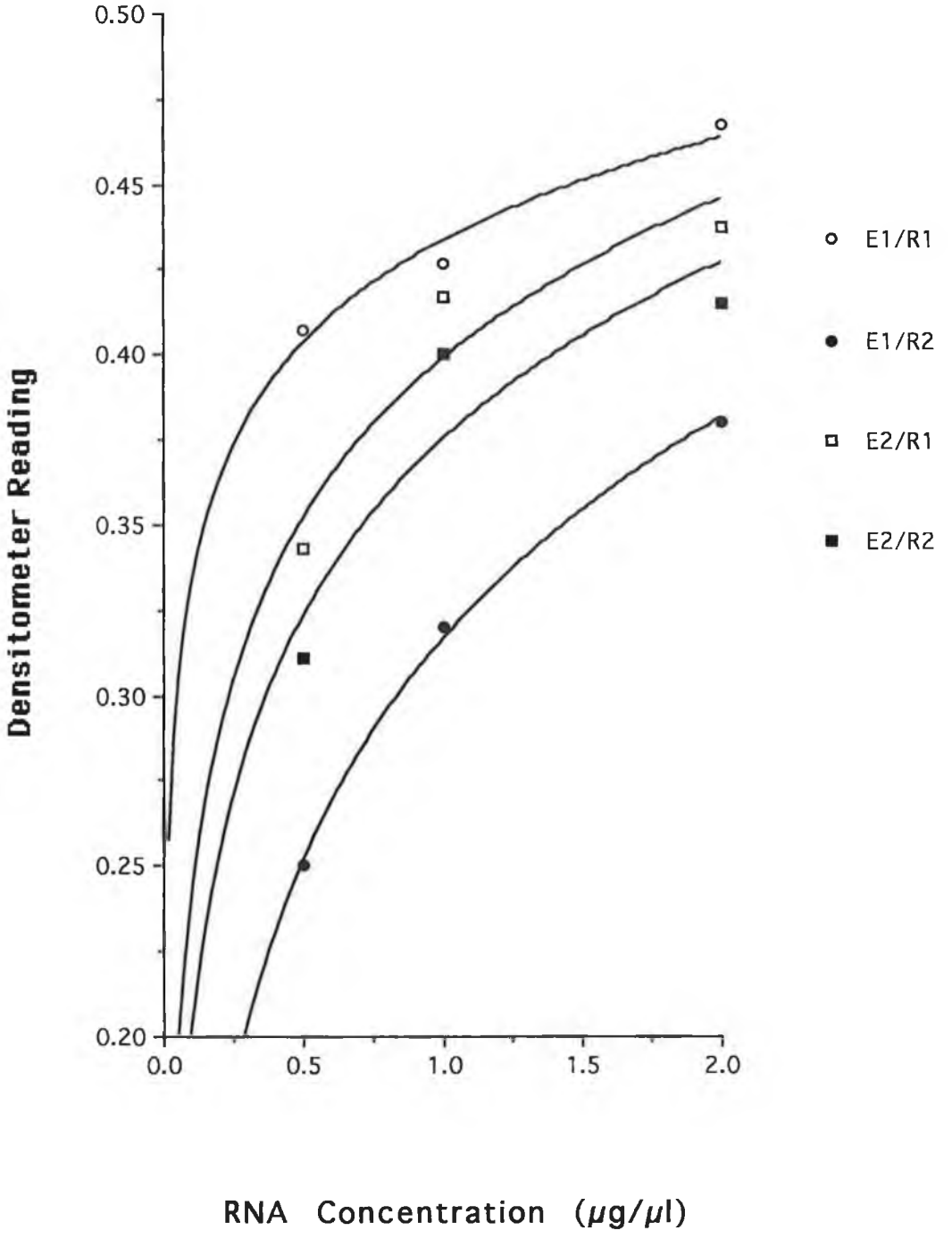


Fig. 3.5.1.10.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 25 cycles of amplification of Topoisomerase II cDNA derived from DLKP/VP-8 cultured cells.

Figs. 3.5.1.11 - 3.5.1.14 Topoisomerase IIα mRNA levels in DLKP, DLKPA, DLKP/VP-3 and DLKP/VP-8 were studied by semi-quantitative PCR analysis using a range of three RNA concentrations (0.5 μg/μl, 1.0 μg/μl and 2.0 μg/μl). This was done to establish if the resulting Topoisomerase IIα bands were indicative of the corresponding starting RNA concentrations. 100 μl PCR sample volumes were set up to amplify Topoisomerase IIα cDNA, the target gene transcript. β-actin, acting as an endogenous control and as a means to normalise the intensity of the Topoisomerase IIα bands, was co-amplified with the Topoisomerase IIα cDNA. 10 μl aliquots were removed after 20, 25 and 30 cycles of amplification. The resulting products were separated by gel electrophoresis through a 4 % gel and were identified with respect to molecular weight markers (ran on each gel) ranging from 587 bp to 8 bp in size.

This analysis was performed for each of the cell lines (mentioned above) a total of four times *i.e.* on duplicate stocks of cDNA formed from two RNA extracts of independent cell line stocks - designated E1/R1, E1/R2, E2/R1 and E2/R2. All reactions were performed in duplicate.

Tables 3.5.1.11.1 - 3.5.1.14.1 Topoisomerase IIα band intensities (Figs. 3.5.1.11 - 3.5.1.14, respectively) were analysed by densitometry. The resulting data was corrected by subtracting background readings and normalising with β-actin (the endogenous control).

Figs. 3.5.1.11.2 - 2.5.1.14.2 Relationship between the RNA concentrations and the corrected densitometry readings of the bands resulting after gel electrophoresis of the RT-PCR products (Figs. 3.5.1.11 - 3.5.1.14). The data plotted represents the bands produced after 25 cycles of PCR amplification, as this was found to be within the exponential phase of product accumulation with this primer pair and was suggested as a suitable cycle number at which analysis could be performed (see 3.4).

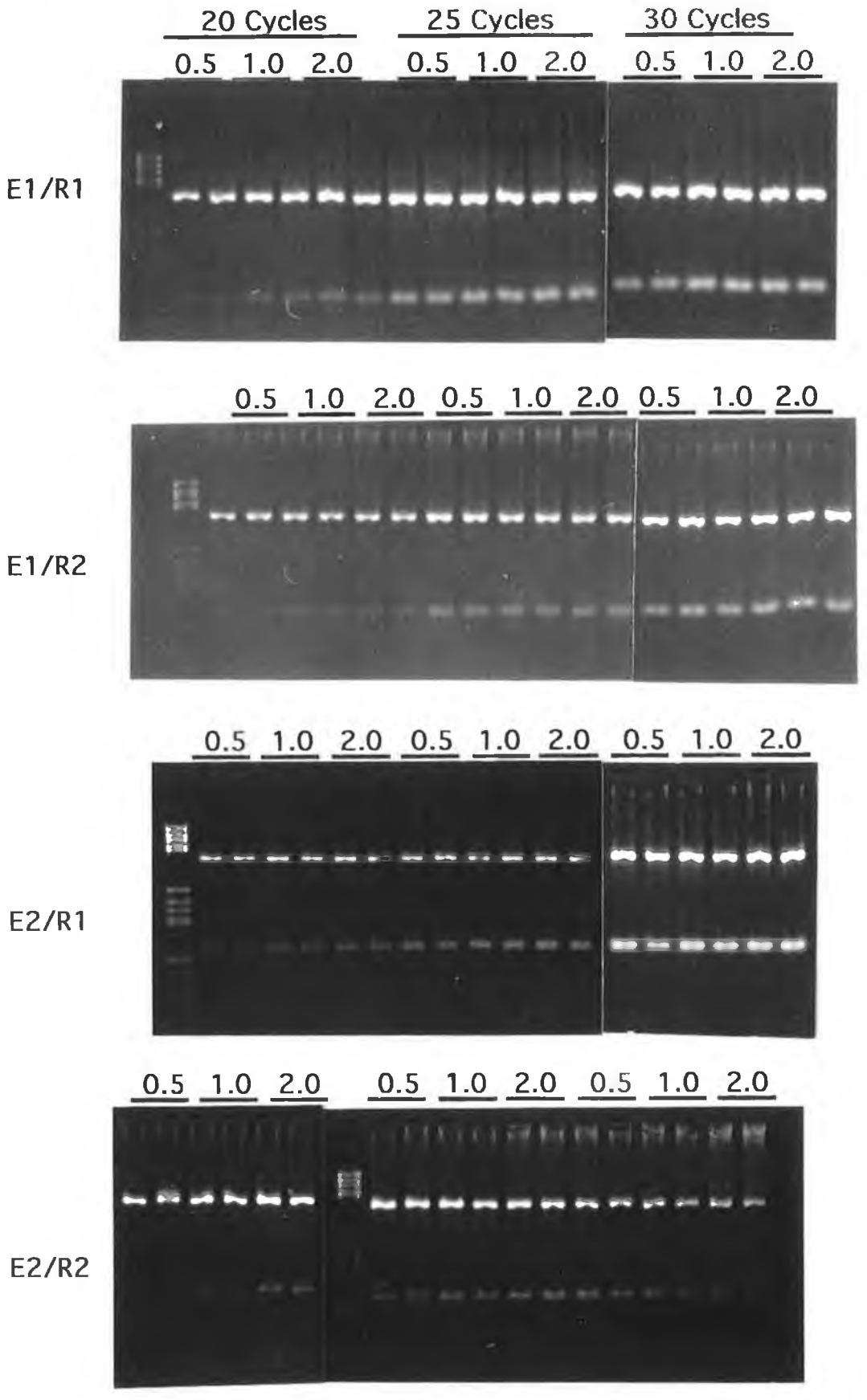


Fig. 3.5.1.11 Gel electrophoresis of the products of Topoisomerase IIα mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKP cultured cells and removing samples after three different PCR cycle time points. This analysis was done a total of four times.

Topo IIα (DLKP)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15			
	20	0.084	0.167	0.292
	25	0.336	0.363	0.431
	30	0.528	0.652	0.789
E1 / R2	15			
	20	0.100	0.250	0.417
	25	0.333	0.430	0.460
	30	0.464	0.500	0.542
E2 / R1	15			
	20	0.056	0.072	0.117
	25	0.287	0.332	0.393
	30	0.478	0.519	0.545
E2 / R2	15			
	20	0.191	0.250	0.371
	25	0.366	0.478	0.515
	30	0.411	0.508	0.566

Table 3.5.1.11.1 Analysis, by densitometry, of Topoisomerase IIα band intensities for DLKP, as in Fig. 3.5.1.11

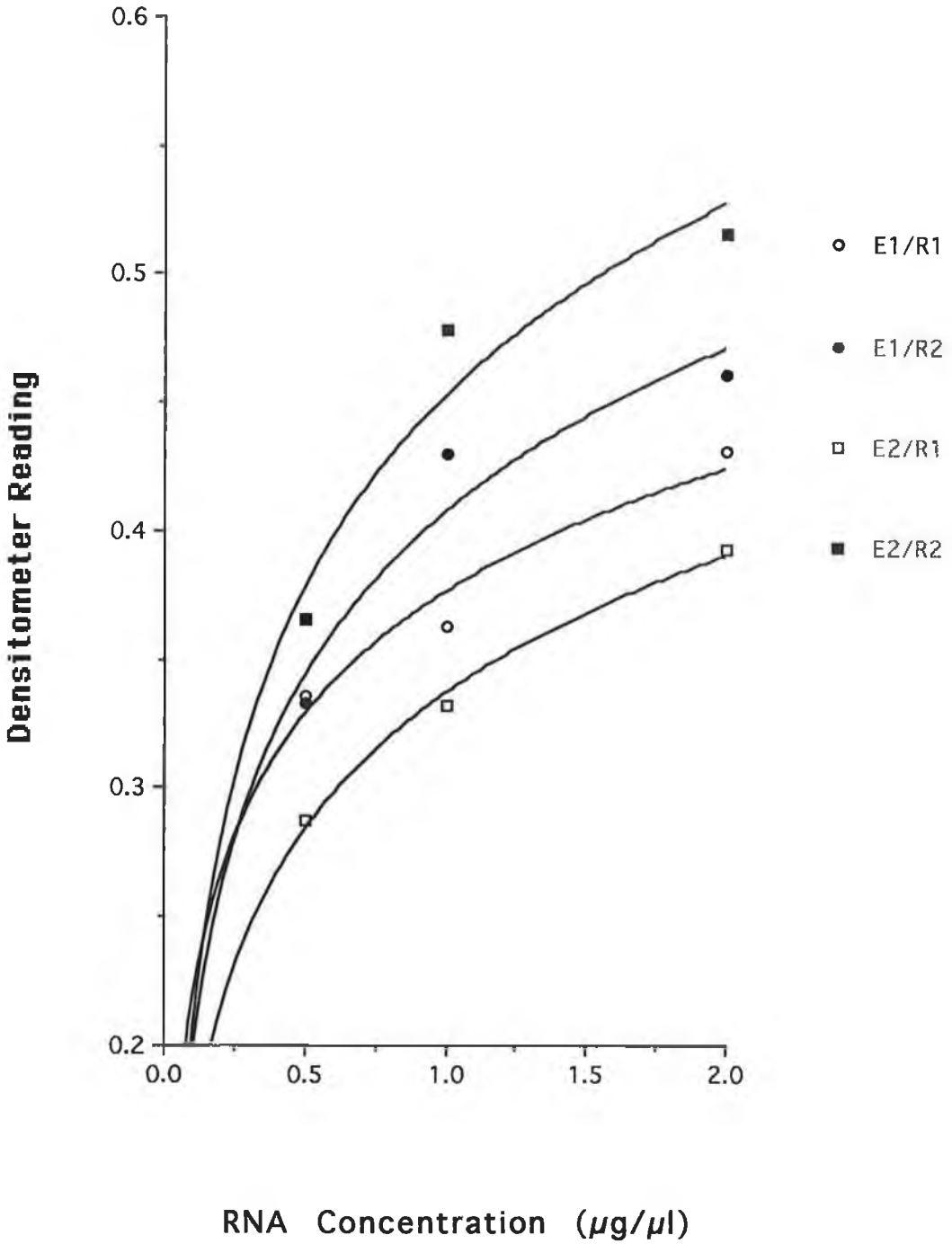


Fig. 3.5.1.11.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 25 cycles of amplification of Topoisomerase IIα cDNA derived from DLKP cultured cells.

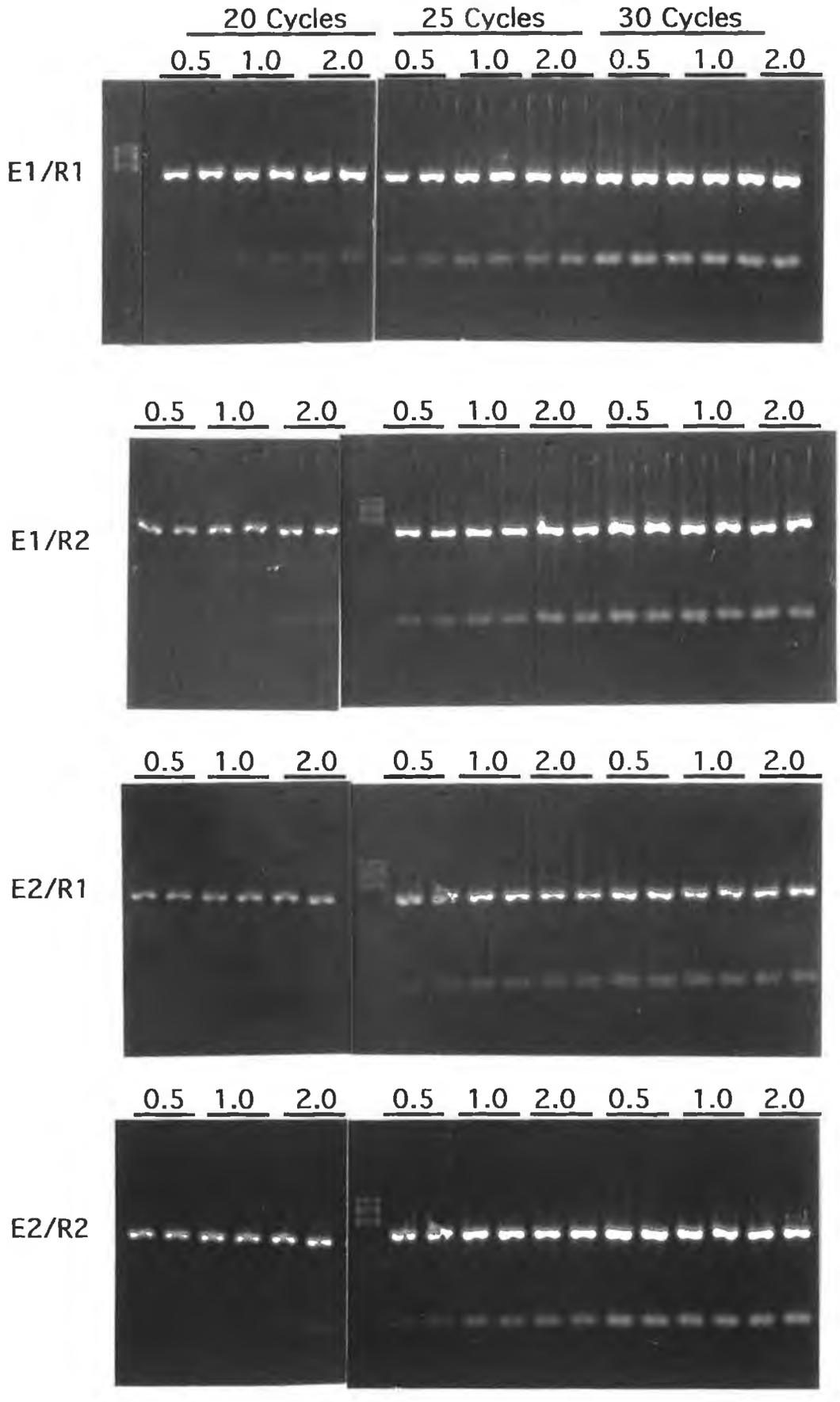


Fig. 3.5.1.12 Gel electrophoresis of the products of Topoisomerase IIα mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKPA cultured cells and removing samples after three different PCR cycle time points. This analysis was done a total of four times.

Topo IIα (DLKPA)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15			
	20	0.105	0.250	0.304
	25	0.225	0.367	0.407
	30	0.548	0.652	0.692
E1 / R2	15			
	20	0.000	0.000	0.052
	25	0.299	0.378	0.409
	30	0.405	0.438	0.598
E2 / R1	15			
	20	0.000	0.000	0.200
	25	0.191	0.250	0.345
	30	0.356	0.432	0.446
E2 / R2	15			
	20	0.083	0.149	0.184
	25	0.147	0.292	0.361
	30	0.349	0.371	0.481

Table 3.5.1.12.1 Analysis, by densitometry, of Topoisomerase IIα band intensities for DLKPA, as in Fig. 3.5.1.12

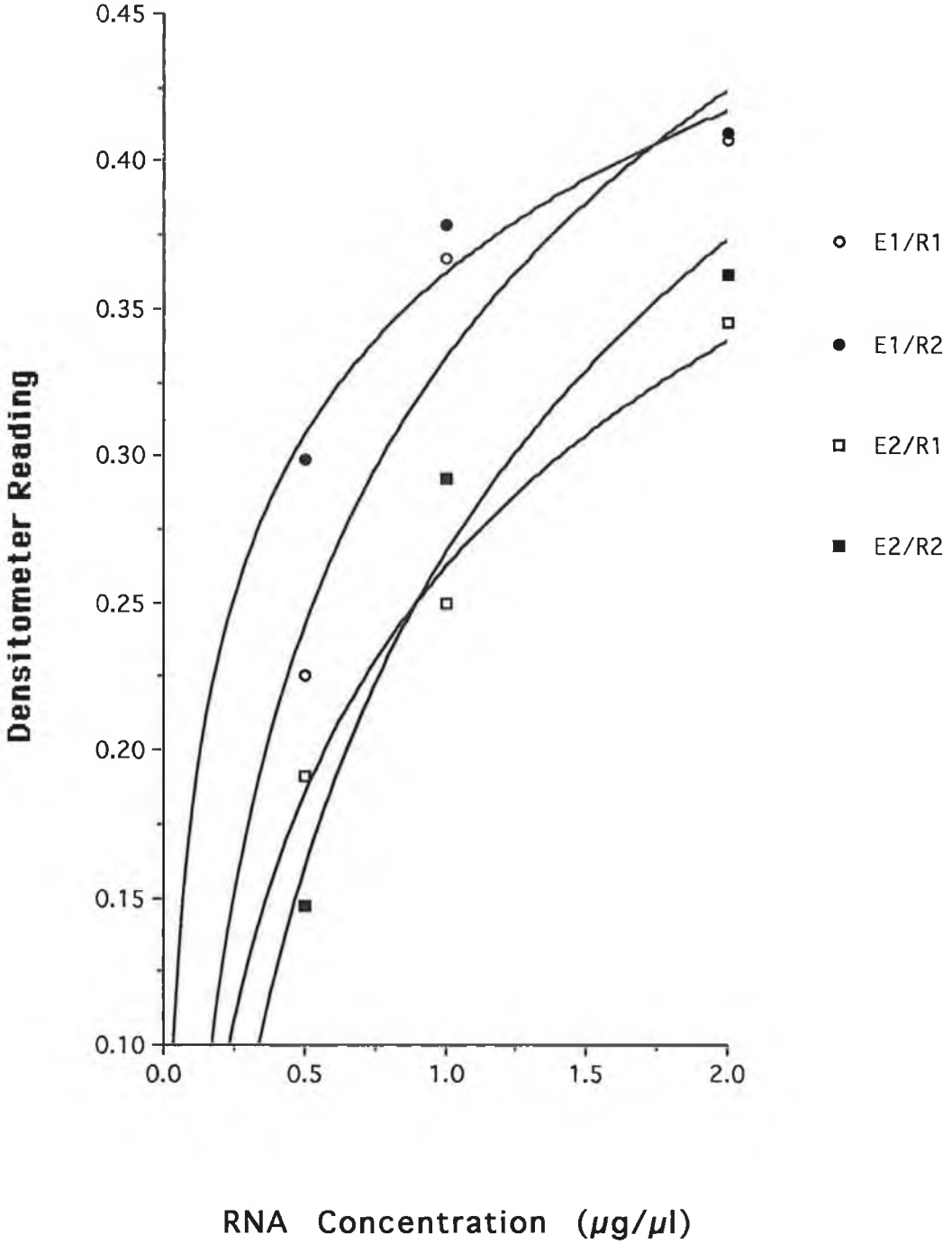


Fig. 3.5.1.12.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 25 cycles of amplification of Topoisomerase IIα cDNA derived from DLKPA cultured cells.

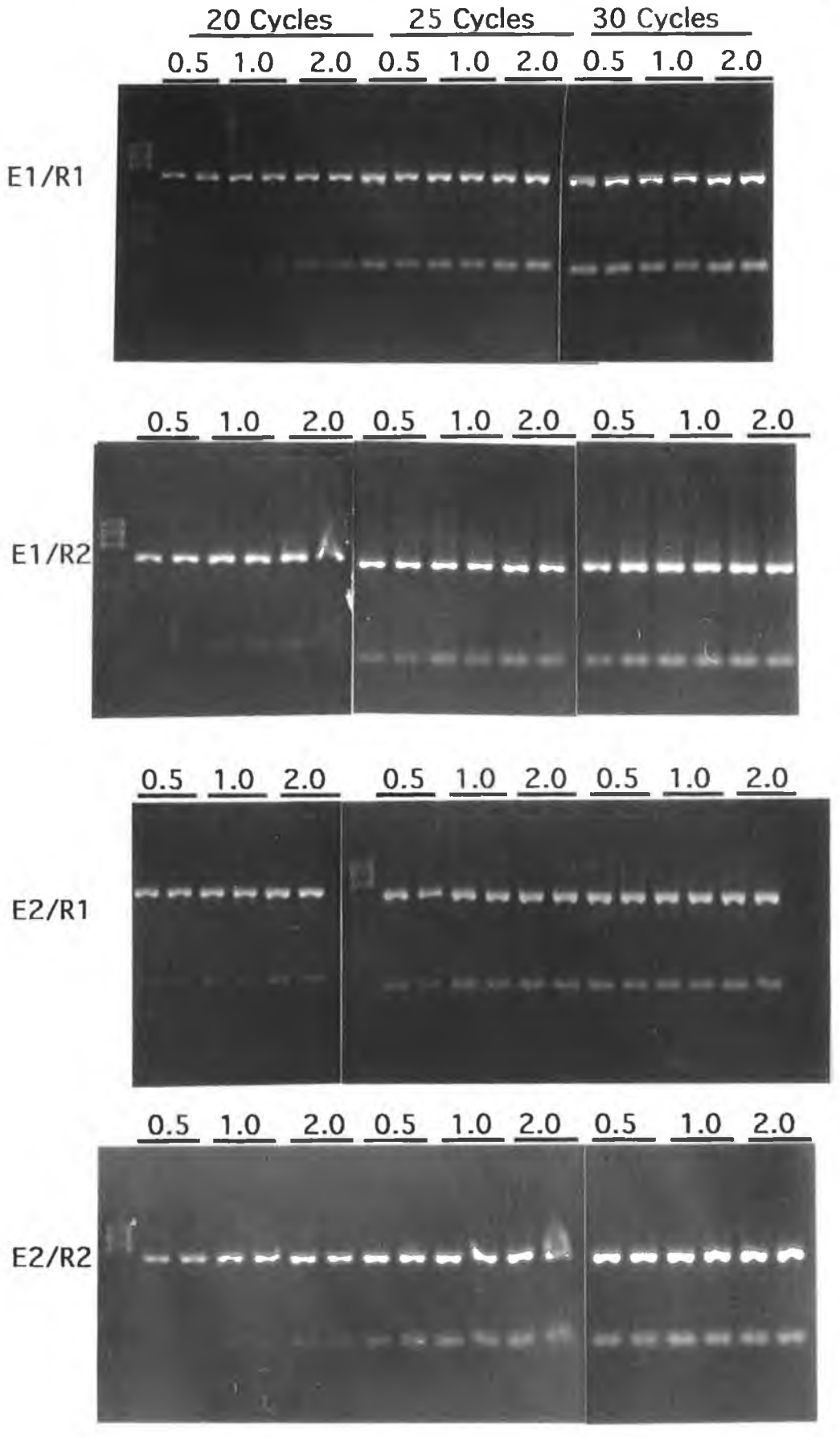


Fig. 3.5.1.13 Gel electrophoresis of the products of Topoisomerase IIα mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKP/VP-3 cultured cells and removing samples after three different PCR cycle time points. This analysis was done a total of four times.

Topo IIα (DLKP/VP-3)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15			
	20	0.238	0.268	0.422
	25	0.494	0.502	0.589
	30	0.555	0.664	0.698
E1 / R2	15			
	20	0.000	0.101	0.223
	25	0.385	0.407	0.481
	30	0.519	0.533	0.640
E2 / R1	15			
	20	0.101	0.175	0.282
	25	0.429	0.484	0.519
	30	0.533	0.649	0.677
E2 / R2	15			
	20	0.118	0.141	0.273
	25	0.308	0.417	0.440
	30	0.342	0.471	0.500

Table 3.5.1.13.1 Analysis, by densitometry, of Topoisomerase IIα band intensities for DLKP/VP-3, as in Fig. 3.5.1.13

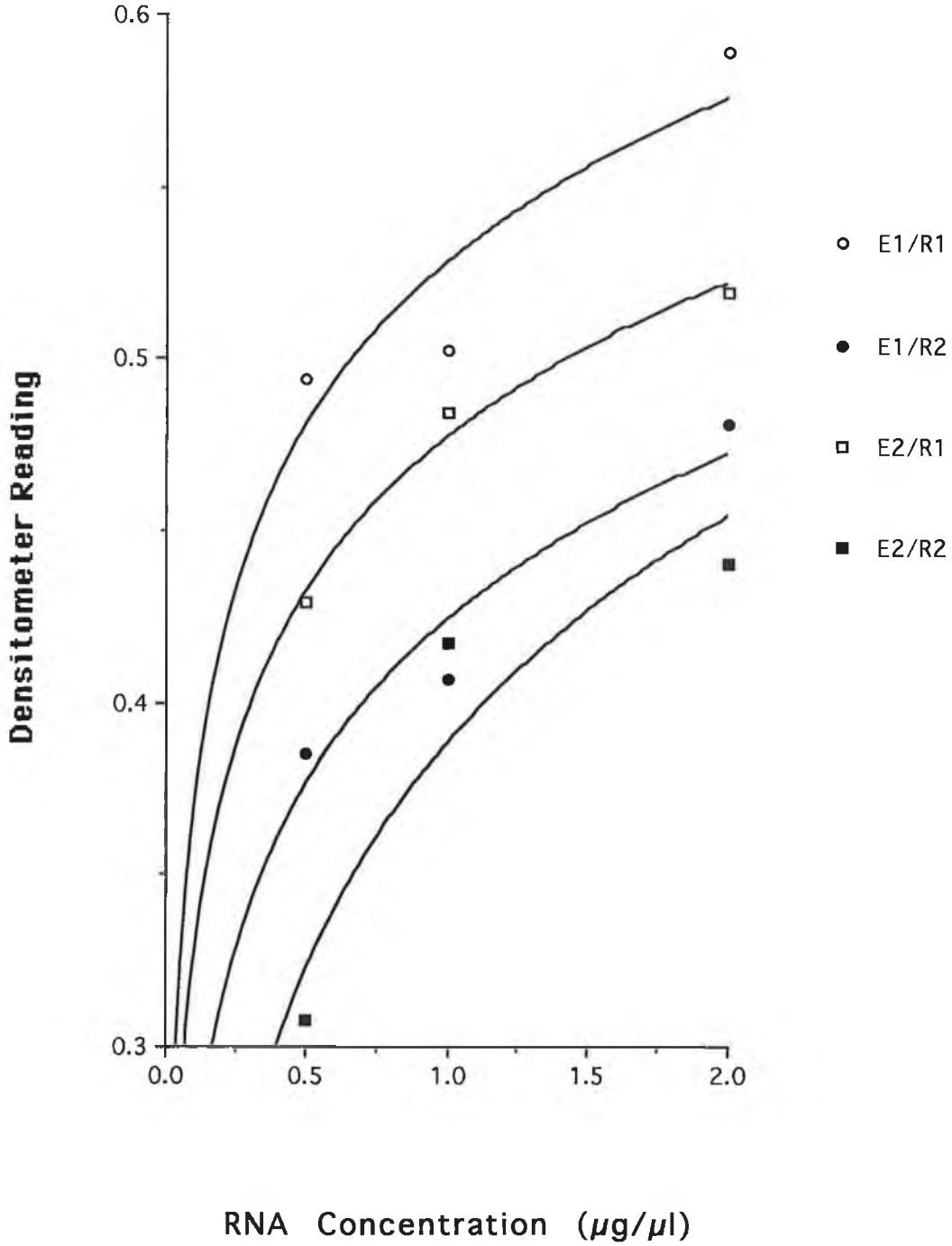


Fig. 3.5.1.13.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 25 cycles of amplification of Topoisomerase IIα cDNA derived from DLKP/VP-3 cultured cells.

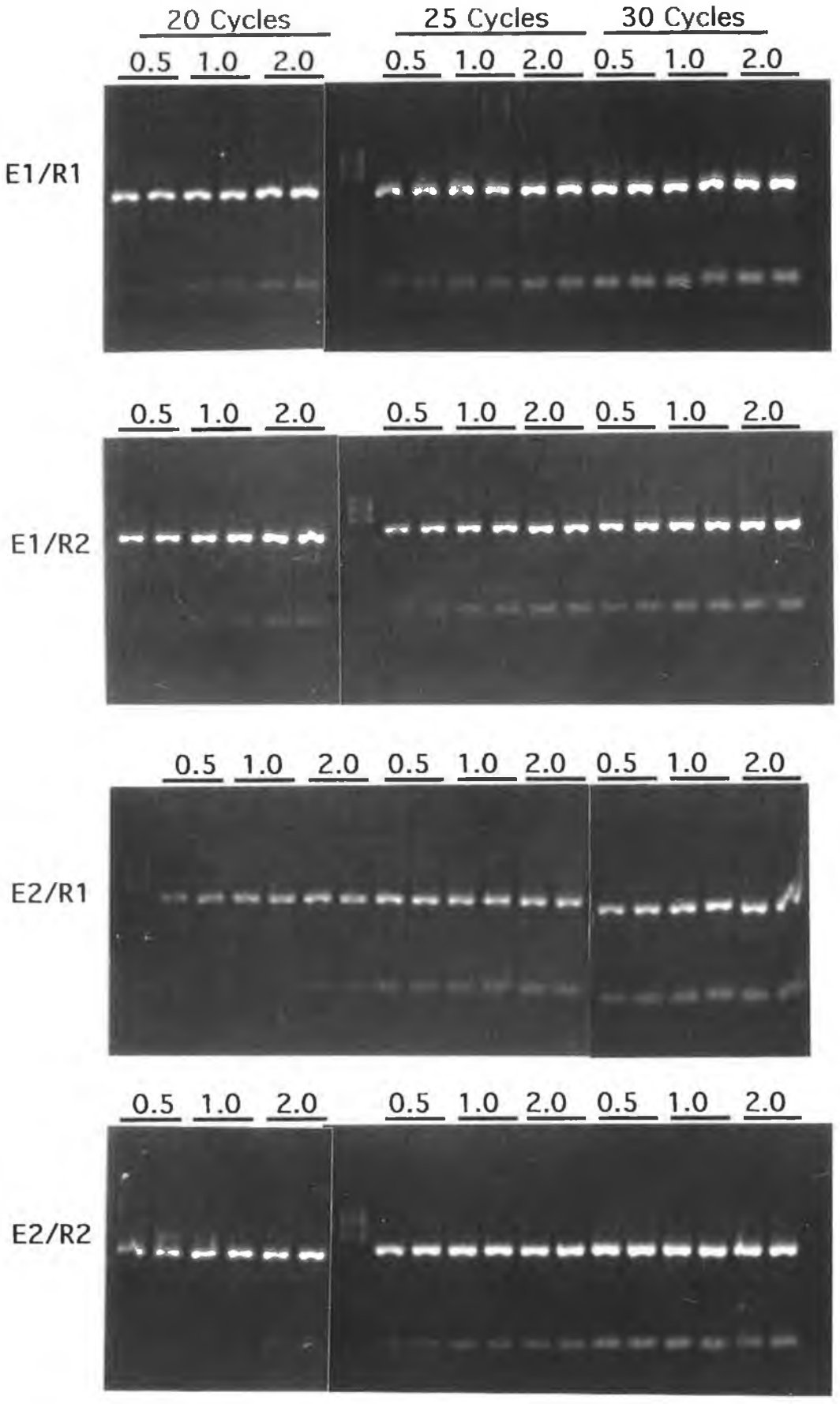


Fig. 3.5.1.14 Gel electrophoresis of the products of Topoisomerase IIα mRNA analysis by RT-PCR, using three template RNA concentrations (0.5, 1.0, 2.0 μg/μl) extracted from DLKP/VP-8 cultured cells and removing samples after three different PCR cycle time points. This analysis was done a total of four times.

Topo IIα (DLKP/VP-8)		RNA Concentration (μg / μl)		
Extraction/Run	Cycles	0.5	1.0	2.0
E1 / R1	15			
	20	0.150	0.250	0.332
	25	0.228	0.404	0.436
	30	0.542	0.615	0.654
E1 / R2	15			
	20	0.046	0.083	0.119
	25	0.177	0.313	0.345
	30	0.412	0.475	0.475
E2 / R1	15			
	20	0.111	0.150	0.191
	25	0.149	0.217	0.323
	30	0.528	0.556	0.565
E2 / R2	15			
	20	0.087	0.116	0.143
	25	0.227	0.333	0.400
	30	0.543	0.572	0.572

Table 3.5.1.14.1 Analysis, by densitometry, of Topoisomerase IIα band intensities for DLKP/VP-8, as in Fig. 3.5.1.14

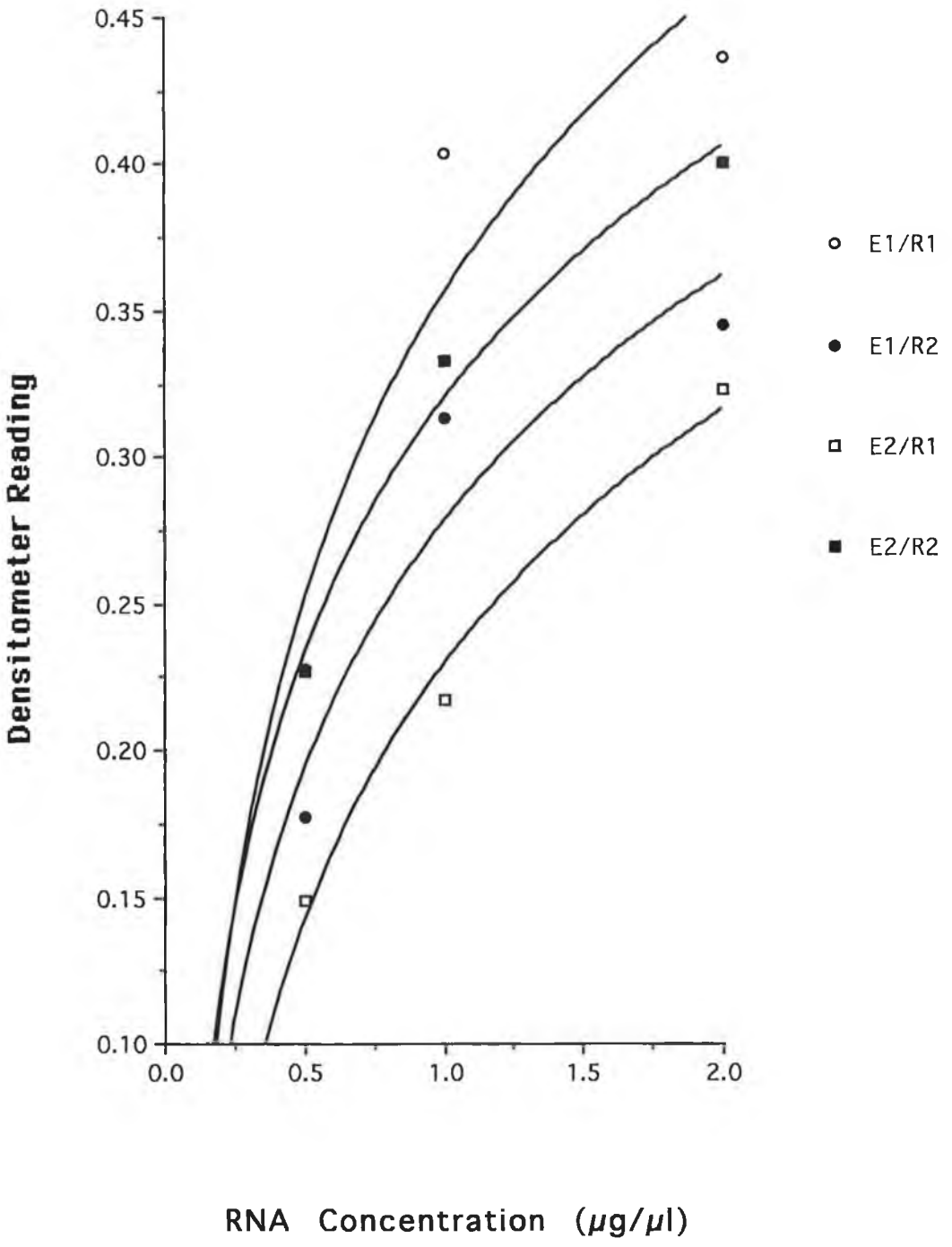


Fig. 3.5.1.14.2 Relationship between RNA concentrations and densitometry readings of resulting band intensities, after 25 cycles of amplification of Topoisomerase IIα cDNA derived from DLKP/VP-8 cultured cells.

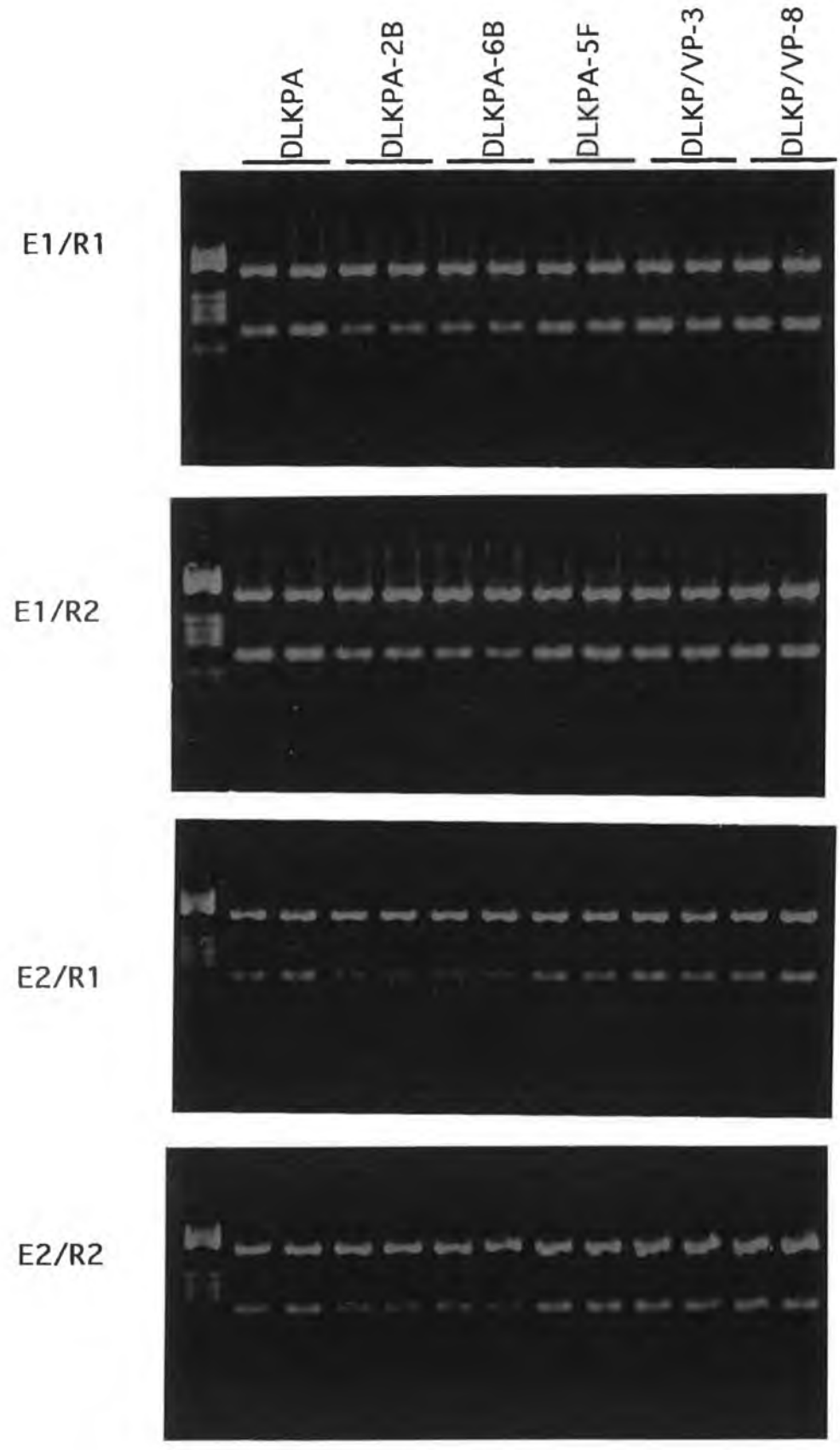


Fig. 3.5.1.15 MDR 1 mRNA levels in DLKPA, DLKPA-2B, DLKPA-6B, DLKPA-5F, DLKP/VP-3 and DLKP/VP-8 cells were studied, simultaneously, using 1.0 $\mu g/\mu l$ RNA as template in the RT reaction and performing 20 cycles of PCR amplification. The β-actin (383 bp) and MDR 1 (157 bp) amplification products were separated by electrophoresis through a 4 % agarose gel. Their sizes were verified by comparison to molecular weight markers, ranging from 587 bp to 8 bp (left-hand lane). The analysis was performed for each of the cell lines (mentioned above) a total of four times *i.e.* on duplicate stocks of cDNA formed from two RNA extracts of independent cell line stocks. These were designated E1/R1, E1/R2, E2/R1 and E2/R2. All reactions were performed in duplicate. The results were analysed by densitometry (Table 3.5.1.15.1).

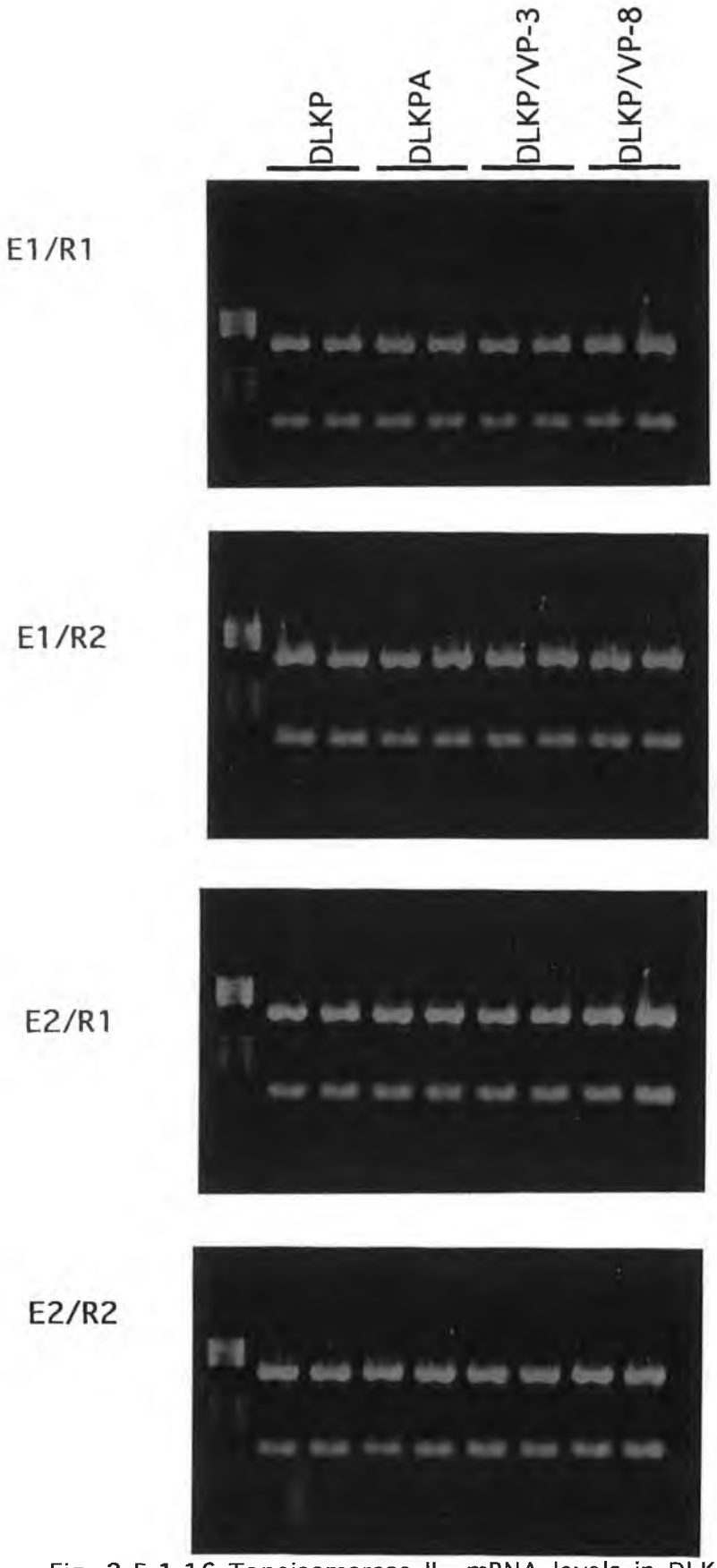


Fig. 3.5.1.16 Topoisomerase IIα mRNA levels in DLKP, DLKPA, DLKP/VP-3 and DLKP/VP-8 cells were studied, simultaneously, using 1.0 μg/μl RNA as template in the RT reaction and performing 25 cycles of PCR amplification. The β-actin (383 bp) and Topoisomerase IIα (139 bp) amplicons were separated by electrophoresis through a 4 % agarose gel. Their sizes were verified by comparison to molecular weight markers, ranging from 587 bp to 8 bp (left-hand lane). The analysis was performed for each of the cell lines (mentioned above) a total of four times i.e. on duplicate stocks of cDNA formed from two RNA extracts of independent cell line stocks. These were designated E1/R1, E1/R2, E2/R1 and E2/R2. All reactions were performed in duplicate. The results were analysed by densitometry (Table 3.5.1.16.1).

Cell Line/Clone	E1/R1	E1/R2	E2/R1	E2/R2
DLKPA	0.600	0.610	0.494	0.488
DLKPA-2B	0.420	0.497	0.338	0.310
DLKPA-6B	0.486	0.490	0.318	0.362
DLKPA-5F	0.610	0.608	0.492	0.487
DLKP/VP-3	0.604	0.606	0.490	0.485
DLKP/VP-8	0.600	0.603	0.487	0.484

Table 3.5.1.15.1 Densitometry analsyis (after background subtraction and normalising with β-actin) of MDR 1 mRNA levels in MDR variants of DLKP, after 20 cycles of PCR amplification.

Cell Line/Clone	E1/R1	E1/R2	E2/R1	E2/R1
DLKP	0.646	0.648	0.678	0.666
DLKPA	0.640	0.644	0.670	0.662
DLKP/VP-3	0.642	0.645	0.672	0.658
DLKP/VP-8	0.642	0.641	0.669	0.657

Table 3.5.1.16.1 Densitometry analsyis (after background subtraction and normalising with β-actin) of Topoisomerase IIα mRNA levels in DLKP and its MDR variants, after 25 cycles of PCR amplification.

Assessing the variations between products from different RT-PCR analyses from the same batch of RNA *e.g.* E1/R1 and E1/R2 and between different batches of RNA *i.e.* E1 and E2 from the same cell line was quite difficult, for two reasons. Firstly, inconsistant printing may cause bias in the photographs which would hinder visual analysis (negatives were favoured for densitometry analysis). Secondly, it seemed that although the relative values obtained by densitometry analysis were consistent, the actual values differed greatly, even on repeat analysis of the same gel). This implies that readings cannot by directly compared from one gel (negative) to another. Since inconsistent printing from one photograph to another affects the β-actin bands as well as the MDR 1 bands, some visual assessment of the reproducibility was made.

By visual analysis of MDR 1, Topoisomerase II and Topoisomerase IIα mRNA levels, the reproducibility of the these studies was generally found to be quite consistent between RNA extracts (E1 and E2), and between different RT-PCR analyses (R1 and R2). An example of this is Fig. 3.5.1.2. In some cases, *e.g.* Fig. 3.5.1.3, good reproducibility between studies on a given extract was found, but not from one extract to another. The lower levels of RNA apparently in one extract by comparison to another were generally found with both β-actin and MDR 1. As all measurements of RNA concentration were performed in the same way (by spectrophotometry), it is unlikely that this difference is due to different RNA concentrations being included in respective RT reactions. This suggested either slight degradation of the RNA prior to analysis, or inconsistent printing of the resulting negatives were responsible for this difference. (Differences in printing were generally also observed with the molecular weight marker). Overall, these, generally consistent results, suggest that both the RT and PCR reactions are reproducible.

3.5.2 Analysis of Densitometry Efficiency

From many of the preceding results, although the densitometry readings often were linearly related to the intensities of the bands they represented, the relationship, according to these readings was not proportional to the starting RNA concentrations. This suggested that possibly the data produced by the densitometer was not completely accurate.

In an attempt to check the accuracy of the densitometer for the analysis of products differing by approximately two-fold, serial dilutions of DNA of which the actual concentrations were known (Lambda DNA and molecular weight marker V) were electrophoresed on agarose gels, photographed and analysed. The DNA concentrations were plotted with the corresponding densitometry data. Each DNA concentration was electrophoresised and analysed in duplicate. This study was repeated four times for lambda and twice for molecular weight marker V. Photographs of the resulting gels after lambda DNA electrophoresis are shown in Fig. 3.5.2.1. Although approximately two-fold concentration differences are visible on the gel, this was not reflected in the densitometry readings, as illustrated in Fig. 3.5.2.2. A plot of similar shape resulted from both analysis of the molecular weight marker V DNA (Fig. 3.5.1.3).

Results from this study suggest that the analysis by densitometry, and not the RT or PCR efficiencies, was the limiting factor when studying MDR 1, Topoisomerase II and Topoisomerase IIα transcript levels, as described in 3.5. The plotted results can be used, therefore, as a standard curve from which the relative concentrations of an unknown RNA can be estimated, if it is within the range of these plots. Due to another limitation of the densitometer, however, if a certain gel is analysed repeatedly, although the same trend of values may result (*i.e.* the same relative values), the absolute values may vary. Therefore, if an unknown sample is to be estimated from such a standard curve, it should be electrophoresed and analysed by densitometry with the "standards".

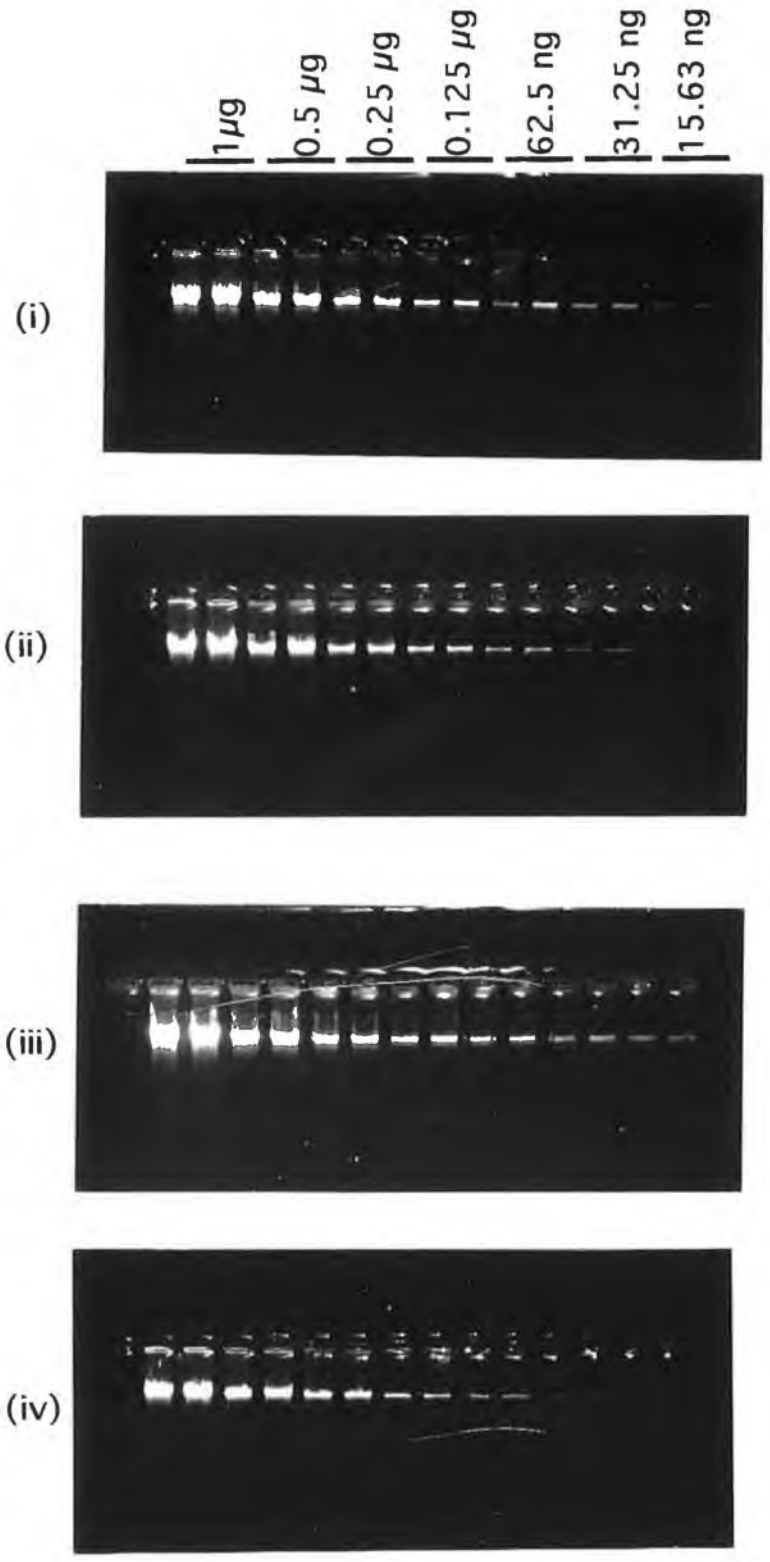
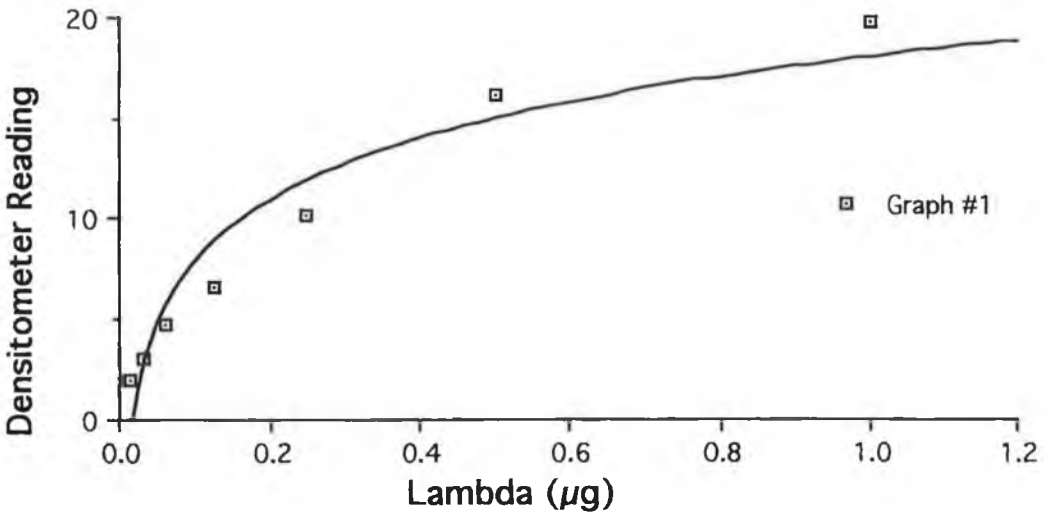


Fig. 3.5.2.1 Gel electrophoresis of lambda DNA serial dilutions. Four independent serial dilutions (i)-(iv), ranging from 1 µg to 15.63 ng of DNA (1 µg, 0.5 µg, 0.25 µg, 0.125 µg, 62.5 ng, 31.25 ng and 15.63 ng) were made and were ran out on a 1 % agarose gel, in duplicate.

(i)



(ii)

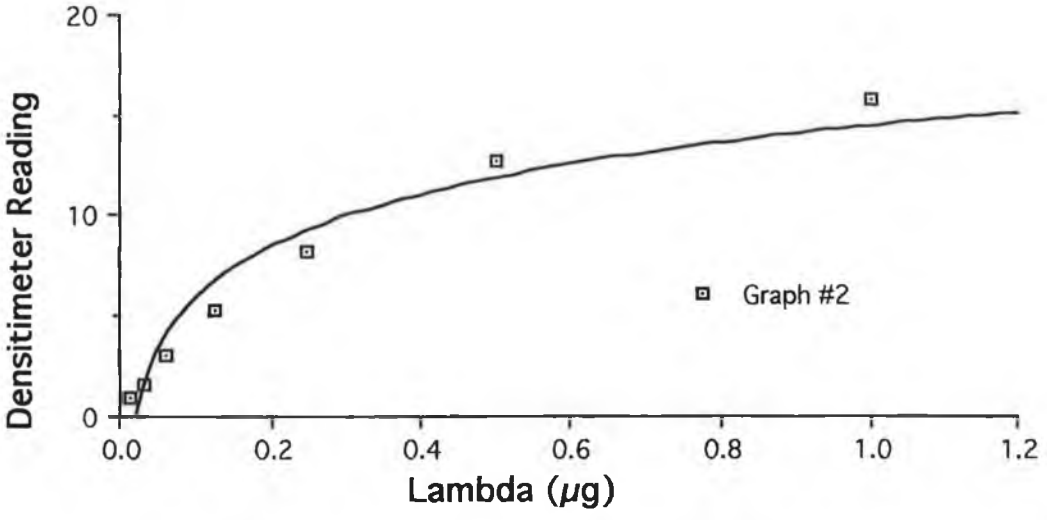
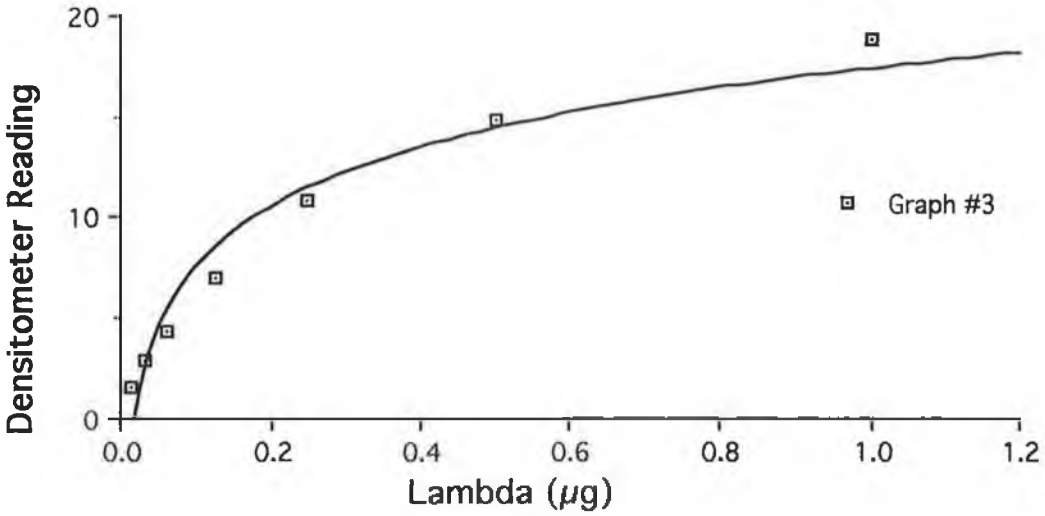


Fig. 3.5.2.2 Relationship between the lambda DNA concentration and intensity of the resulting bands represented by the densitometry readings (after background subtraction). The values plotted are the means for each of the duplicate readings. Graphs (i), (ii), (iii) and (iv) correspond to gels (i), (ii), (iii) and (iv) in Fig. 3.5.2.1, respectively.

(iii)



(iv)

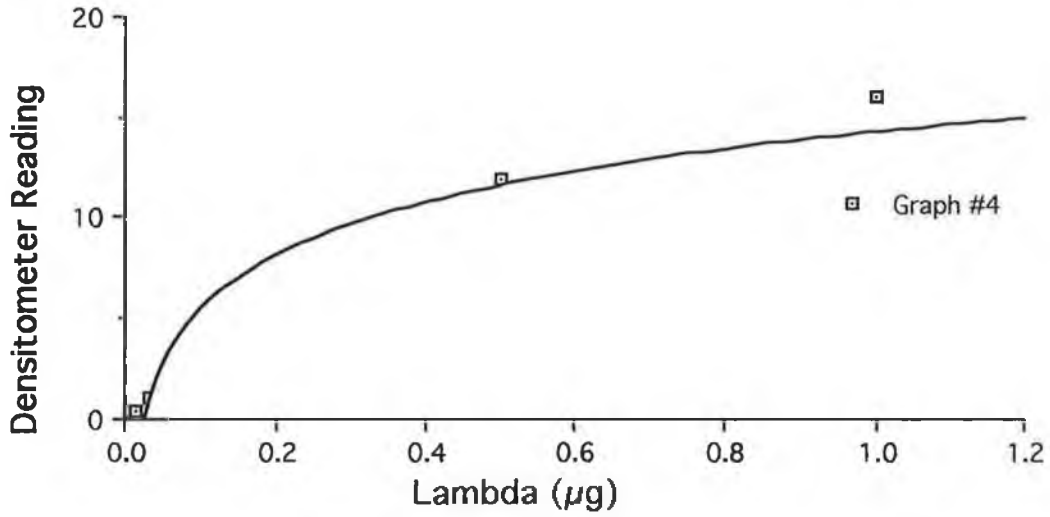


Fig. 3.5.2.2 CONTINUED

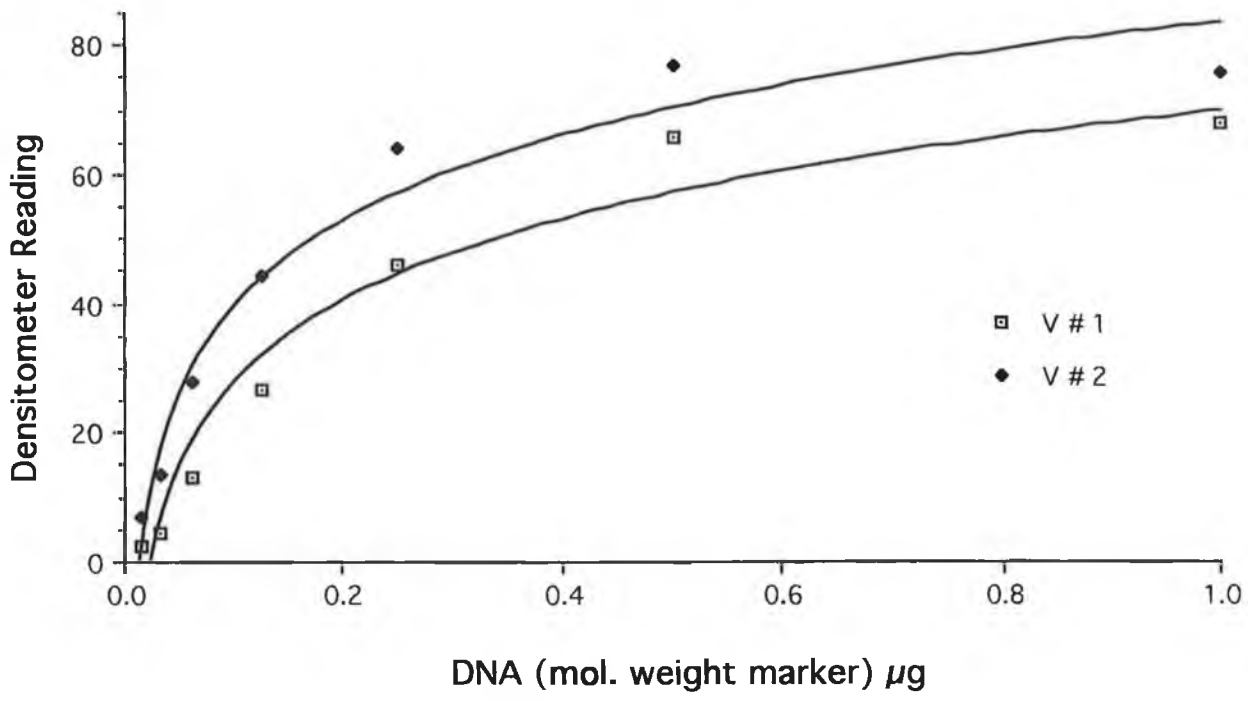


Fig. 3.5.2.3 Serial dilutions of a molecular weight marker (V, Boehringer Mannheim), ranging from 1 μg to 15.63 ng (1 μg, 0.5 μg, 0.25 μg, 0.125 μg, 62.5 ng, 31.25 ng and 15.63 ng) were analysed by densitometry after electrophoresis through a 1 % agarose gel. The values plotted are the DNA (molecular weight marker) concentrations *versus* the means of the corresponding densitometry readings, after background subtraction.

3.5.3 Semi-Quantitative Analysis of MDR 1 mRNA using a range of RNA concentrations and cDNA amounts

In order to verify that the densitometer, and not the efficiency of the RT and/or PCR reactions, was the major limiting factor in this semi-quantitative analysis, a range of RNA concentrations and resulting cDNA amounts were analysed. If similar shaped graphs were produced from this analysis as were produced when testing out the densitometer (as in 3.5.2), and yet the bands were visually as expected, this would strongly suggest that the densitometer is the "limiting factor" in these studies, and not either the RT or PCR reactions.

DLKPA-2B and DLKPA-5F were the two cell lines included in this study of MDR 1 mRNA, as they seemed (based on the preliminary data presented above) to possess relatively low and high levels of this gene transcript, respectively. 20 cycles of PCR amplification were performed throughout this phase of the study. In all cases, the densitometry readings plotted were the result of background subtraction and normalising with β-actin (Gel (i), in each figure). Amplifications of the MDR 1 and β-actin transcripts, independently, were also done in most cases (Gels (ii) and (iii), respectively) to ensure, by comparison to the gels resulting after co-amplification of these transcripts, that neither amplified product was limited due to the presence of the other (Gel (i)).

The initial run for DLKPA-2B (E1/R1 *i.e.* extract no. 1; RT-PCR analysis no. 1) was performed on only two RNA concentrations (0.5 μg/μl and 2.0 μg/μl) to establish if a difference in band intensities was apparent (Fig. 3.5.3.1). As indicated in Fig. 3.5.3.1.1, the results after densitometry analysis were indicative of a linear relationship between RNA concentrations and between cDNA amounts, but not proportionally. However, by visual analysis of the MDR 1 bands, in particular, this relationship between the bands frequently seemed to be proportional to the starting RNA and cDNA concentrations. Using similar RNA concentrations (0.5 μg/μl, 1.0 μg/μl and 2.0 μg/μl

and the three different amounts of cDNA), a similar trend was noted for DLKPA-5F at the lowest cDNA concentrations. However, because this cell line expressed relatively high levels of MDR 1 compared to DLKPA-2B, the resulting band intensities were much stronger with this cell line, and so not as easily analysed. The results from this are shown in Figs. 3.5.3.2 and 3.5.3.2.1. Probably due to the greater intensities of the MDR 1 bands formed on analysis of the DLKPA-5F RNA, the relationship between RNA concentration and densitometry readings was even less obvious (both by densitometry and visual analysis). When plotted, the curves resulting from the three RNA concentrations using 5 μl cDNA reached a plateau almost immediately. A range-finding experiment was therefore performed for this cell line, after which it was realised that lower concentrations of RNA template were more suitable for its semi-quantitative study. In the following experiments, 0.125 μg/μl, 0.25 μg/μl and 0.5 μg/μl of RNA extracted from the DLKPA-5F cell line were analysed. The higher RNA concentrations, 0.5 μg/μl, 1.0 μg/μl and 2.0 μg/μl were used throughout, when studying the DLKPA-2B cell line.

In the resulting experiments on the DLKPA-2B cell line, Figs. 3.5.3.3 - 3.5.3.5.1, a linear relationship between both RNA concentration and densitometry reading, and cDNA amount and densitometry reading, was observed, although not proportionally. Again, by visual analysis a proportional relationship frequently did seem to exist. This was seen for example in Fig. 3.5.3.3 where fold-differences were apparent with both different RNA and cDNA concentrations. (Similar results were produced for DLKPA-5F). Although the bands produced on the gel were weaker for the two studies on the second RNA extracts, compared to the first, after normlising with β-actin, similar curves resulted. This suggests that the MDR 1 mRNA is expressed at the same levels in both cell lines, as is the β-actin gene transcript. The reduced levels of both in the second RNA extracts may be indicative of partial degradation. The importance of including an endogenous control, such as β-actin, is emphasised by the fact that normalising the MDR 1 band with the corresponding β-actin in

both extracts, gave similar results. The results from studies using 2.5 μl cDNA and 5.0 μl cDNA are almost parallel in all cases and seem to represent the actual gel bands more accurately, by comparison to those resulting from 1 μl cDNA. This suggests that the bands produced by the latter are possibly too weak to be detected by the densitometer.

As with the DLKPA-2B cell line, the bands produced on analysis of the DLKPA-5F second RNA extract (Figs. 3.5.3.8.1 and 3.5.3.9.1), were weaker, although relatively the same as those from the first extract (Figs. 3.5.3.6.1 and 3.5.3.7.1). As in most of the DLKPA-2B studies, the curves resulting when plotting DLKPA-5F RNA concentration *versus* densitometry readings are parallel. This, again, supports the idea that the RT and PCR reactions are equally efficient with each RNA concentration and cDNA amount, and that it is the densitometry analysis that is preventing the proportional relationship be identified.

From these studies, the levels of MDR 1 mRNA seemed to be more than four-fold that in the DLKPA-2B cells. This is because the initial concentration of RNA analysed for DLKPA-2β was four times that for DLKPA-5F, and yet slightly stronger bands seemed to be produced for DLKPA-5F. However, as these products were not run out on a gel together no definite statement about the relationship between their RNA levels can be made.

Figs. 3.5.3.1 - 3.5.3.8 MDR 1 mRNA levels in DLKPA-2B and DLKPA-5F were studied by semi-quantitative PCR analysis using a range of three RNA concentrations (except for DLKPA-2B, Fig. 3.5.3.1, where two RNA concentrations were analysed) and three amounts of resulting cDNA (equivalent to 1 μl, 2.5 μl and 5.0 μl), formed during the RT reaction in each case. This was done to establish if the MDR 1 bands, resulting after RT-PCR, were indicative of the corresponding starting RNA concentrations and cDNA amounts. After preliminary analyses were performed (Fig. 3.5.3.1 for DLKPA-2B, and Fig. 3.5.3.2 for DLKPA-5F), RNA from each of the cell lines was studied a total of four times *i.e.* duplicate stocks of cDNA were formed from two RNA extracts of independent cell line stocks - designated E1/R1, E1/R2, E2/R1 and E2/R2. The RNA template concentration for DLKPA-2B cells were 0.5 μg/μl, 1.0 μg/μl and 2.0 μg/μl. For DLKPA-5F, the RNA concentrations were 0.125 μg/μl, 0.25 μg/μl and 0.5 μg/μl.This study involved (i) co-amplifying β-actin (383 bp) and MDR 1 cDNA (157 bp), and individually amplifying (ii) MDR 1 and (iii) β-actin cDNA, respectively. 10 μl aliquots of the products resulting after 20 cycles of PCR amplification were separated by gel electrophoresis through a 4 % agarose gel. Bands were identified with respect to molecular weight markers (left-hand lane in each gel) ranging from 587 to 8 base pairs in size. All reactions were performed in duplicate.

Figs. 3.5.3.1.1 - 3.5.3.9.1 Relationship between template RNA concentrations and densitometry readings after correction (by background subtraction and normalising with β-actin) of the bands resulting from RT-PCR analysis of MDR 1 mRNA, for each of the cDNA dilutions (as illustrated in Figs. 3.5.3.1 - 3.5.3.8).

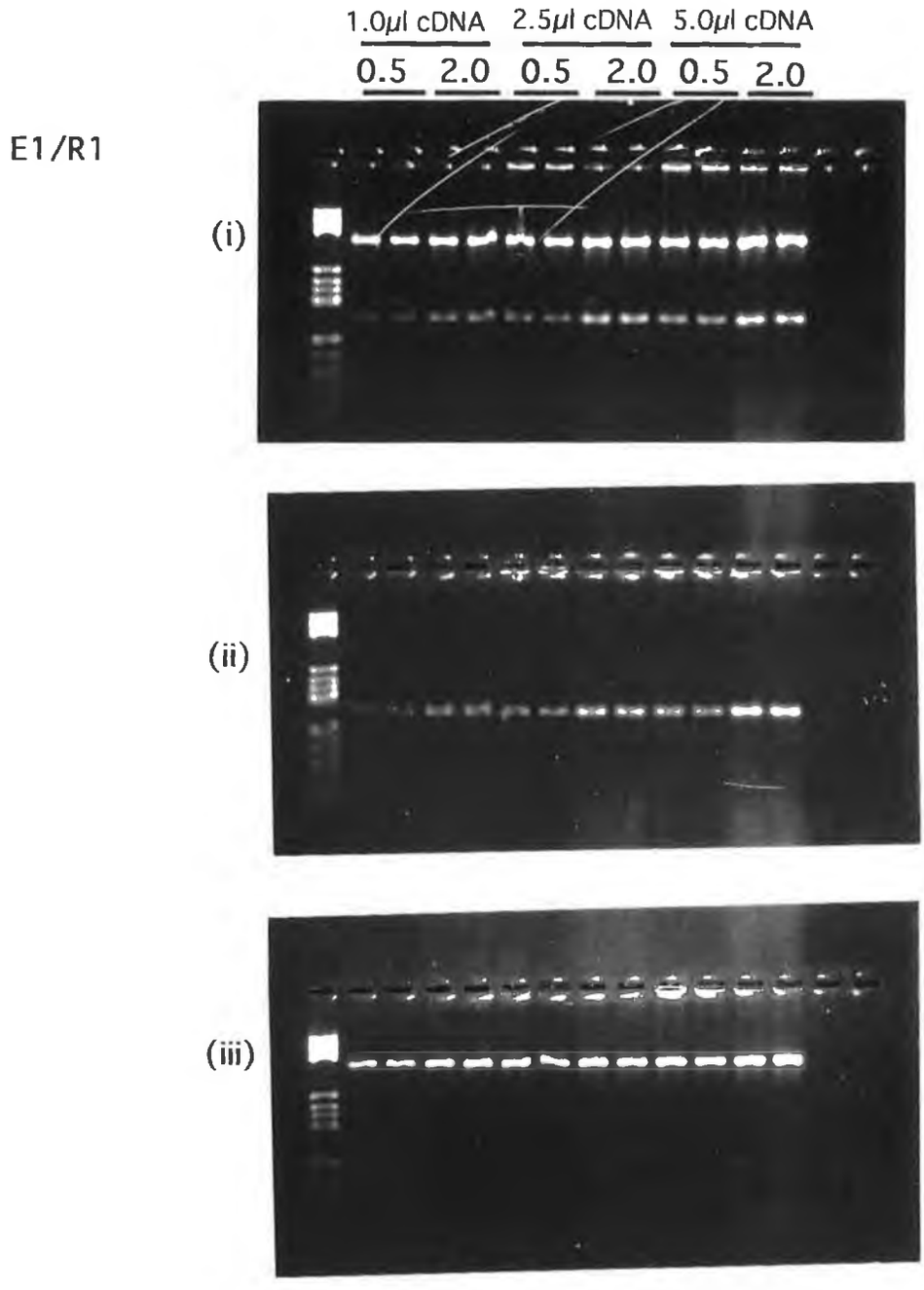


Fig. 3.5.3.1 Gel electrophoresis of products resulting from RT-PCR amplification of β-actin (383 bp) and MDR 1 (157 bp) cDNA formed on RNA templates extracted from DLKPA-2B cultured cells. ((i) is co-amplification of β-actin and MDR 1; (ii) and (iii) are independant amplifications of MDR 1 and β-actin, respectively). The amounts of cDNA (1 μl, 2.5 μl and 5.0 μl) formed on RNA templates at two concentrations (0.5 μg/μl and 2.0 μg/μl) were analysed (E1/R1).

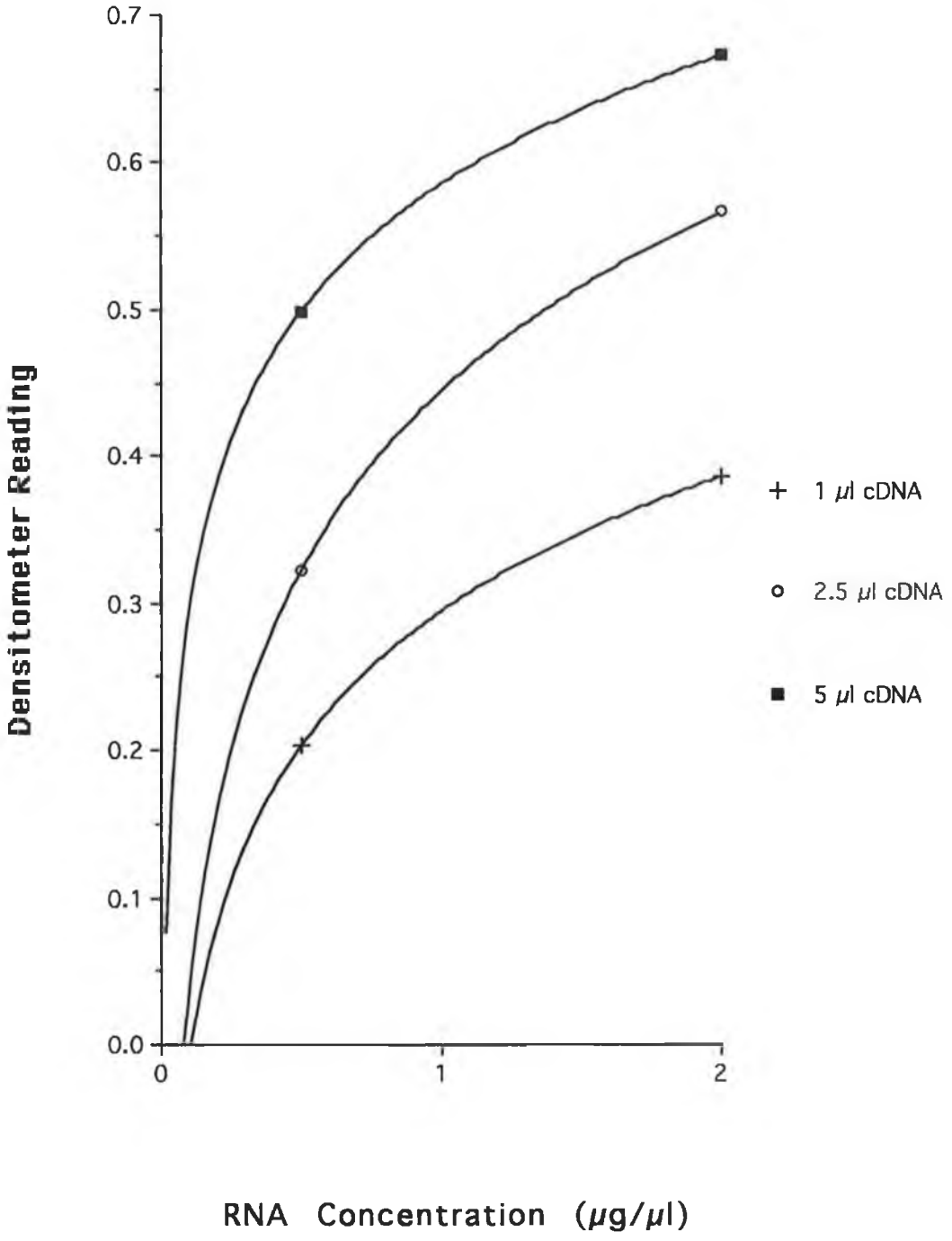


Fig. 3.5.3.1.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-2B, as in Fig. 3.5.3.1.

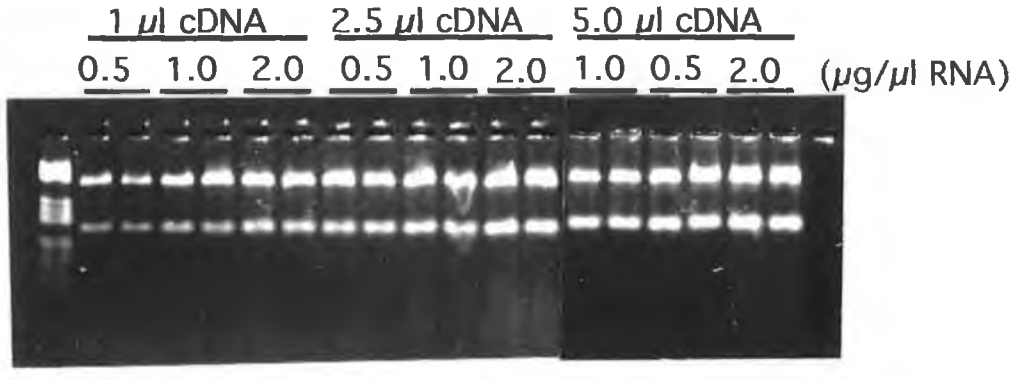


Fig. 3.5.3.2 Gel electrophoresis of products resulting from RT-PCR co-amplification of β-actin (383 bp) and MDR 1 (157 bp) cDNA formed on RNA templates extracted from DLKPA-5F cultured cells. Amounts of cDNA (1 µl, 2.5 µl and 5.0 µl) formed on RNA templates at three concentrations (0.5 µg/µl, 1.0 µg/µl and 2.0 µg/µl) were analysed. Increasing band intensities with increasing RNA concentrations and cDNA amounts, were noted. This was most obvious at lower RNA and cDNA concentrations.

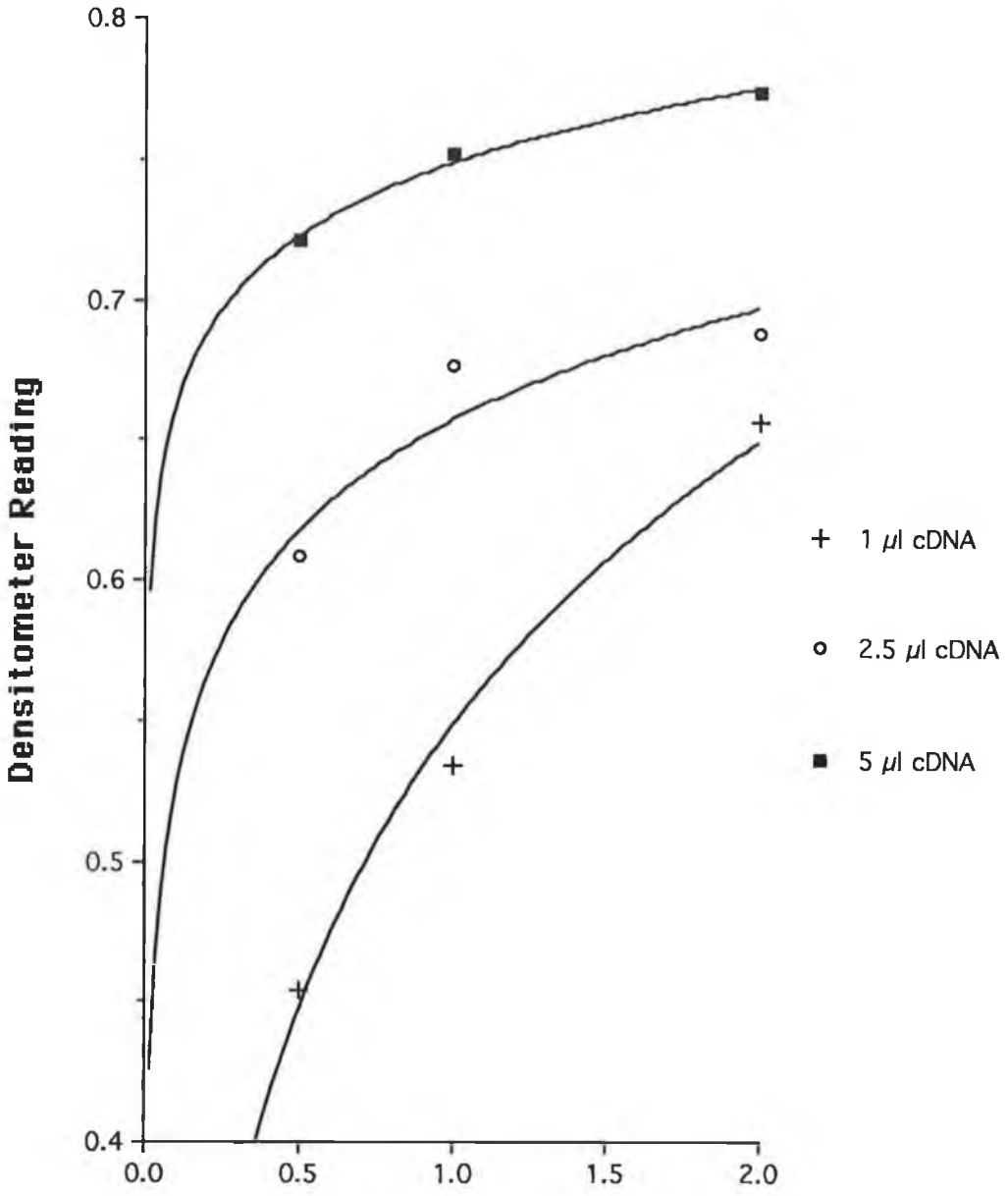


Fig. 3.5.3.2.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-5F, as in Fig. 3.5.3.2.

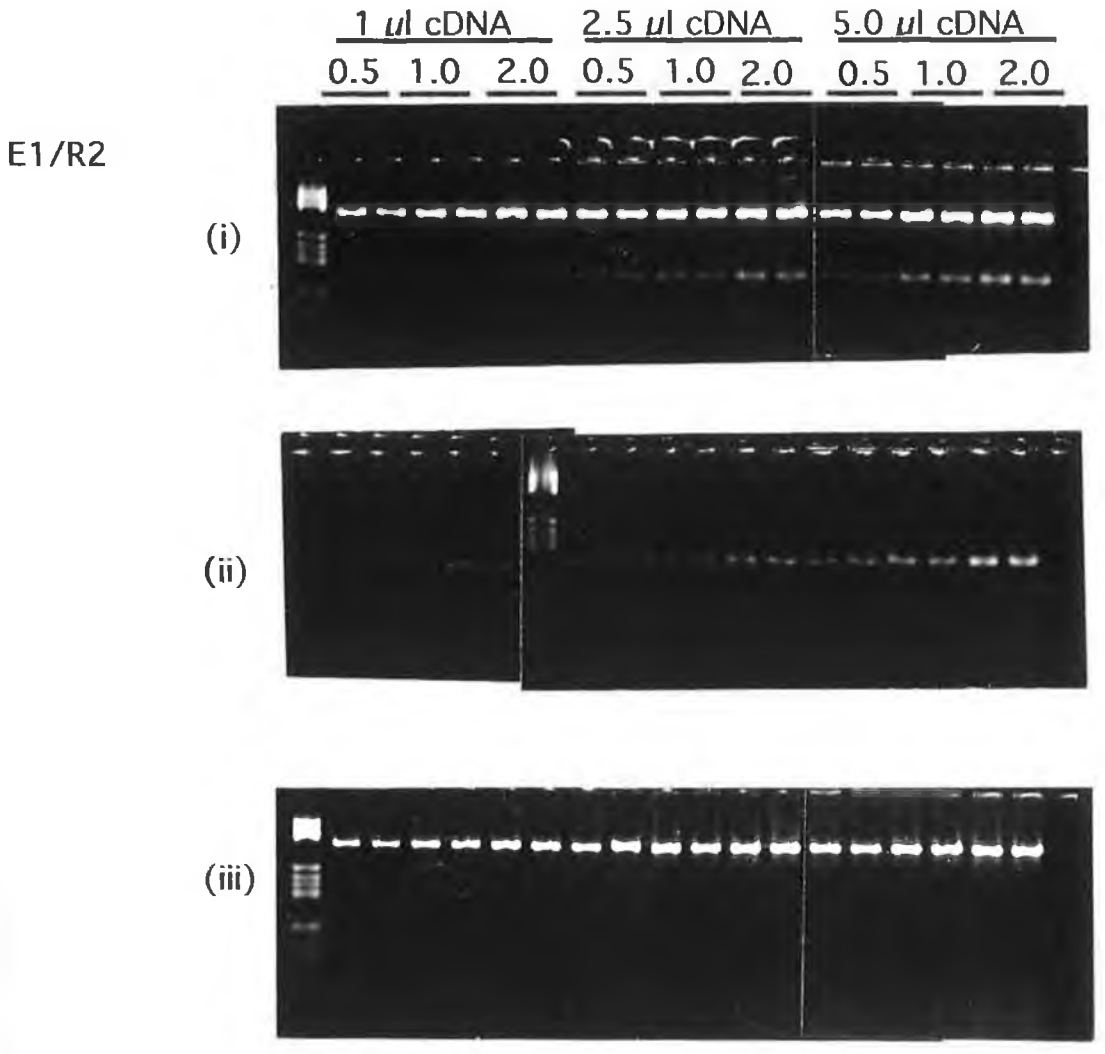


Fig. 3.5.3.3 Gel electrophoresis of products resulting from RT-PCR amplification of β-actin (383 bp) and MDR 1 (157 bp) cDNA formed on RNA templates extracted from DLKPA-2B cultured cells. ((i) is co-amplification of β-actin and MDR 1; (ii) and (iii) are independant amplifications of MDR 1 and β-actin, respectively). The amounts of cDNA (1.0 µl, 2.5 µl and 5.0 µl) formed on RNA templates at two concentrations (0.5 µg/µl, 1.0 µg/µl and 2.0 µg/µl) were analysed (E1/R2).

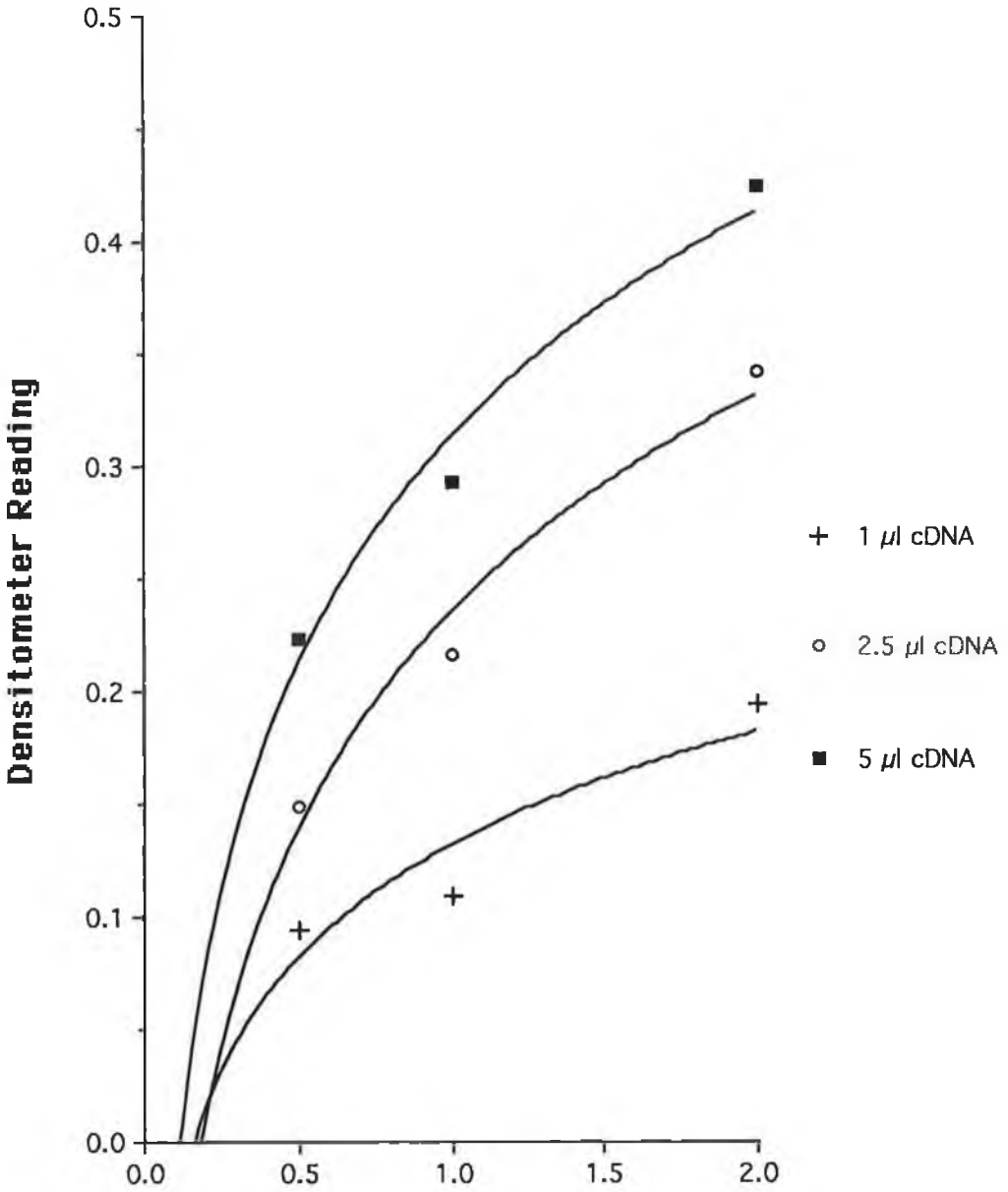


Fig. 3.5.3.3.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-2B (E1/R2), as in Fig. 3.5.3.3.

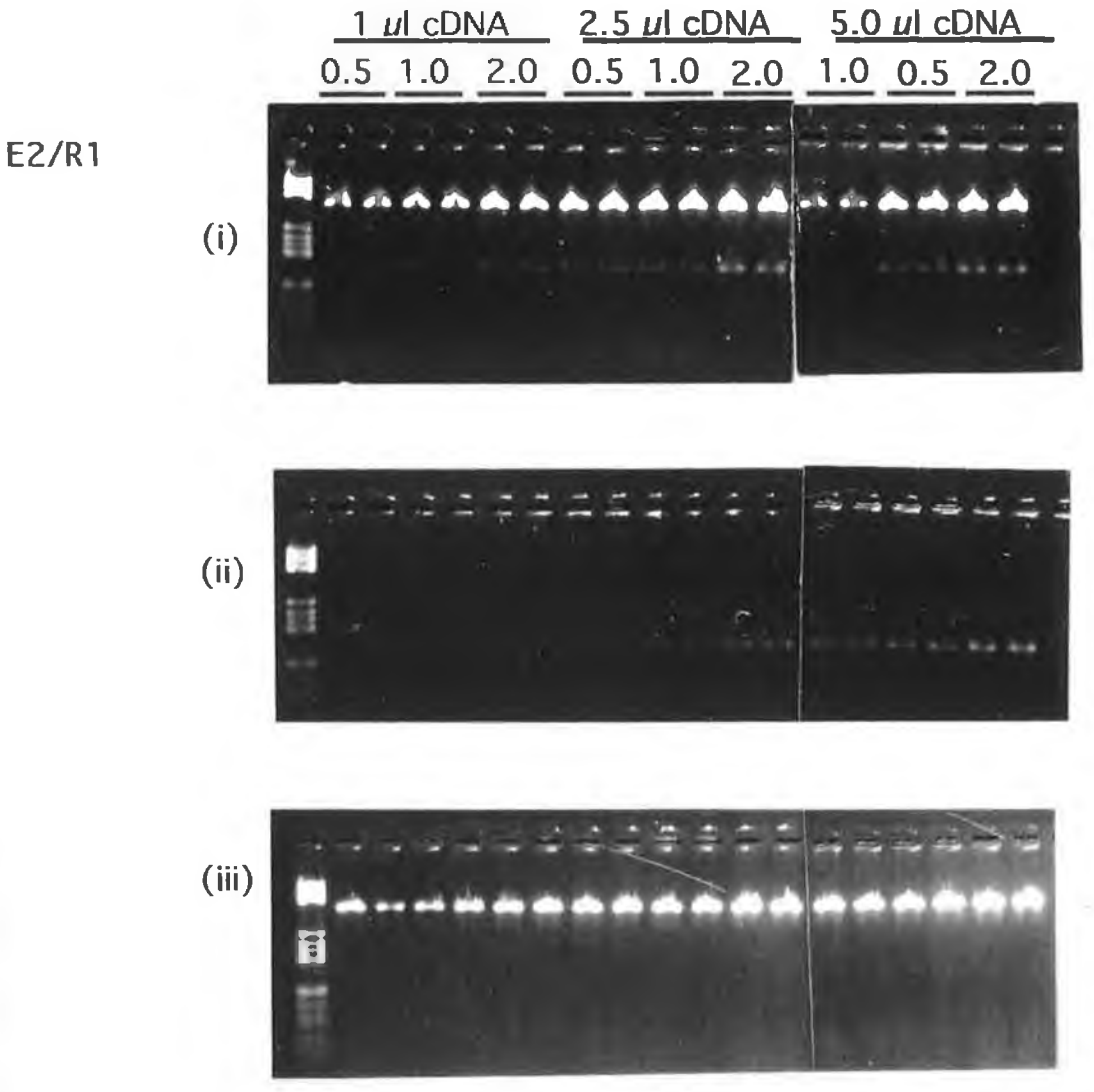


Fig. 3.5.3.4 Gel electrophoresis of products resulting from RT-PCR amplification of β-actin (383 bp) and MDR 1 (157 bp) cDNA formed on RNA templates extracted from DLKPA-2B cultured cells. ((i) is co-amplification of β-actin and MDR 1; (ii) and (iii) are independant amplifications of MDR 1 and β-actin, respectively). The amounts of cDNA (1.0 µl, 2.5 µl and 5.0 µl) formed on RNA templates at two concentrations (0.5 µg/µl, 1.0 µg/µl and 2.0 µg/µl) were analysed (E2/R1).

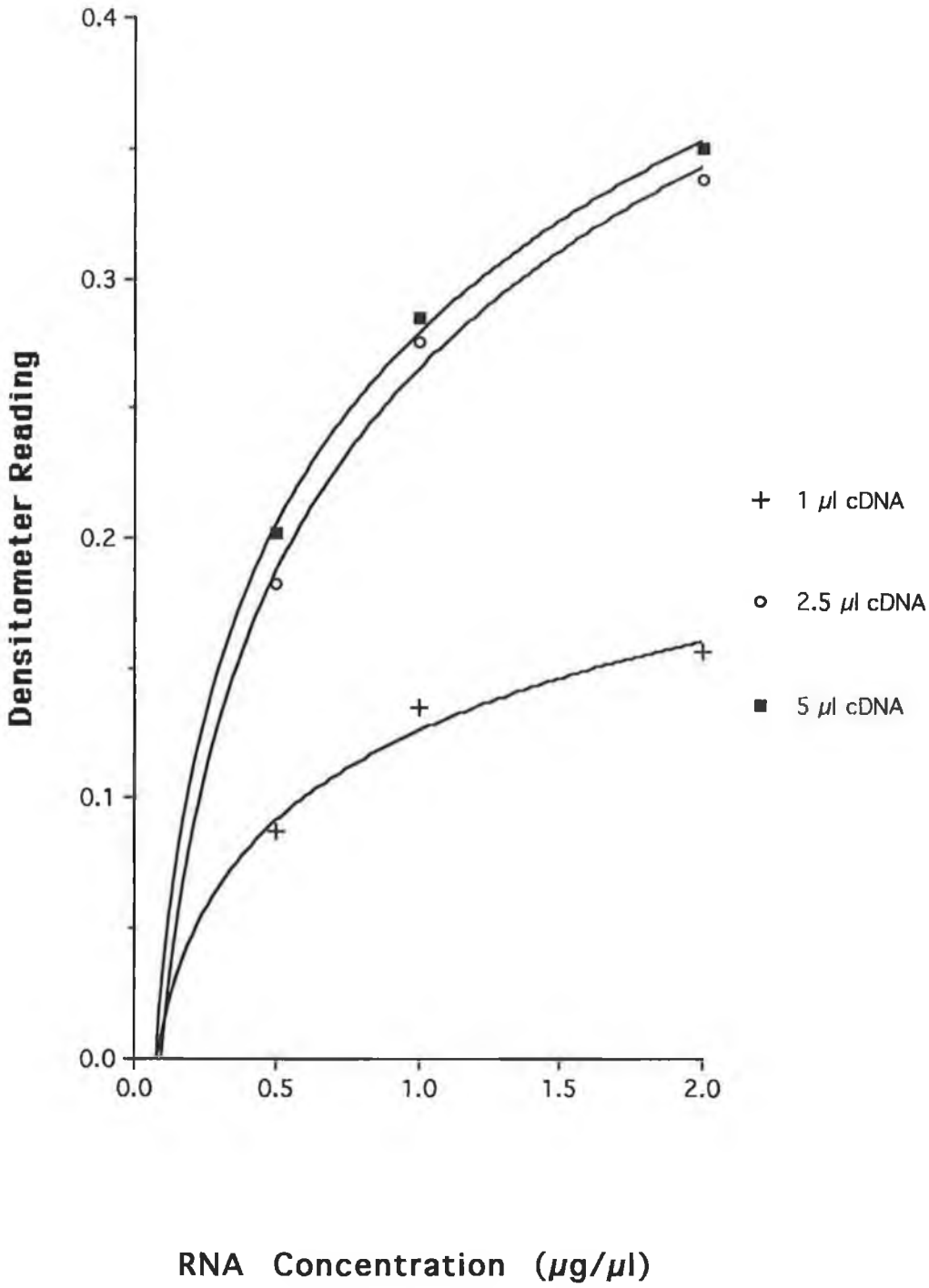


Fig. 3.5.3.4.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-2B (E2/R1), as in Fig. 3.5.3.4.

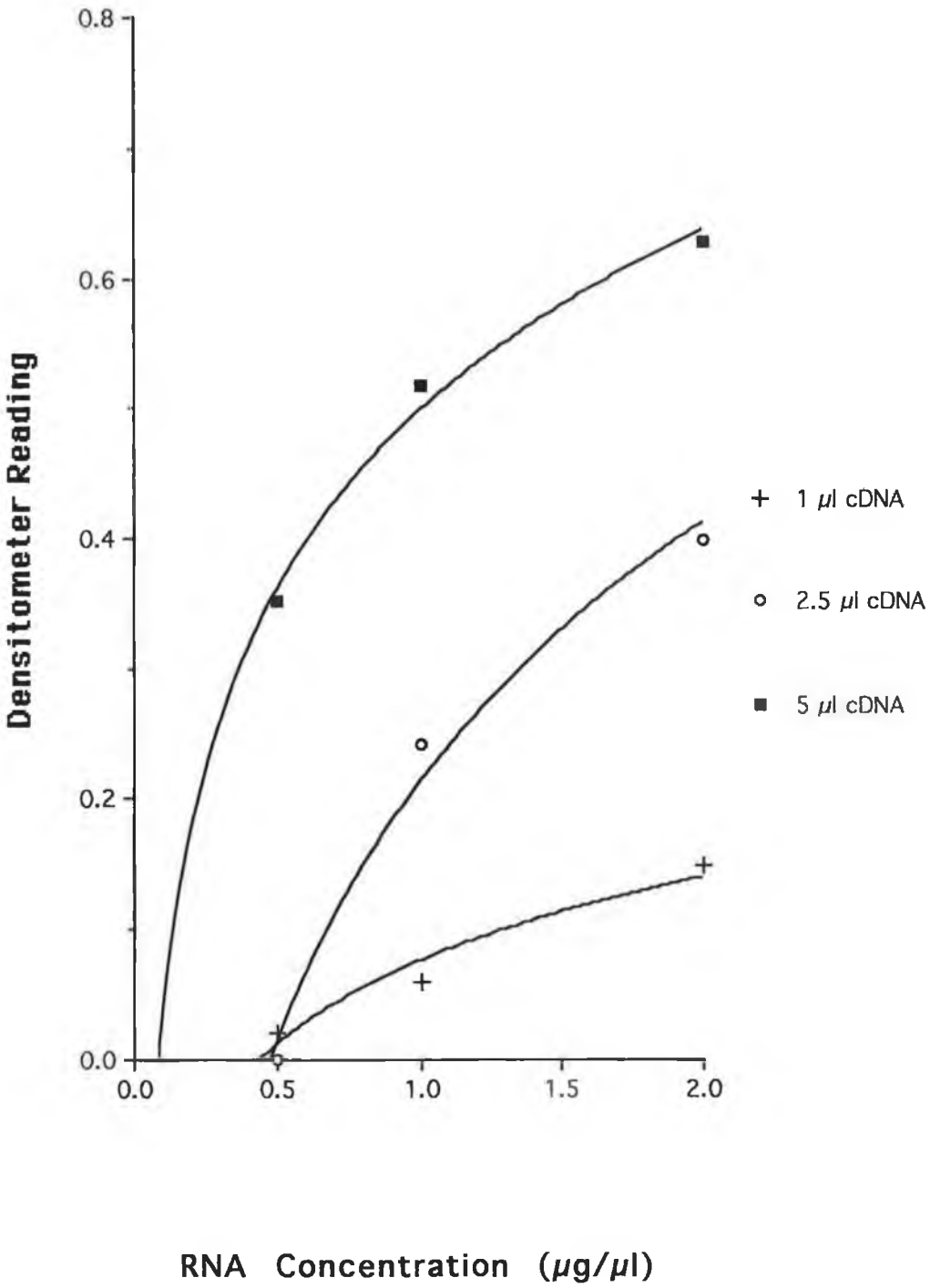


Fig. 3.5.3.5.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-2B (E2/R2).

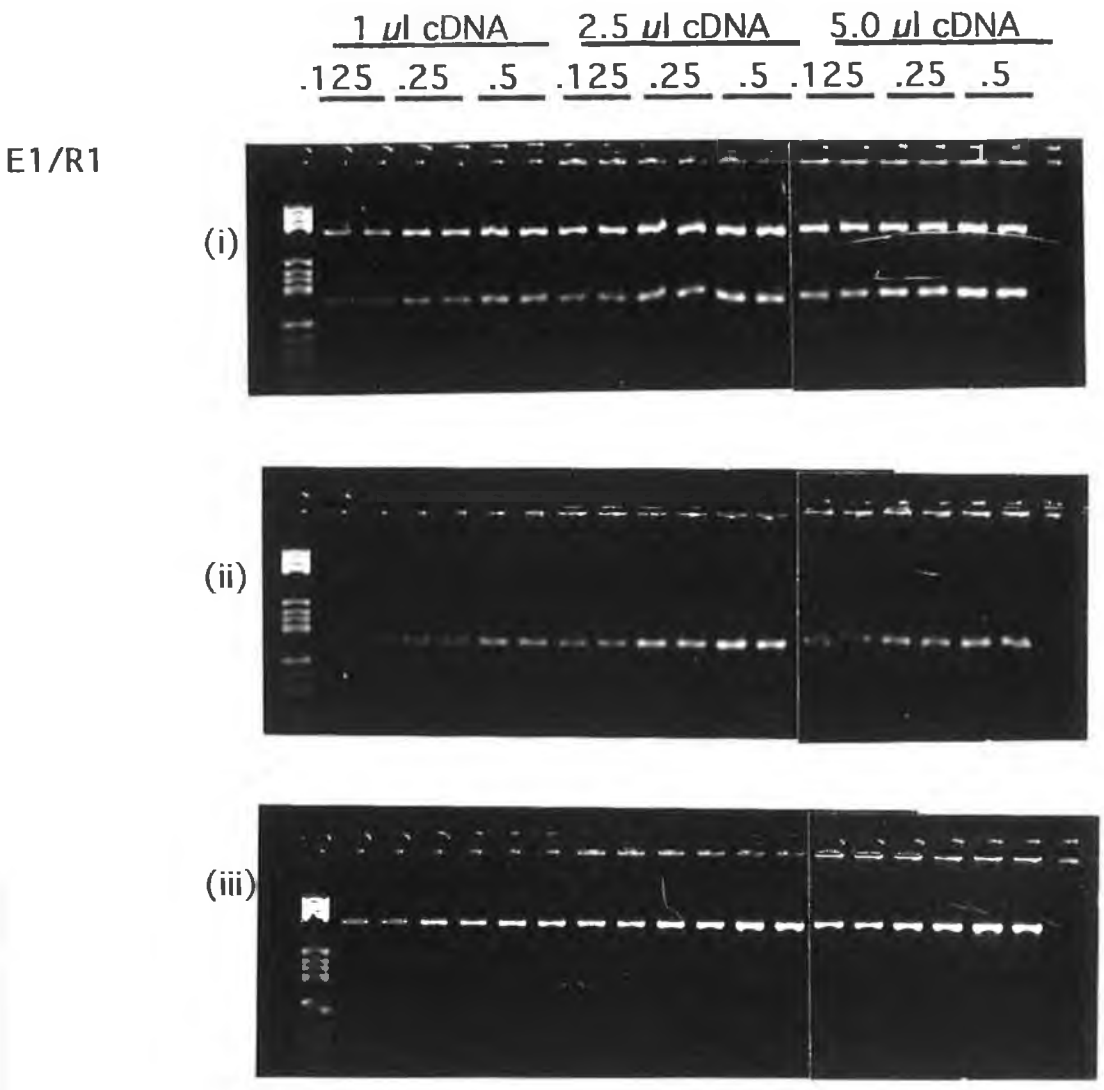


Fig. 3.5.3.6 Gel electrophoresis of products resulting from RT-PCR amplification of β-actin (383 bp) and MDR 1 (157 bp) cDNA formed on RNA templates extracted from DLKPA-5F cultured cells. ((i) is co-amplification of β-actin and MDR 1; (ii) and (iii) are independant amplifications of MDR 1 and β-actin, respectively). The amounts of cDNA (1.0 µl, 2.5 µl and 5.0 µl) formed on RNA templates at two concentrations (0.125 µl, 0.25 µl and 0.5 µl) were analysed (E1/R1).

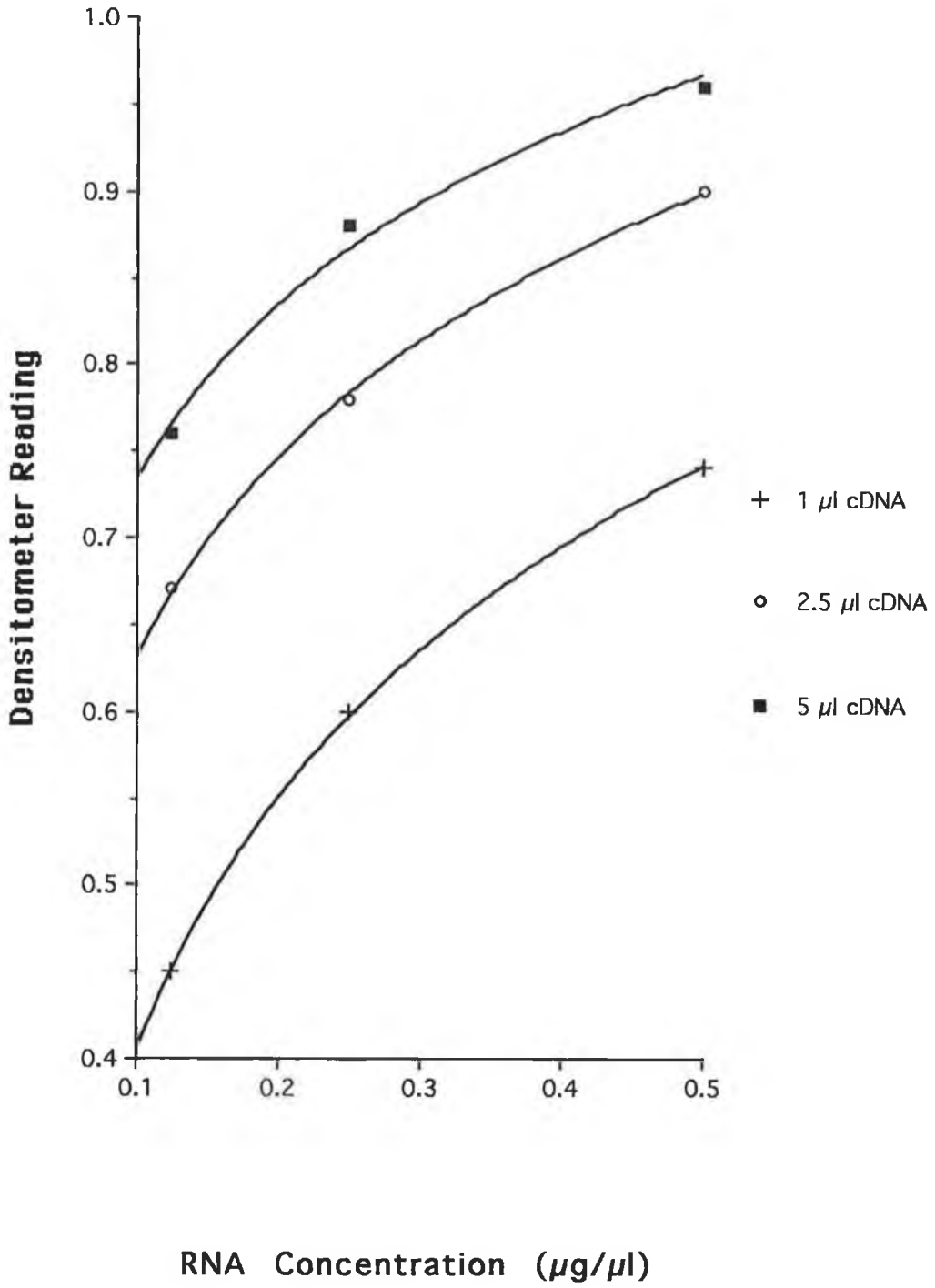


Fig. 3.5.3.6.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-5F (E1/R1), as in Fig. 3.5.3.6.

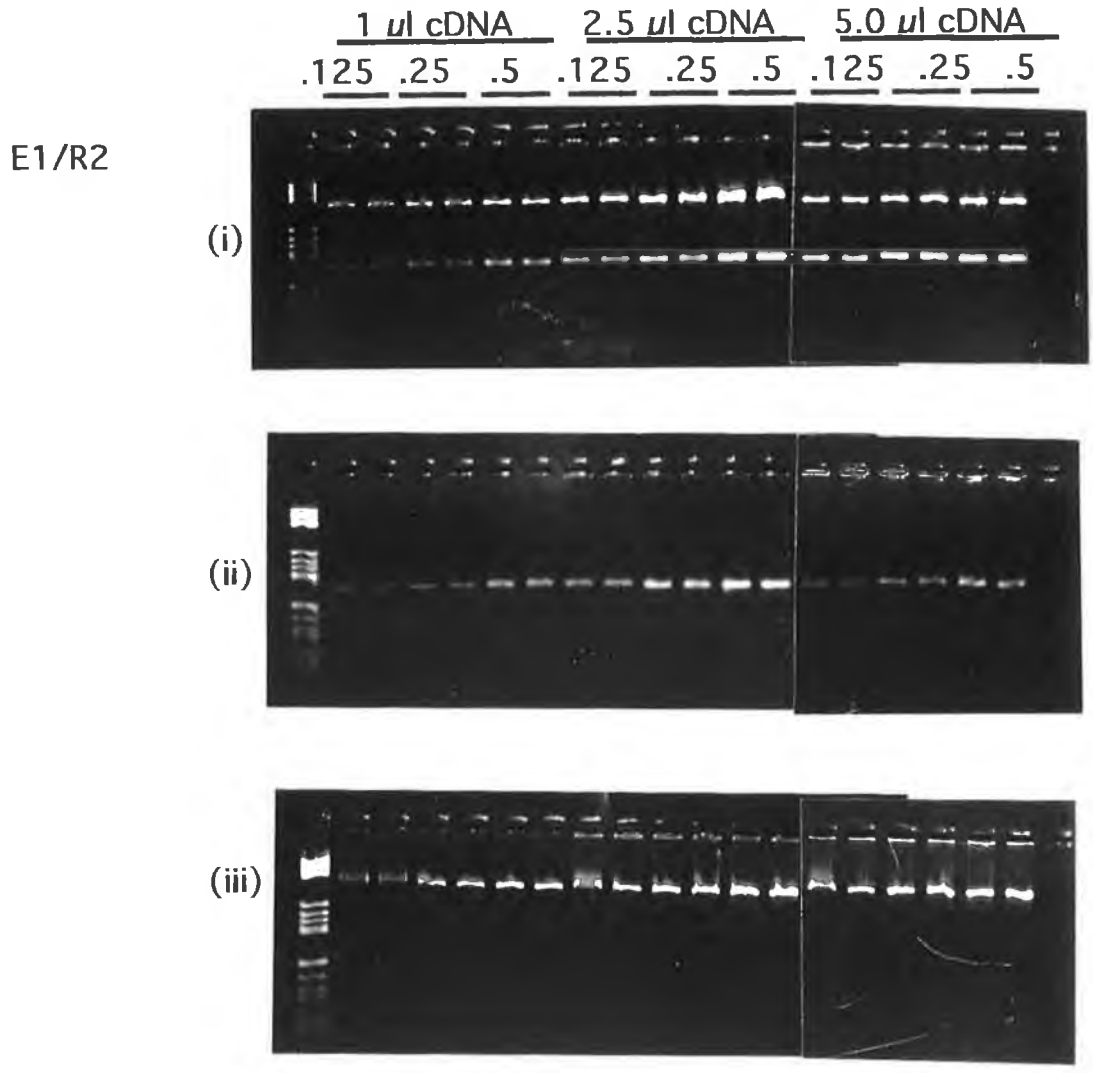


Fig. 3.5.3.7 Gel electrophoresis of products resulting from RT-PCR amplification of β-actin (383 bp) and MDR 1 (157 bp) cDNA formed on RNA templates extracted from DLKPA-5F cultured cells. ((i) is co-amplification of β-actin and MDR 1; (ii) and (iii) are independant amplifications of MDR 1 and β-actin, respectively). The amounts of cDNA (1.0 µl, 2.5 µl and 5.0 µl) formed on RNA templates at two concentrations (0.125 µl, 0.25 µl and 0.5 µl) were analysed (E1/R2).

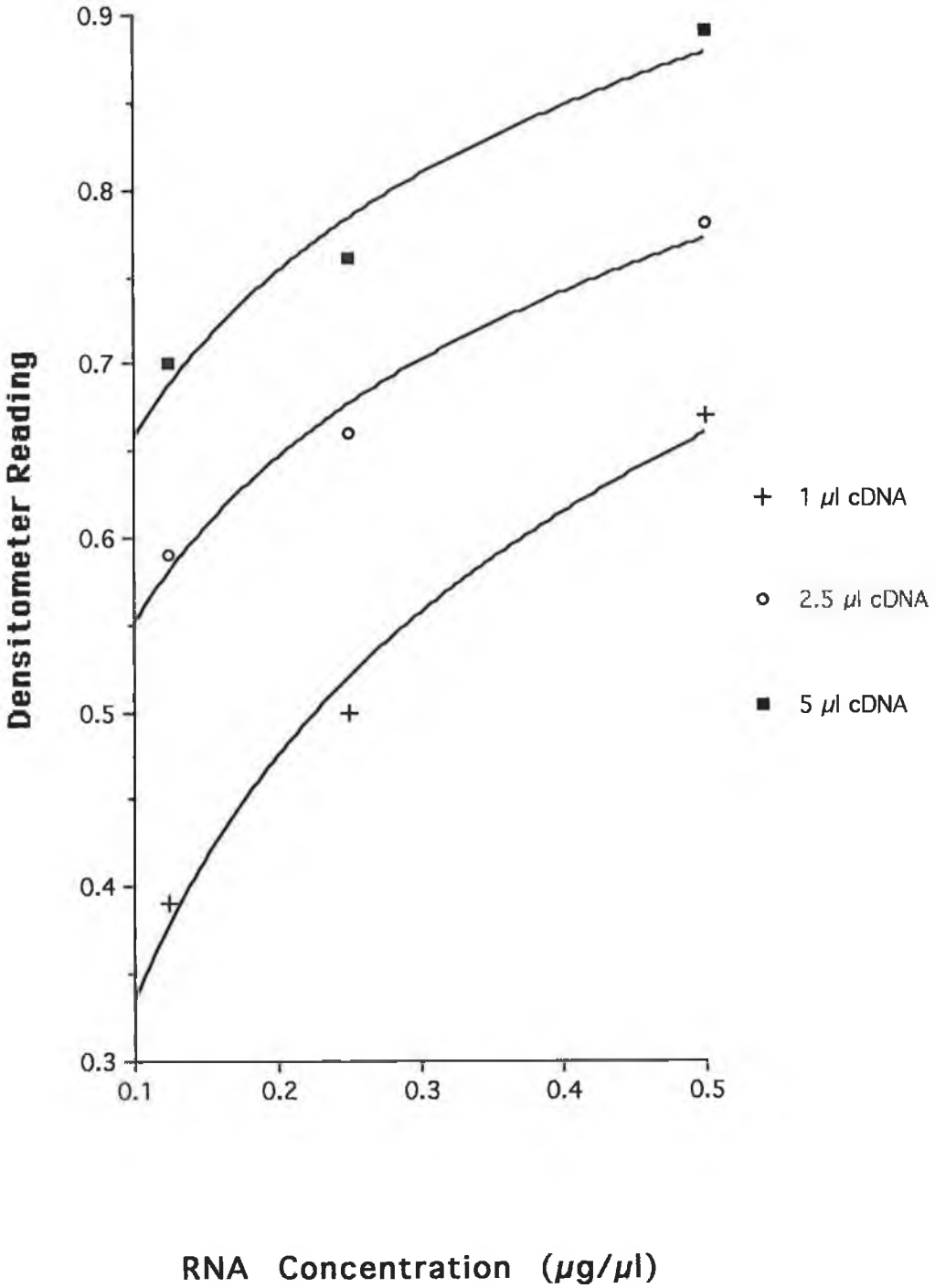


Fig. 3.5.3.7.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-5F (E1/R2), as in Fig. 3.5.3.7.

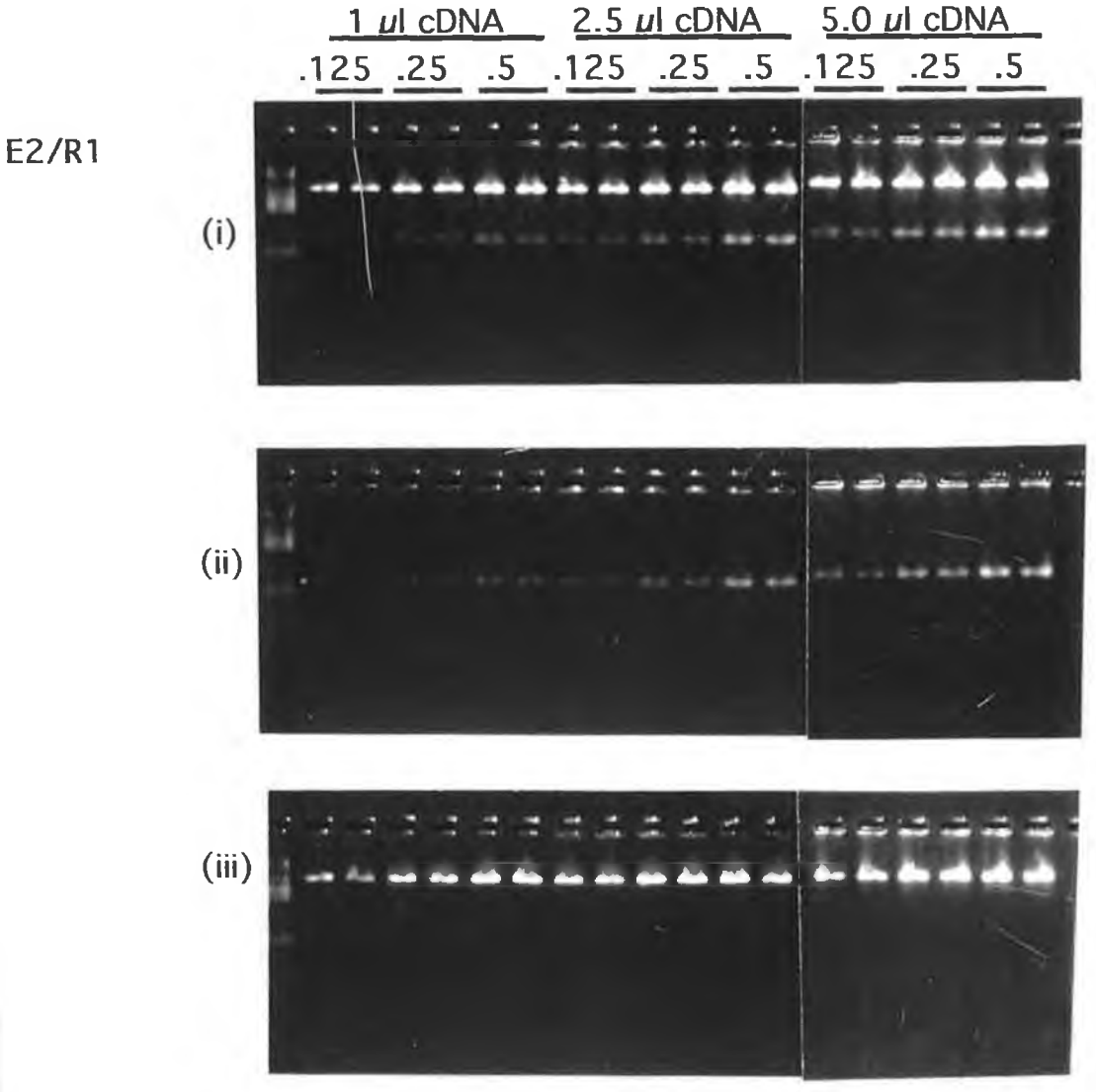


Fig. 3.5.3.8 Gel electrophoresis of products resulting from RT-PCR amplification of β-actin (383 bp) and MDR 1 (157 bp) cDNA formed on RNA templates extracted from DLKPA-5F cultured cells. ((i) is co-amplification of β-actin and MDR 1; (ii) and (iii) are independant amplifications of MDR 1 and β-actin, respectively). The amounts of cDNA (1.0 µl, 2.5 µl and 5.0 µl) formed on RNA templates at two concentrations (0.125 µl, 0.25 µl and 0.5 µl) were analysed (E2/R1).

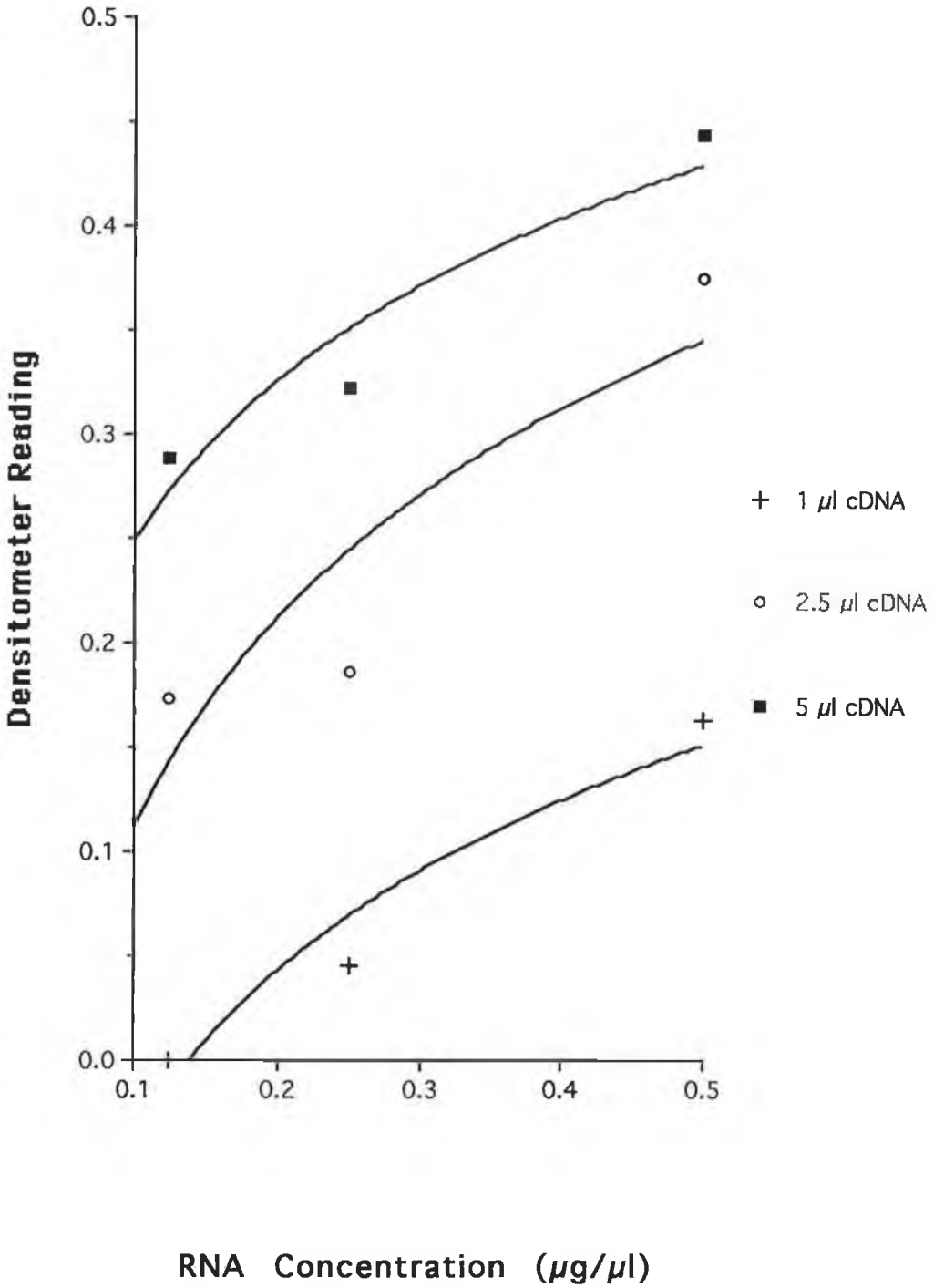


Fig. 3.5.3.8.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-5F (E2/R1), as in Fig. 3.5.3.8.

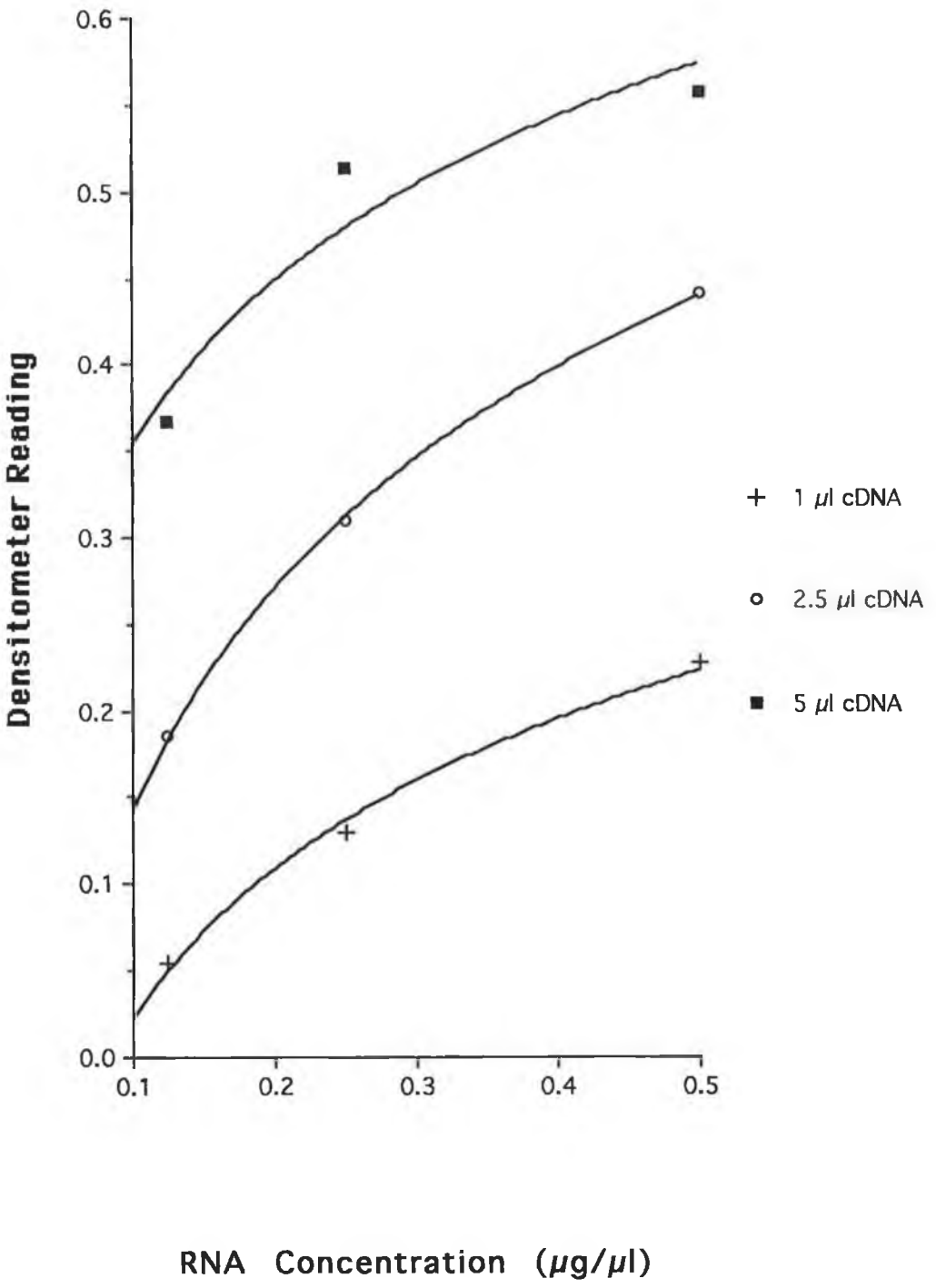


Fig. 3.5.3.9.1 Analysis, by densitometry, of MDR 1 band intensities for DLKPA-5F (E2/R2).

3.6 Quantitative RT-PCR

Quantitative analysis of MDR 1 mRNA transcript levels were attempted, using an exogenous control/standard (constructed by Dr. Carmel Daly, as described in 2.11). This control, which is amplified during the PCR reaction with the same primers (Table 3.1) as cDNA formed on the endogenous MDR 1 mRNA template (but with a different intervening sequence between the primers, results in the formation of a 331 bp product. This product is easily distinguished from the endogenous 157 bp amplified product, when separated by gel electrophoresis through a 4 % agarose gel.

The aim of using such an exogenous competitive control is to calculate the absolute levels of the target mRNA. This was done by titrating a (constant) unknown amount of cDNA template to be quantified against a dilution series of known concentrations of exogenous control. Since the amount of control is known, it may be assumed that when the PCR products resulting from the endogenous mRNA and control are equal, the amount of the specific mRNA under analysis in the original sample is equal to the amount of template added. From this, the number of molecules of target mRNA is, theoretically, deducible using simple mathematics.

The amount of exogenous control in grams is known. Grams are converted to moles using the constant 309, which is the average molecular weight of the A, T, C and G bases (molecular weight of bases: A = 312.2; C = 288.2; G = 328.2; T = 303.2). Molecules of control per ml is then calculated, using the following formula (Pharmacia GenQuant System):

$$\text{Molecules/ml} = \text{Conc. } (\mu g/ml) \times 6.023 \times 10^{23} \; / \; \text{Molecular Weight} \times 10^{6}$$

(As an additional refinement, this formula can be further modified by accounting for its phosphorylated or phosphorylated

state of the sequence. If precise calculations are required, the individual base molecular weights and the molecular weight of Uracil (289.2) in the mRNA being quantified, can be taken into account).

A preliminary experiment, using this exogenous control to analyse the amounts of the MDR 1 transcript in RNA extracted (0.25 μg/μl RNA; 2.5 μl cDNA) from DLKPA-5F cells is illustrated in Fig. 3.6.1. The amounts of exogenous control included were 2 ng/μl, 1 ng/μl, 0.5 ng/μl, 0.25 ng/μl, 0.125 ng/μl, 0.063 ng/μl and 0.031 ng/μl. Each PCR reaction was set up in duplicate. As indicated in Fig. 3.6.1.1(i), both the exogenous control and MDR 1 cDNA densitometry readings, and those of the exogenous control when amplified separately (Fig. 3.6.1.1.(ii)), when plotted, produce curves similar in form to that of the DNA controls used to analyse the efficiency of the densitometer (see Section 3.5.2). This, again, suggests that the densitometer analysis is not completely accurate and that the gel photographs should also be analysed visually. From this experiment, however, it seems that the internal control range of concentrations chosen was too great, and that a lower concentration range is required to quantify the MDR 1 mRNA levels in this DLKPA-5F extract.

In subsequent PCR reactions, the range of exogenous control concentrations was reduced to between 0.25 ng/μl - 0.002 ng/μl. The exogenous control concentrations were titrated against cDNA formed on mRNA templates extracted from DLKPA-2B and DLKPA-5F cell lines. To act as an endogenous control for the cDNA, β_2-microglobulin was also amplified. This control was favoured over β-actin in these experiments as it is more easy distinguished from the control due to their size differences (exogenous control = 331 bp; β-actin = 383 bp; β_2-microglobulin = 114 bp). This analysis was performed on a number of RNA and cDNA concentrations.

By visual analysis, the amount of MDR 1 mRNA in this concentration of DLKPA-2B total RNA is approximately equivalent to 0.016 ng/μl exogenous control, and that of DLKPA-5F,

approximately equivalent to 0.031 ng/μl exogenous control. From this, the amount of MDR 1 mRNA in the DLKPA-5F cells is therefore estimated to be approximately twice that in the DLKPA-2B cells. However, it must also be taken into account that the concentration of initial DLKPA-5F template was is 4 times that of the DLKPA-2B cells. Overall, the MDR 1 mRNA levels in DLKPA-5F cells is estimated to be almost 8 times that in the DLKPA-2B cells.

Densitometry analysis was also performed on these bands so that an exogenous control standard curve could be drawn, against which the MDR 1 mRNA levels in the DLKPA-2B and DLKPA-5F extracts could be extrapolated *i.e.* the amount of exogenous control which produces the same densitometry reading determined by the point of intersection of the corresponding lines on the graph. This (of course assuming accurate readings by the densitometer) was done to give an indication of the results obtainable. The results from this analysis are shown in Fig. 3.6.2.1.

The concentration of the exogenous control at the point where the curves intersect was, therefore, calculated using the assumption that at the point of intersection the equation of both curves are equal.

DLKPA-2B:

Exogenous Control Curve Eqn.: $y = 23.37 + 5.5 \log(X)$
MDR 1 Curve Eqn.: $y = 5.19 - 4.0 \log(X)$

(where y = Densitometry reading and x = Exogenous Control concentration)

At the point of intersection -

$23.37 + 5.5 \log(x) = 5.19 + 4.0 \log(x)$

$\log(x) = -1.91$

$x = 0.012$

i.e. Exogenous Control Conc. = 0.012 ng/μl

DLKPA-5F:

Exogenous Control Curve Eqn.: $y = 23.78 + 7.25 \log(X)$
MDR 1 Curve Eqn.: $y = 8.58 - 1.85 \log(X)$

(where y = Densitometry reading and x = Exogenous Control concentration)

At the point of intersection -

$$23.78 + 7.25 \log(x) = 8.58 + 1.85 \log(x)$$
$$\log(x) = -1.6$$
$$x = 0.025$$

i.e. Exogenous Control Conc. = 0.025 ng/μl

The relationship between the MDR 1 mRNA concentration in the DLKPA-2B (using 1.0 μg/μl initial RNA template) and DLKPA-5F cells (using 0.25 μg/μl initial RNA template) is similar to that observed from these gels by visual analysis, although by densitometry analysis the overall MDR 1 mRNA levels in the DLKPA-5F cells is, apparently, slightly more than 8 times that in the DLKPA-2B cells.

Using the formula:

$$\textbf{Molecules/ml} = \frac{\textbf{Conc. } (\mu\textbf{g/ml}) \textbf{ X } 6.023 \textbf{ X } 10^{23}}{\textbf{Molecular Weight X } 10^{6}}$$

the number of exogenous control molecules at this point can be calculated.

DLKPA-2B:
molecules/ml = $0.012 \times 6.023 \times 10^{23} / 1.02 \times 10^{5} \times 10^{6} = \underline{7.09 \times 10^{10}}$

DLKPA-5F:
molecules/ml = $0.025 \times 6.023 \times 10^{23} / 1.02 \times 10^{5} \times 10^{6} = \underline{1.47 \times 10^{11}}$

Figs. 3.6.1 and 3.6.2 Gel electrophoresis of products formed after co-amplification of an MDR 1 exogenous competitive control (331 bp) with MDR 1 (157 bp) and β_2-microglobulin cDNA formed on endogenous mRNA templates extracted from (i) DLKPA-2B and (ii) DLKPA cell lines. Endogenous MDR 1 mRNA is the target to be quantified and β_2-microglobulin, the endogenous positive control. Serial dilutions of exogenous control were amplified with a constant amounts of the target cDNA to be quantified. The left-hand lane of each gel contains the DNA molecular weight marker consisting of 22 fragments of the following base-pair sizes: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11 and 8 base pairs, by which the amplicons could be identified.

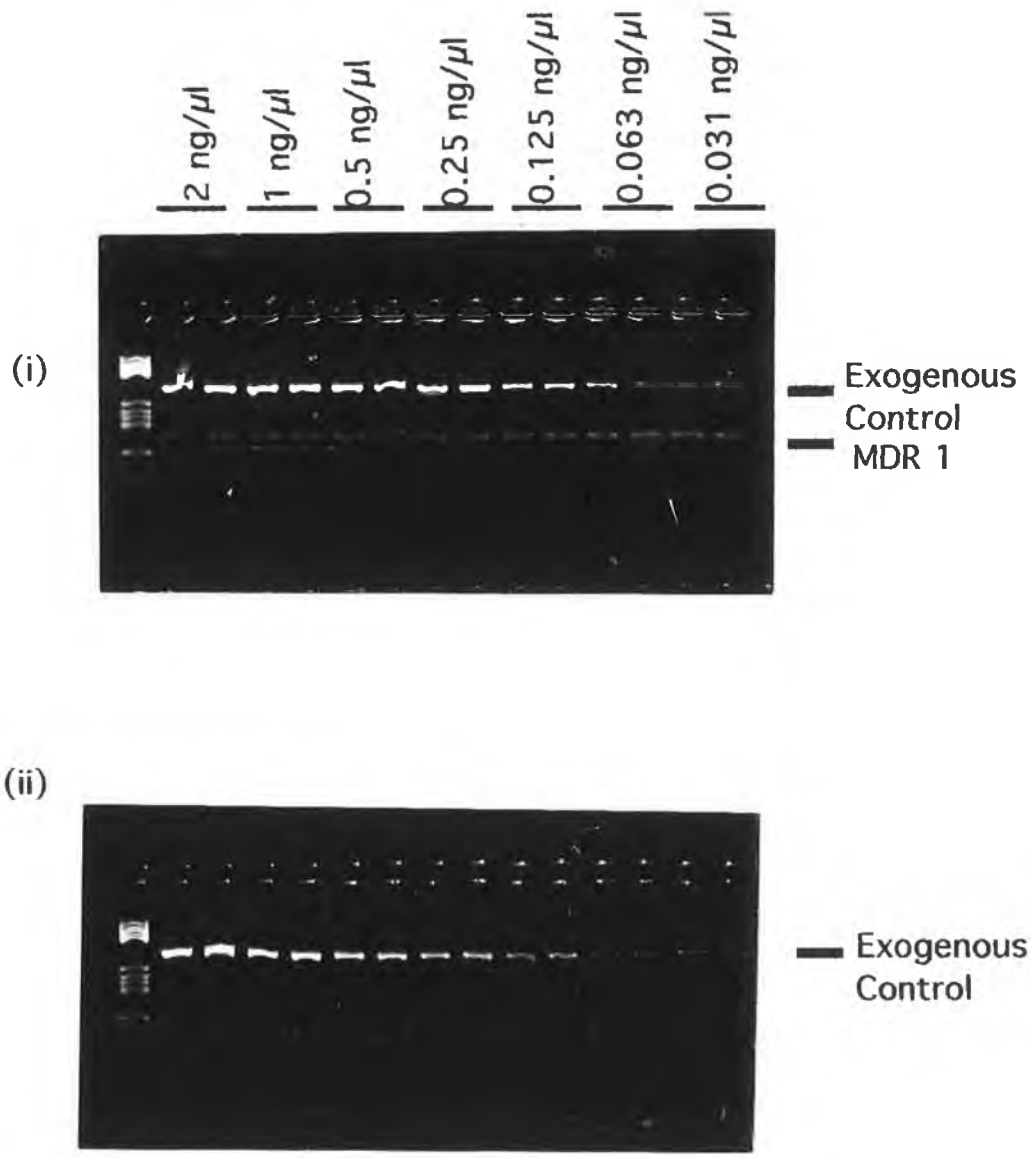


Fig. 3.6.1 (i) Gel electrophoresis of products formed after co-amplification of an MDR 1 exogenous competitive control (331 bp) with cDNA formed on endogenous MDR 1 mRNA templates (157 bp), extracted from DLKPA-5F cells using 2.5 μl of cDNA formed on a 0.25 μg/μl RNA template. Serial dilutions of exogenous control, ranging from 2 ng/μl to 0.031 ng/μl were amplified, in duplicate, with a constant concentration of the target MDR 1 cDNA to be quantified. From this preliminary analysis, it seems that the concentrations of exogenous control are too great for estimating the endogenous MDR 1 concentration. A range of lower concentrations should apparently be used.
(ii) The same range of concentrations of exogenous competitive control (2 ng/μl to 0.031 ng/μl) were amplified independently, to establish if co-amplification with the endogenous MDR 1 gene product limited its amplification in any way.

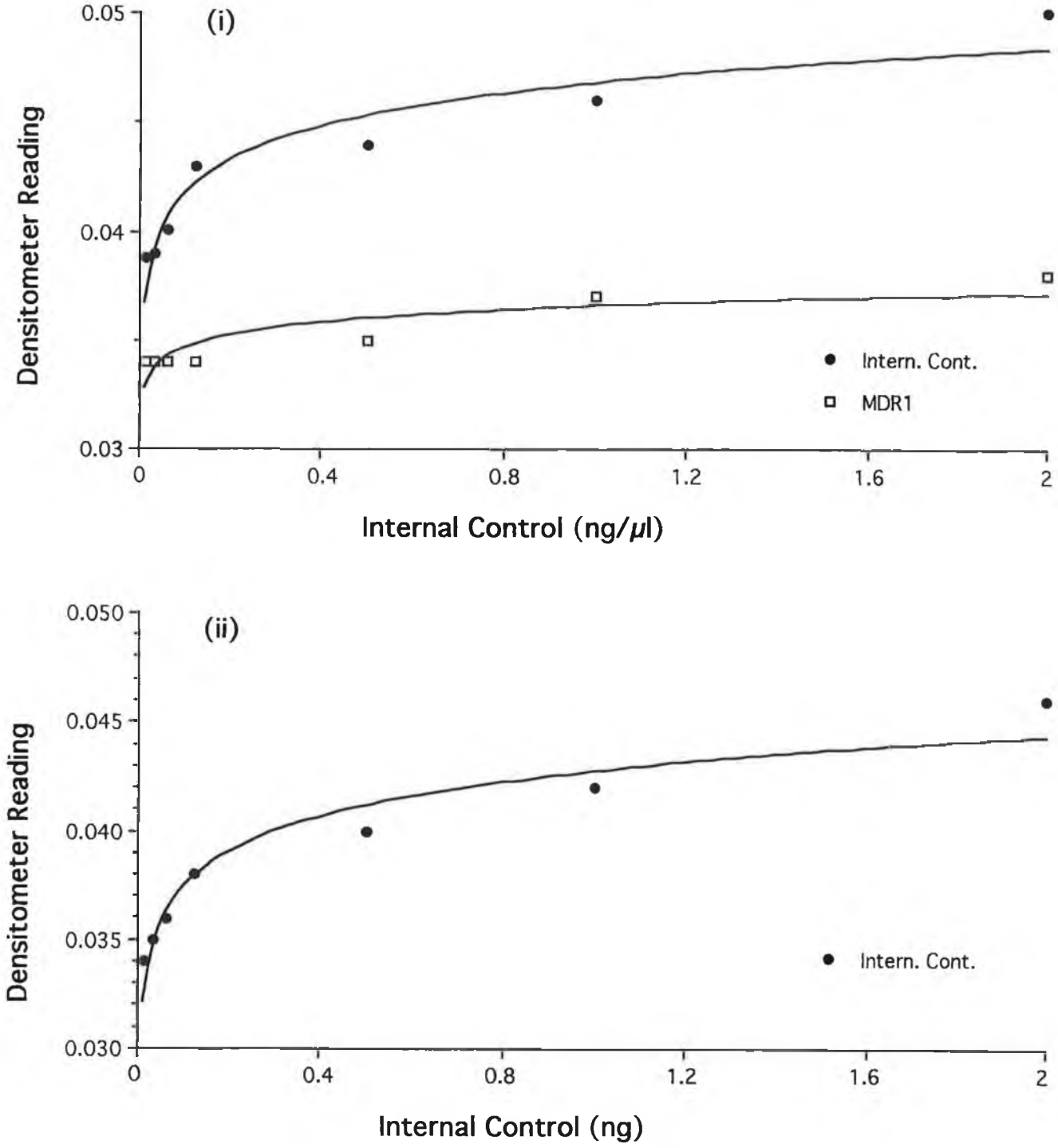


Fig. 3.6.1.1 (i) Relationship between concentrations of exogenous control and densitometry readings (after background subtraction) of the intensities of the resulting bands. The densitometry readings of the corresponding bands resulting from target MDR 1 cDNA amplification in each sample, were also plotted on this graph (*i.e.* analysis of Fig. 3.6.1(i)).
(ii) Plot of exogenous control concentrations versus corresponding densitometry readings, after background subtraction (as illustrated in Fig. 3.6.1(ii)).

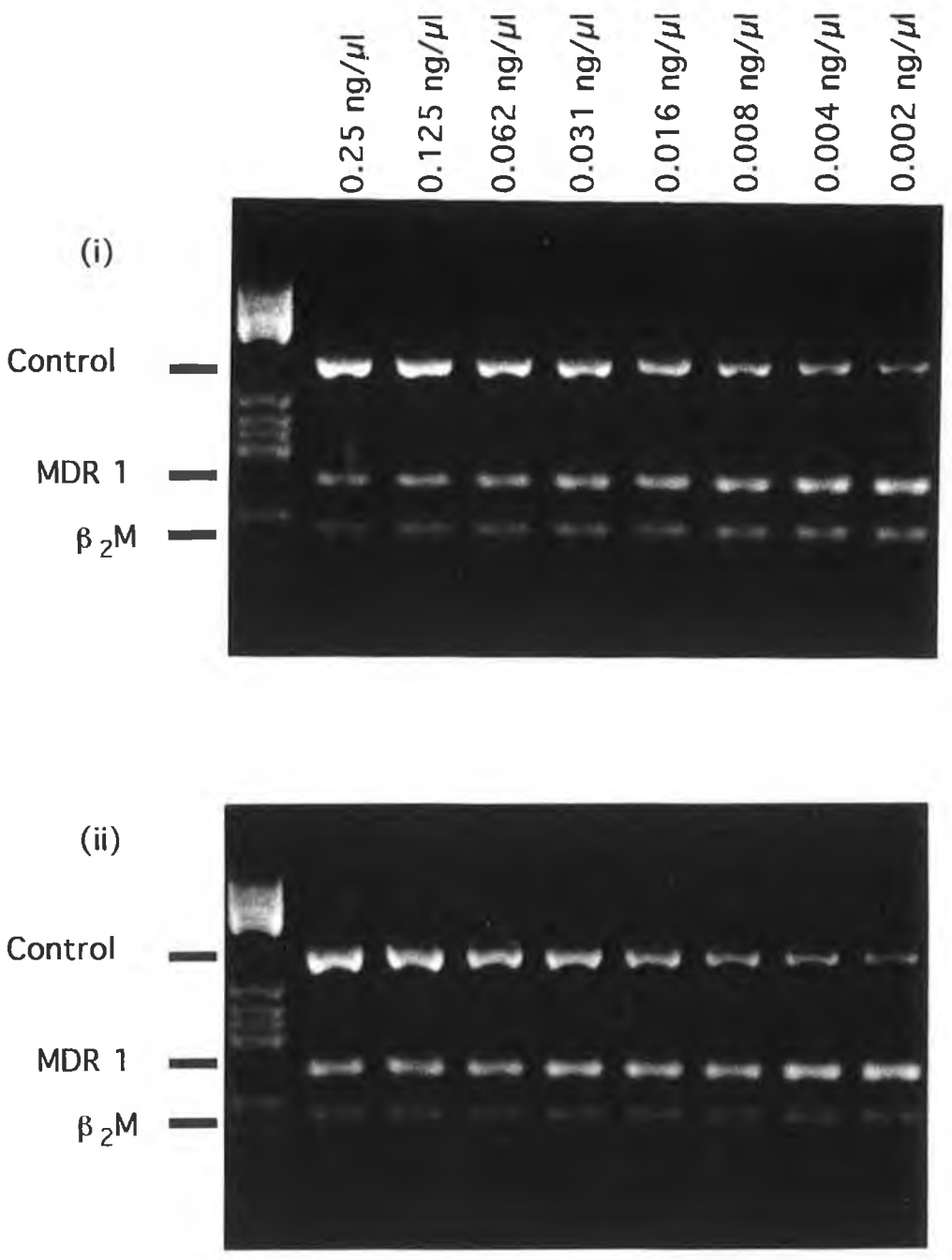


Fig. 3.6.2 2.5 μl of cDNA formed on a 1.0 μg/μl RNA template, in the case of (i) DLKPA-2B and on a 0.25 μg/μl RNA template extracted from (ii) DLKPA-5F cells were co-amplified, respectively, with the exogenous competitive control.

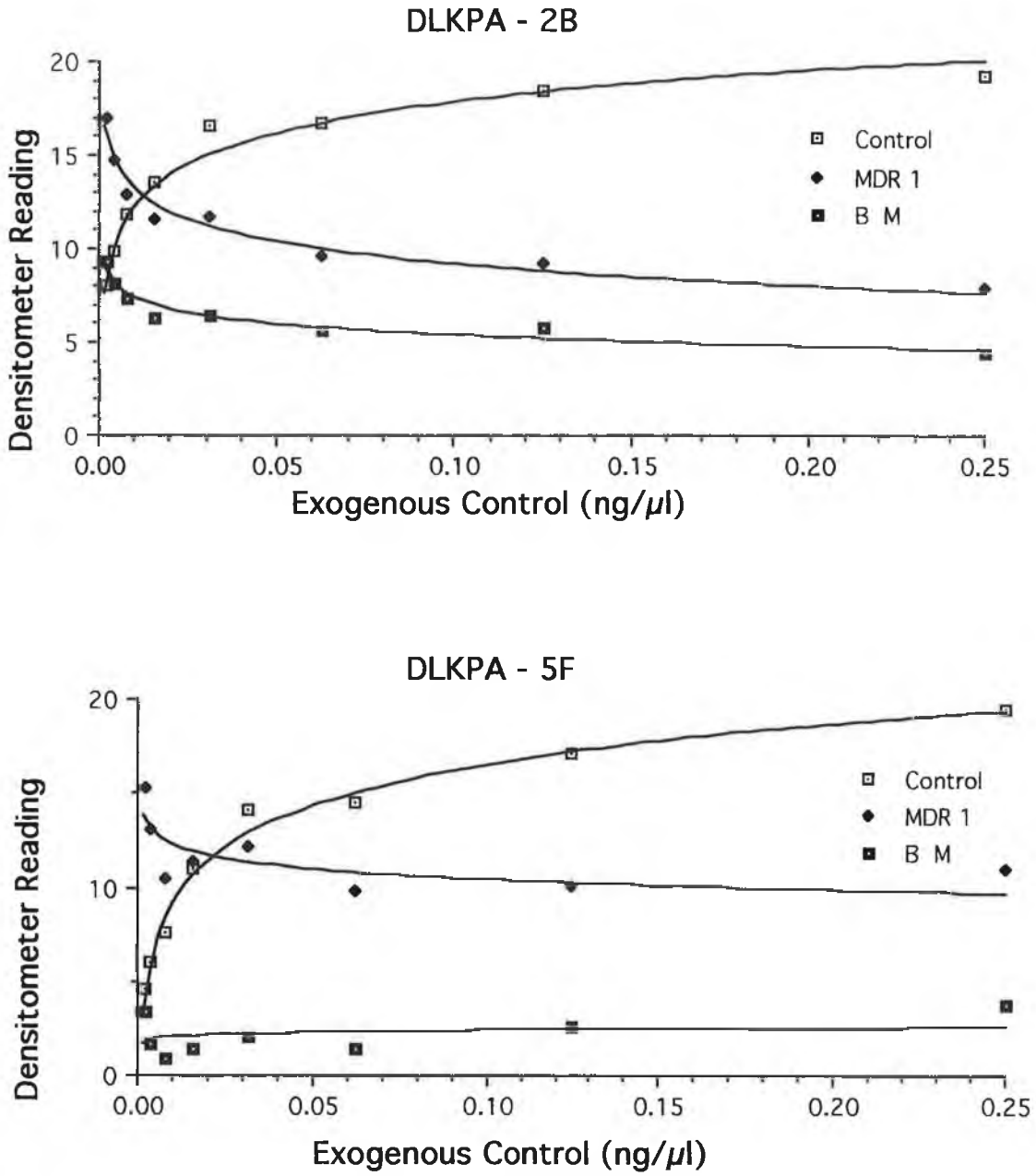


Fig. 3.6.2.1 Densitometry analysis of results illustrated in Fig 3.6.1 when titrating a range of known concentrations in exogenous control (MDR 1) against a constant amount of cDNA (2.5 µl) formed on 1.0 µg/µl RNA template in the case of DLKPA-2B, and 0.25 µg/µl in the case of DLKPA-5F.

3.7 Protein Analysis using Western Blotting Techniques.

Alterations in the expression of certain proteins associated with multiple drug resistance were investigated by western blot analysis, to establish if changes noted at the gene transcript level were indicative of the corresponding protein levels. Levels of p-glycoprotein were studied in DLKP, its multiple drug resistant variant DLKPA, clones isolated from DLKPA, including DLKPA-2B, DLKPS-6B and DLKPA-5F and VP-16-selected MDR variants, DLKP/VP-3 and DLKP/VP-8. Alterations in Topoisomerase II protein levels, and that of its α subunit were analysed in DLKP, DLKPA, DLKP/VP-3 and DLKP/VP-8.

3.7.1 Western Blot Analysis of P-glycoprotein Levels

Western blot analysis of p-glycoprotein was performed on cellular membrane preparations using the anti-p-glycoprotein antibody, C219, to detect this 170 kDa protein. The results (Fig. 3.7.1; Table 3.7.1.1) suggest that this protein is over-expressed in all MDR cell lines studied, to a greater or lesser extent. P-glycoprotein was not detected in the sensitive form of the cell line, DLKP.

CELL LINE / CLONE	P-gp LEVEL
DLKP/VP-3	0.14
DLKP/VP-8	0.07
DLKP	0.00
DLKPA	0.03
DLKPA-2B	0.09
DLKPA-6B	0.04
DLKPA-5F	0.01

Table 3.7.1.1: Densitometry analysis of p-glycoprotein levels detected by western blotting

The most significant increases in p-glycoprotein levels with multiple drug resistance were detected in the VP-16

selected cell lines. DLKP/VP-3, the lesser resistant variant expressed higher levels of this protein than the more resistant cell line, DLKP/VP-8. Elevated levels of p-glycoprotein expression in the adriamycin-selected cell line and its cloned populations were apparent, but to a lesser extent than in the VP-16 selected cell lines. As with the VP-16 selected cell lines, the levels of p-glycoprotein did not correlate with their multiple drug resistant ranking order. DLKPA-5F, the most resistant clone seemed to express lowest levels of immunodetectable p-glycoprotein, followed by DLKPA, the heterogenous population of the resistant variants. The greatest increases in p-glycoprotein in the DLKPA variants was found to be for DLKPA-2B, the least resistant clone.

3.7.2 Western Blot Analysis of Topoisomerase II levels.

Levels of the nuclear enzyme Topoisomerase II and its α subunit were analysed in nuclear extracts from variants of the DLKP cell line. This analysis was done using a polyclonal antibody raised against Topoisomerase II which detects both the 170 kDa (Topoisomerase IIα) and 180 kDa (Topoisomerase IIβ) isoforms of the enzyme and a monoclonal antibody specific for the α subunit, respectively. As illustrated in Fig. 3.7.2(i). Results from analysis with the polyclonal antibody showed that all cell lines studied possessed immunologically reactive Topoisomerase II, with reduced levels present in the multiple drug resistant variants, DLKPA and DLKP/VP-3, in comparison to the sensitive cell line (Table 3.7.2.1). A slight increase in the level of this protein was apparent in DLKP/VP-8 nuclear extracts. However, the differences detected are very slight, except in the case of DLKPA.

It is not possible to determine from this western blot (Fig. 3.7.2.1) which Topoisomerase II isoform is altered with the MDR phenomenon, as the isoforms are not clearly distinguishable. However, from the analysis of Topoisomerase IIα, specifically, using the monoclonal antibody directed

against the α subunit of Topoisomerase II*, reduced levels of this sub-unit were detected in the resistant variants, in comparison to DLKP (Fig. 3.7.2(ii); Table 3.7.2.1). This, again, was most obvious in the DLKPA cell line. A significant decrease in Topoisomerase IIα protein levels was also apparent in the most resistant VP-16 selected variant, DLKP/VP-8. Levels of Topoisomerase IIα seemed to be only slightly altered in the DLKP/VP-3 cell line.

Cell Line	Topo II	Topo II α
DLKPA	0.76	0.16
DLKP	0.90	0.52
DLKP/VP-3	0.82	0.44
DLKP/VP-8	0.99	0.37

Table 3.7.2.1: Densitometry analysis of Topoisomerase II and Topoisomerase IIα detection by Western blotting.

* An extra band was observed near the end of the Topoisomerase IIα Western blot. This extra band was also detected in the negative control, suggesting that it is the result of non-specific binding of the secondary antibody.

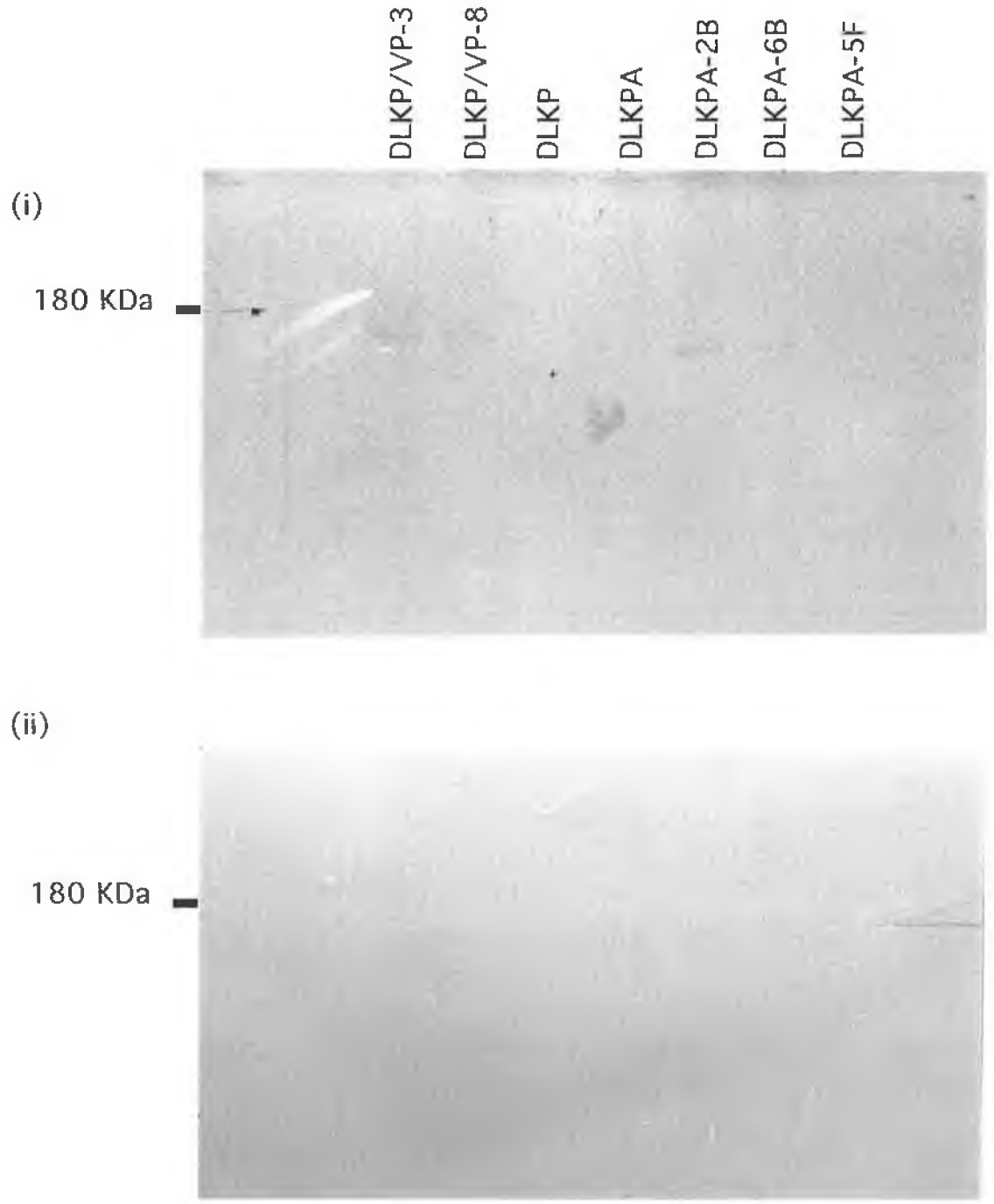


Fig. 3.7.1 Western blot detection of p-glycoprotein in cell membrane preparations from DLKP variants, using C219 antibody. In both (i) the positive blot and (ii) the negative blot, 10 μg of protein was loaded per lane (as indicated for the positive blot). The blotting procedures for both (i) and (ii) were identical, except that the positive blot was incubated with a monoclonal antibody, C219, directed against p-glycoprotein, whilst for the negative blot, the primary antibody was replaced with an equal volume of antibody diluent. The size of the detected protein (170 kDa) was verified using the molecular weight markers (ran in the left-hand lane on the positive blot and the right-hand lane on the negative blot).

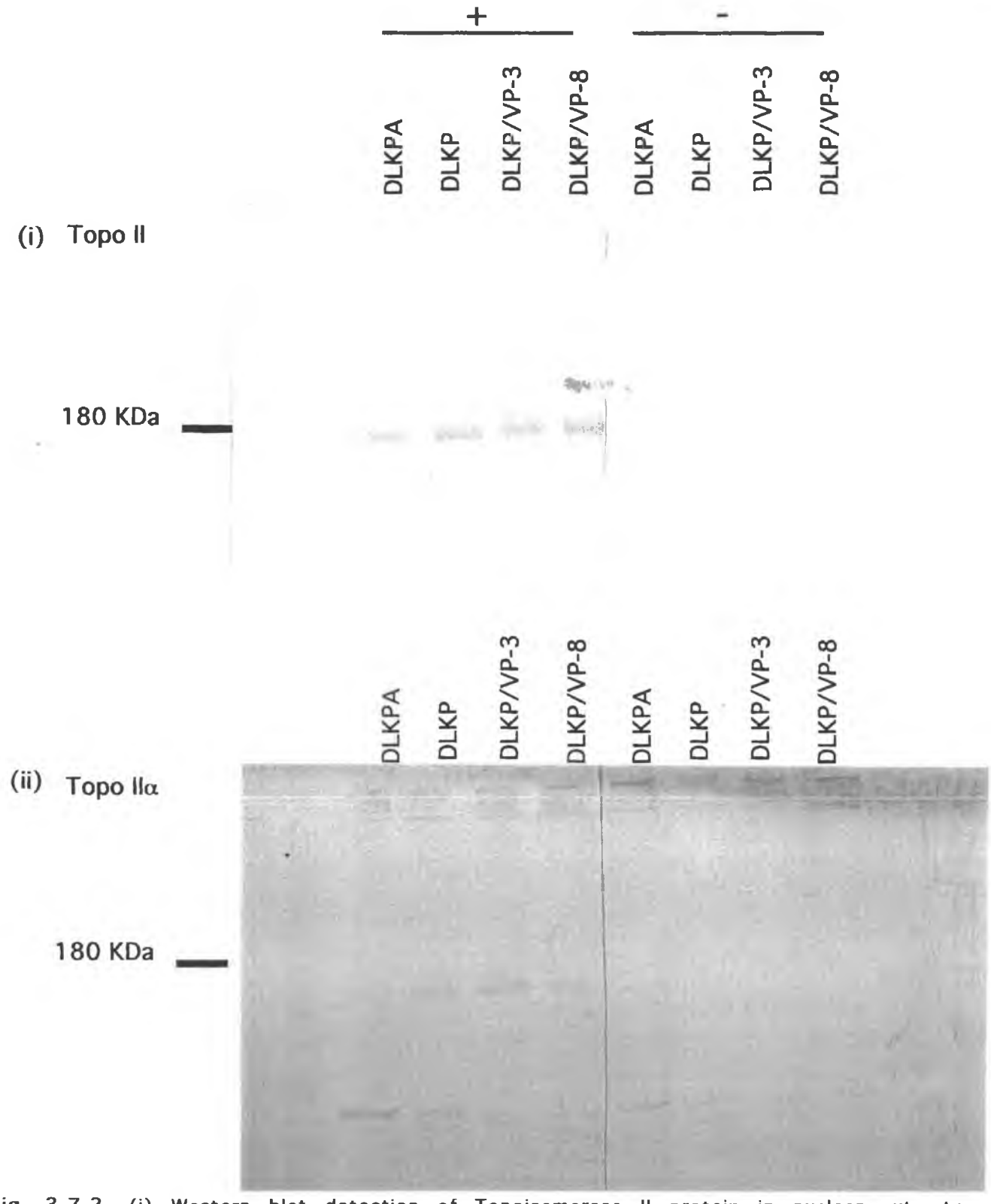


Fig. 3.7.2 (i) Western blot detection of Topoisomerase II protein in nuclear extracts, isolated from DLKP variants. For both the positive (left-hand) and negative (right-hand) blots, 35 μg of protein was loaded per lane. The procedures for both blots were identical, except that the positive blot was incubated with a polyclonal antibody directed against Topoisomerase II, whilst the negative blot was incubated with pre-immune serum. Molecular weight markers were ran in the left-hand lane of the positive blot and the right-hand lane of the negative blot.

(ii) Western blot detection of Topoisomerase IIα in nuclear extracts from DLKP variants, using a monoclonal antibody directed against this protein. For the negative control (right-hand blot), the primary antibody was replaced with an equal volume of pre-immune serum, with the remainder of the blotting procedure performed as for the positive blot (left-hand blot). In all cases, 35 μg of protein was loaded per lane. Molecular weight markers were ran in the left-hand lane of the positive blot and the right-hand lane of the negative blot.

3.8 Induction of MDR1 Gene Expression.

Possible induction of MDR 1 gene expression by exposing cultured cells to a chemotherapeutic drug, for a relatively short period of time, was attempted. This study involved exposing populations of DLKP cells and OAW42 cells to two concentrations of adriamycin (0.1 $\mu g/\mu l$ and 1.0 $\mu g/\mu l$) until obvious signs of stress were apparent, as described by Chaudbary and Roninson (1993), for 72 hours. Possible alterations in MDR 1, Topoisomerase II, MRP or GST π transcript levels were analysed by "*in situ*" RT-PCR using multiplex primers.

As illustrated in Fig. 3.8.1, exposure of DLKP cultured cells to adriamycin resulted in increased cytoplasmic granularity and vacuolar spaces. The cells lost their rounded shape, stretching into long, thin cells with extended processes contacting neighbouring cells. These characteristics were most pronounced at the higher drug concentrations. Similarly, the presence of these concentrations of adriamycin had significant effects on the OAW42 cell line (Fig. 3.8.2). In areas of the flask where the cells had reached near-confluency, the presence of large vacuoles in the cytoplasmic regions were noted. In less densely populated areas, the cells were out-stretched with small protrusions acting as contacts between cells.

As indicated in Fig. 3.8.3(i), no significant changes in the mRNA levels studied were noted with the lung cell line, following exposure to either concentration of drug, when compared to the untreated cells. However, induction of the MDR 1 mRNA levels by adriamycin was apparent in the ovarian cell line, following exposure of two independent stocks of this cell line. The results seemed to correlate with the concentrations of drug added (Fig. 3.8.3(ii) and Fig. 3.8.3(iii)) *i.e.* a slight increase in MDR 1 transcript levels occurred with the presence of 0.1 $\mu g/\mu l$ adriamycin, with a more pronounced effect observed following exposure to the higher drug concentration. mRNA levels of Topoisomerase II, MRP, GST π, or indeed β-actin, were not apparently affected in this way.

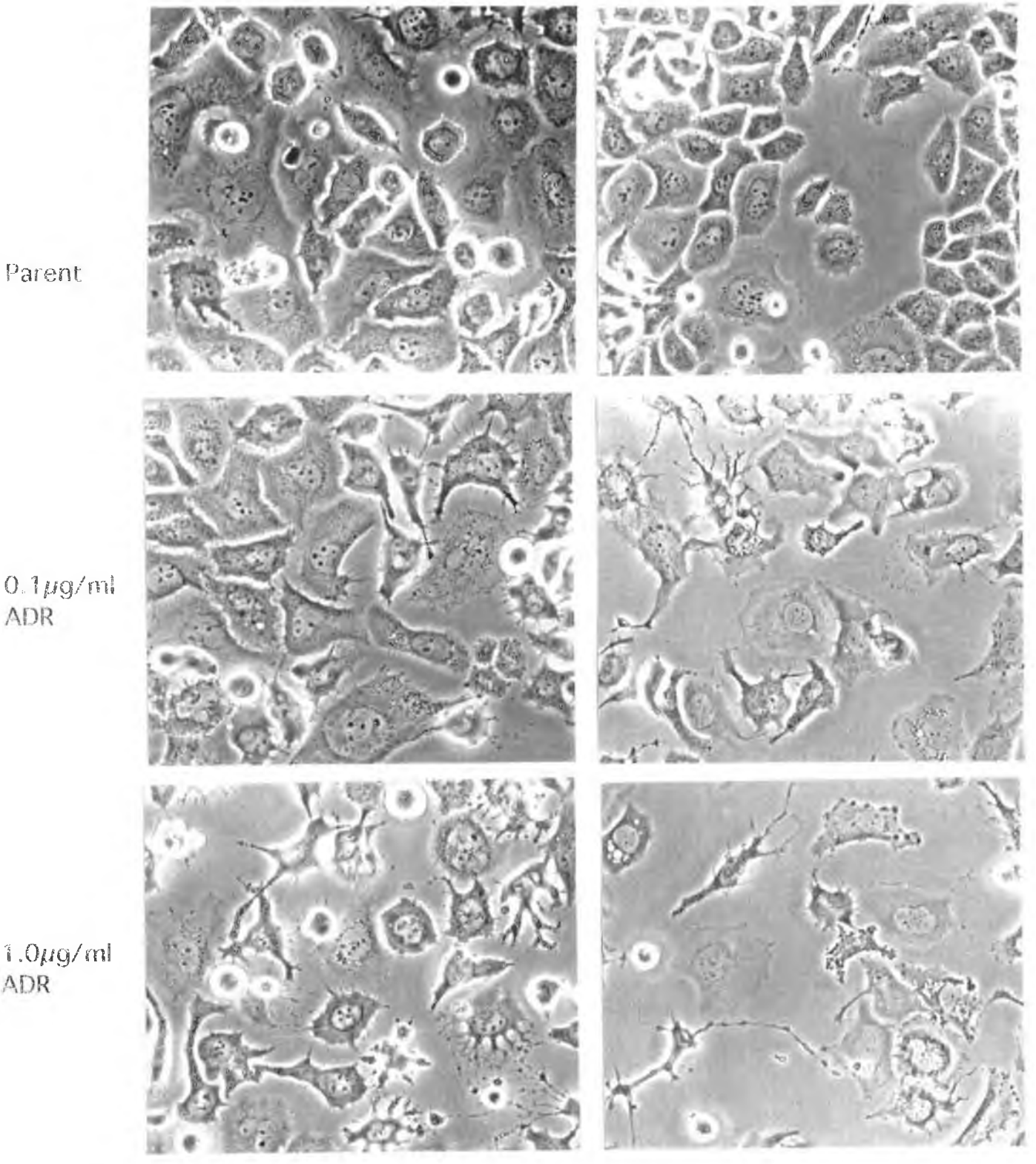


Fig. 3.8.1 Effects of short-term exposure of the drug sensitive lung cell line, DLKP, to increasing concentrations of adriamycin.

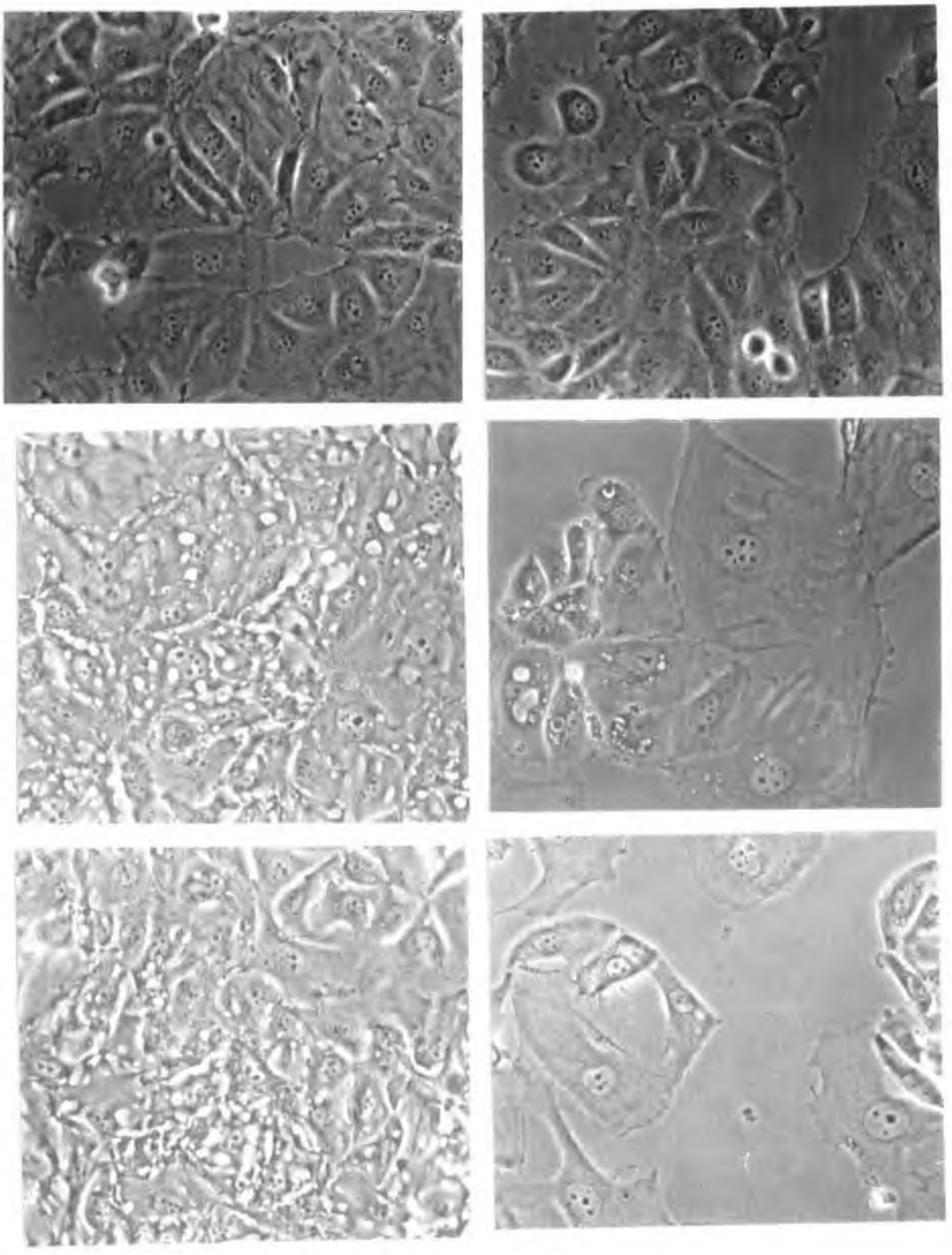


Fig. 3.8.2 Effects of short-term exposure of the drug sensitive ovarian cell line, OAW42, to increasing concentrations of adriamycin.

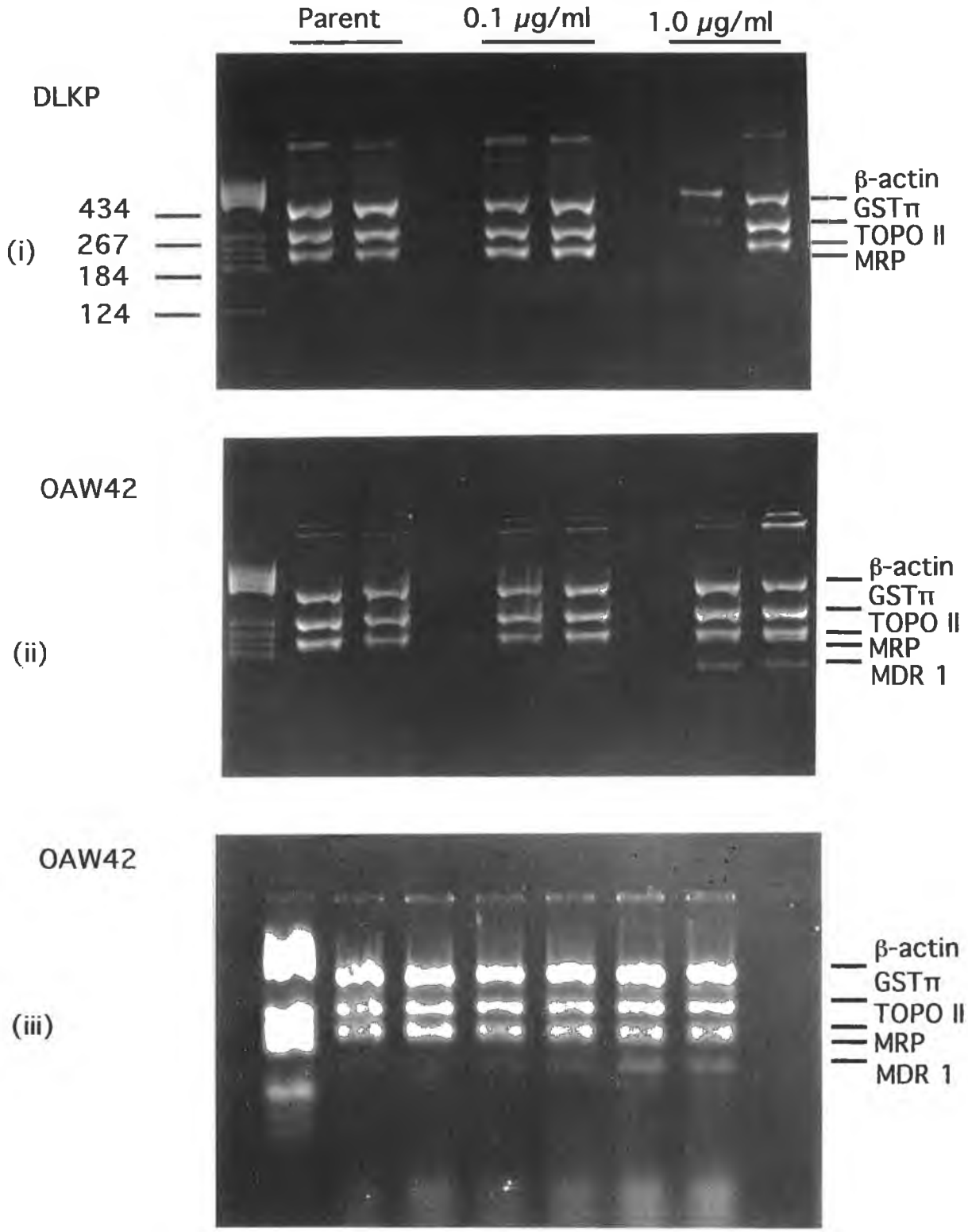


Fig. 3.8.3 Gel electrophoresis of products resulting from *in situ* RT-PCR analysis using multiplex primers to amplify β-actin (383 bp), GST π (270 bp), Topoisomerase II (216 bp), MRP (203 bp) and MDR 1 (157 bp), in cDNA derived from cells following short-term exposure to adriamycin. Sensitive parent cell lines were also analysed as a control. (i) No obvious changes in gene transcript levels were observed in DLKP cells after exposure to adriamycin. (ii) Induction of MDR 1 expression in OAW42 cells seemed to occur. This observation was verified in a repeat experiment (iii) on an independent stock of OAW42 cells.

3.9 Clinical Studies of MDR-Related Factors.

Analysis of transcript levels of MDR-related factors in clinical situations (as well as cultured cells) was evaluated with all of the primer pairs listed in Table 3.1

3.9.1 Analysis of mRNA Transcripts in "Fresh" Tissues.

Amplification of cDNA representing mRNA regions of MDR 1, MRP, GST π, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ and β-actin was attempted with RNA extracted from both normal and tumour lung biopsies. This has been done successfully, in this study, for 8/8 paired lung normal and tumour biopsies (four of which are illustrated as examples in Figs. 3.9.1 - 3.9.4).

Biopsies taken at surgery were placed in ATCC complete medium containing antibiotic (as described in Section 2.4) and were then transported to the laboratory where the RNA extraction procedure was performed in a laminar flow cabinet, as far as possible. Areas of necrosis were removed from the biopsies, which were then rinsed twice in DEPC-treated water. Following this, the tissue was wrapped in tin-foil which had been baked at 180 ^{0}C for at least eight hours. This was then immersed in liquid nitrogen to "snap" freeze the tissue, which was then pulverised by "smashing" the frozen-solid mass. The procedure for extracting RNA from cultured cells was then followed (see Fig. 2.10.1.1).

The MDR 1, MRP, GST π, Topoisomerase I, Topoisomerase II and its Topoisomerase IIα and Topoisomerase IIβ subunit gene transcripts were reverse transcribed and amplified successfully in each of the normal and tumour lung biopsies included in this analysis (Figs. 3.9.1 - 3.9.4). As in the cultured cell studies, amplification of the CYPIA1 region did not occur. In two of the eight cases studied, MDR 1 mRNA regions were apparently over-expressed in tumours, compared to normal tissue (*e.g.* Fig.

3.9.3). In a further three cases (*e.g.* Figs. 3.9.1 and 3.9.4), the levels of this gene transcript seemed to be greater in normal lung than in tumour tissue. No amplification of this region occurred in the remaining three normal or tumour RNA extracts. Whether the lack of amplification of this MDR 1 cDNA region is a true indication of the levels of this gene transcript in some normal and tumour lung tissues is not certain. It is possible that this lack of product formation is due to degradation of the MDR 1 mRNA (or at least, its specific region under study). Why this gene transcript should be affected in this way sooner than MRP, GST π *etc.* has not yet been established.

Although the results from this study are not quantitative (as the PCR reaction was performed for 30 cycles in all cases, and so was not within the exponential phase of PCR product accumulation), it is clear that isolation of intact RNA and analysis of many gene transcript levels can be successfully achieved with both normal and tumour tissues removed at least 1.5 - 4.0 hours before processing. More caution must be exercised, however, when analysing such results, in comparison to those from cultured cells. This is because certain mRNAs in clinical biopies may be degraded in this time period. In the case of cultured cells, the mRNA is generally extracted under ideal conditions *i.e.* from cells which were in their log phase of growth immediately prior to their extraction and so the turn-over of mRNA may be greater. For example, it should be considered that the less intense band representing mRNA (*e.g.* Figs. 3.9.1 and 3.9.4) in the tumour biopsies in comparison to normal lung tissue may be due to mRNA degradation or decreased gene expression.

Figs. 3.9.1.1 - 3.9.1.4 Gel electrophoresis of the. products of MDR 1, MDR 3, MRP, GST π, CYPIA1, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ analysis, by RT-PCR, of RNA extracted from "fresh" normal (N) and tumour (T) lung biopsies. All reactions were set up in duplicate. 30 cycles of PCR amplification were performed. The left-hand lane contained either the DNA molecular weight marker "V" consisting of 22 fragments of the following base pair sizes: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11 and 8, or alternatively, "Phi X" marker digested with Hae III restriction enzyme, resulting in 11 fragments of the following sizes: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 base pairs, with which the target products were sized. The four right-hand lanes on gels (iii) contained negative controls*, where cDNA was replaced in the RT reaction by an equal volume of ultra-pure water.
* The four negative controls shown were the results of MDR 1, MRP, GST π and Topoisomerase II analysis. Negative controls for all other primers were also performed, but are not shown due to a lack of space. As for the negative controls included, no bands resulted.

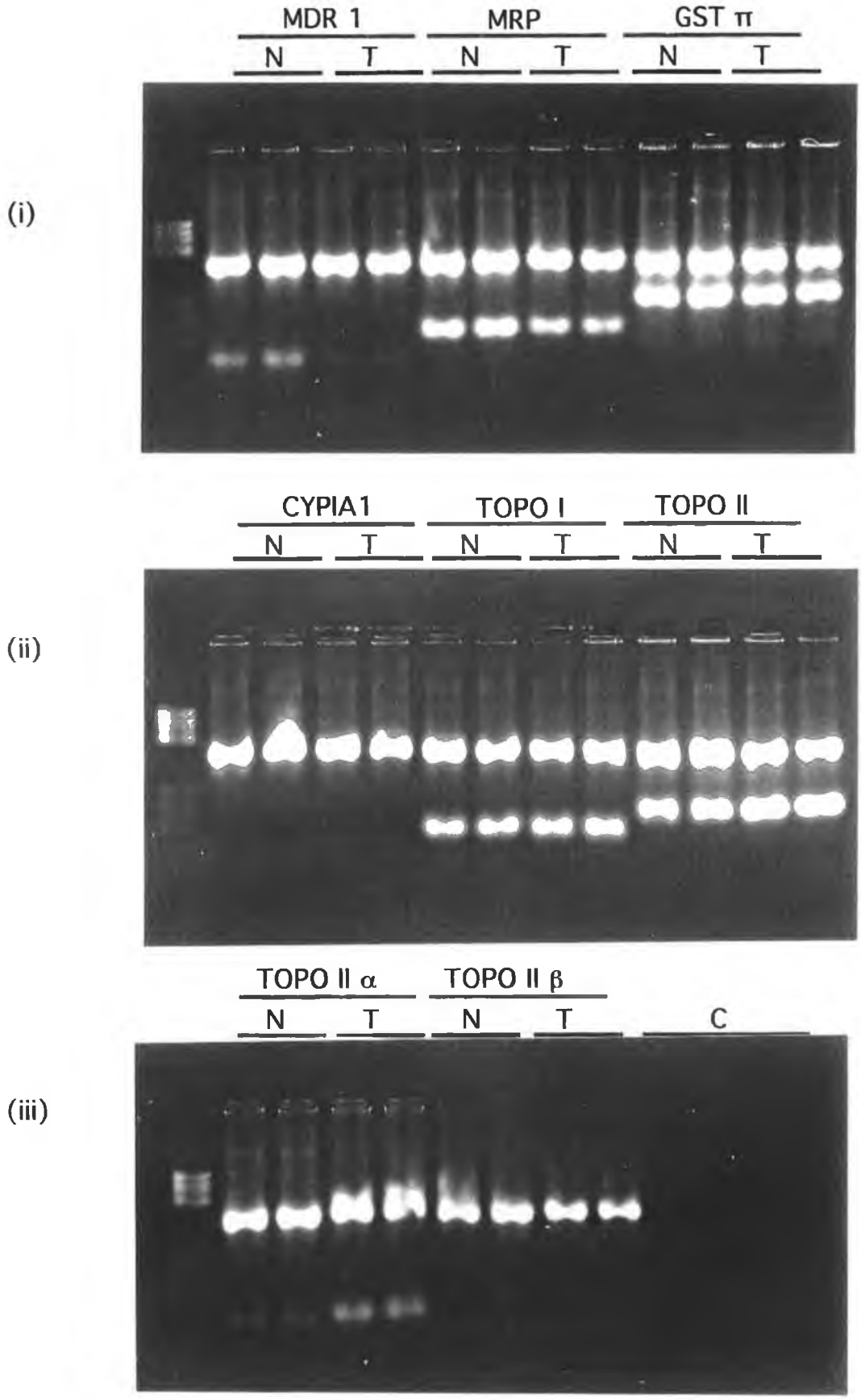


Fig. 3.9.1.1 Gel electrophoresis of products of RT-PCR analysis of mRNA extracted from normal (N) and tumour (T) lung biopsies from a 62 year old female (Hospital No. 7059428). Tissues were processed for RNA extraction within 3.5 hrs of their removal.

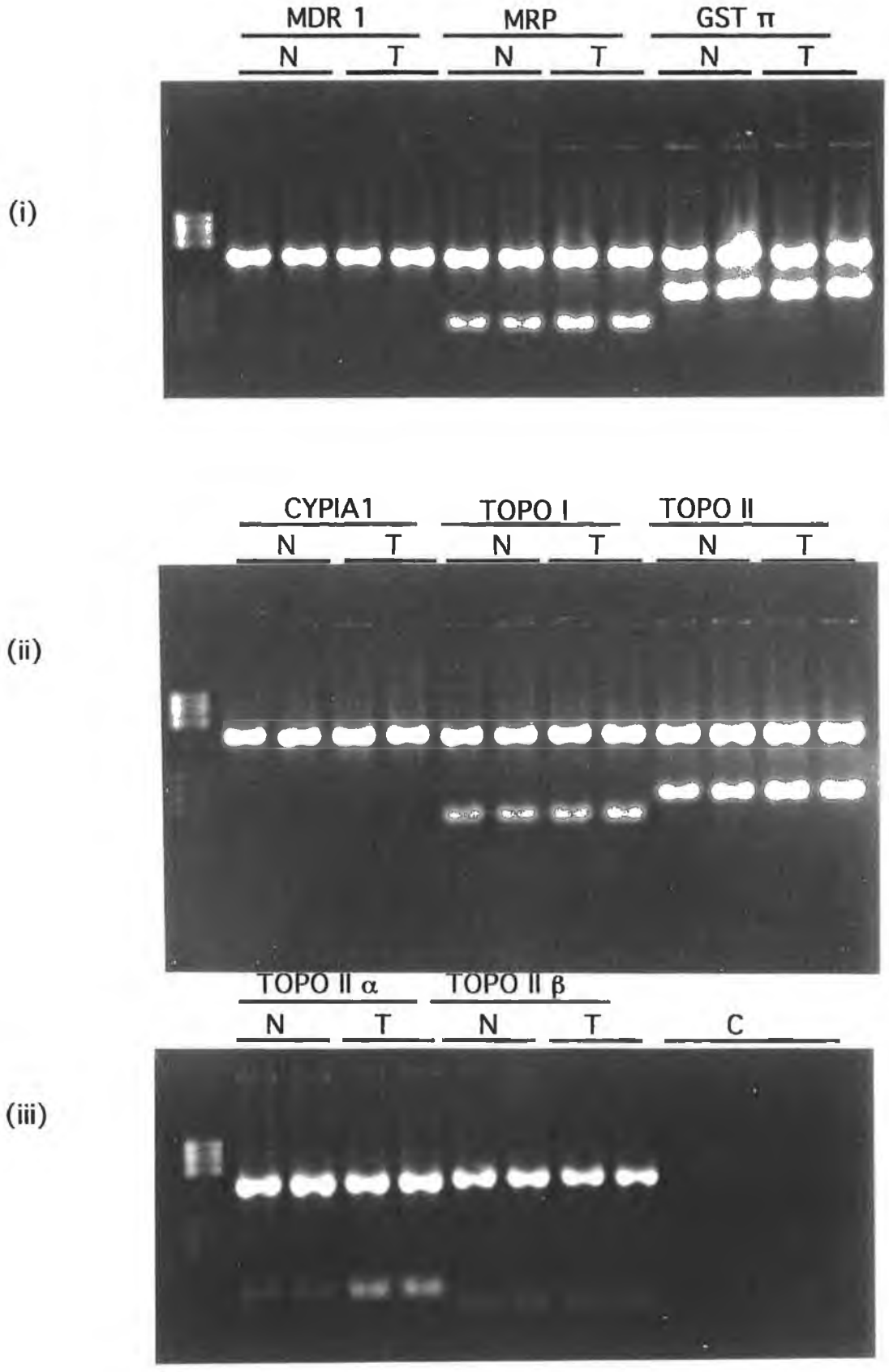


Fig. 3.9.1.2 Gel electrophoresis of products of RT-PCR analysis of mRNA extracted from normal (N) and tumour (T) lung biopsies from a 68 year old male (Hospital No. 2004610). Tissues were processed for RNA extraction within 2 hrs of their removal.

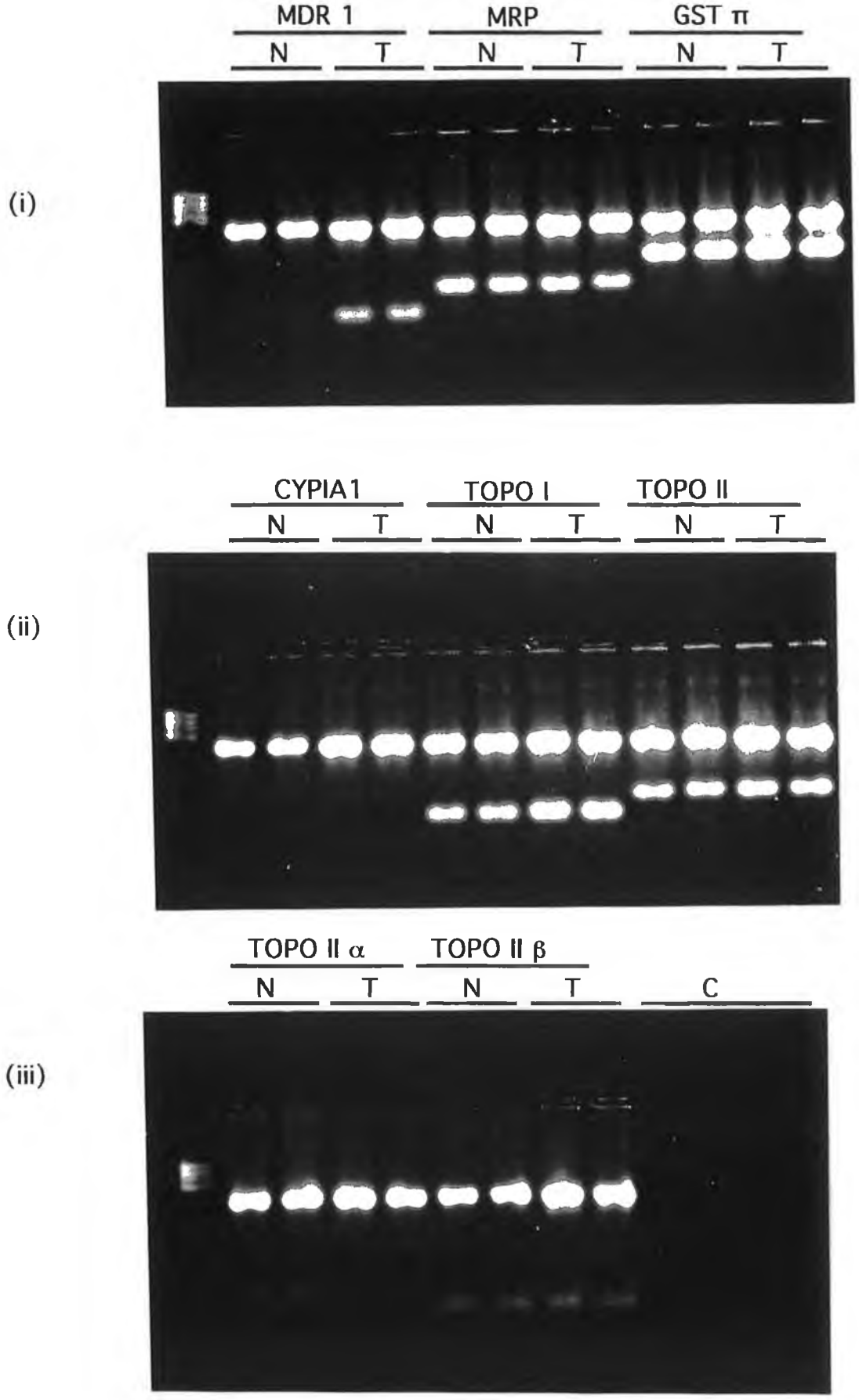


Fig. 3.9.1.3 Gel electrophoresis of products of RT-PCR analysis of mRNA extracted from normal (N) and tumour (T) lung biopsies from a 57 year old male (Hospital No. 397611). Tissues were processed for RNA extraction within 1 hr. 32 min of their removal.

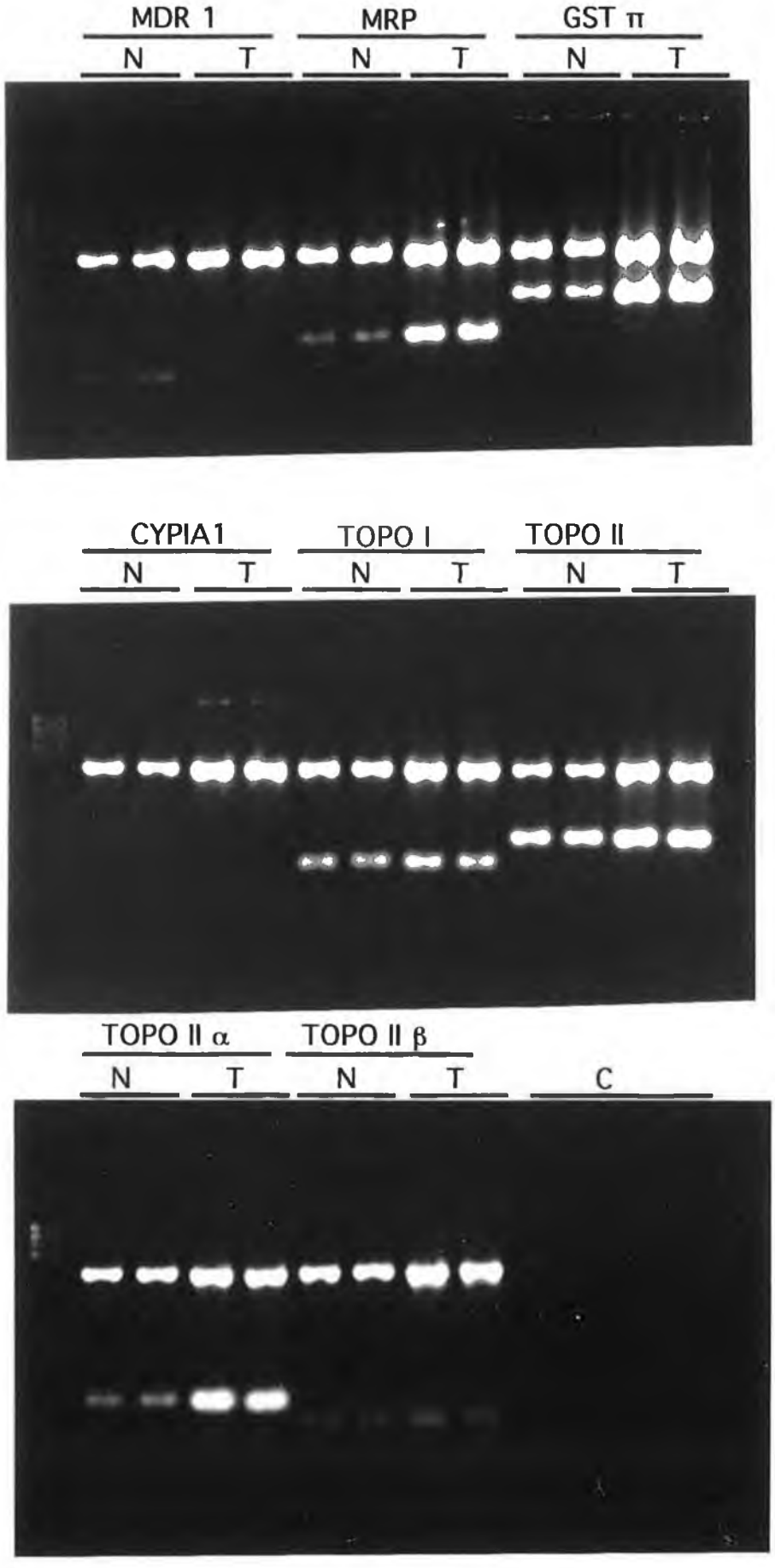


Fig. 3.9.1.4 Gel electrophoresis of products of RT-PCR analysis of mRNA extracted from normal (N) and tumour (T) lung biopsies from a 52 year old male (Hospital No. 405435). Tissues were processed for RNA extraction 4 hrs after their removal.

3.9.2 Analysis of mRNA Transcripts in Paraffin-Embedded Tissues.

Due to the sensitivity of the PCR reaction, retrospective studies of a wide range of pathological archival tissue may be possible. Whether RNA could be extracted routinely, however, from such archival material was uncertain. Jackson *et al.* (1991) reported a 5-day proteinase K incubation for DNA extraction from paraffin-embedded tissue which also produced significant amounts of RNA. Although this was attempted, only successful amplification of β-actin cDNA from paraffin-embedded breast or ovarian tissue was achieved (Fig. 3.9.2). The following procedure was, therefore, developed in an attempt to overcome this problem. (Stringent conditions for RNA handling were adhered to as outlined in 2.10).

Sections of tissue (10 μm thick) were cut from the paraffin block and placed in an autoclaved eppendorf. 200 μl of octane was added to this and placed in a 60 °C water-bath, for 10. mins, to remove the paraffin. The contents of the eppendorf were spun for 10 mins., at maximum speed in a biofuge,at 4 °C resulting in the tissue pelleting at the bottom of the tube and the paraffin forming a wax layer at the top. The wax was removed using a pasteur pipette and the remaining tissue pellet was washed with 70 % ethanol. After re-centrifuging at maximum speed in a biofuge (13,000 g), at 4 °C and removing the ethanol, the tissue pellet was resuspended in 200 μl of digestion buffer consisting of 25 mM EDTA, 100 mM NaCl, 0.5 % SDS, 10 mM Tris-HCL, pH 8.0, to which proteinase K was added to, to result in a concentration of 0.1 mg/ml, immediately prior to its use. The tube was then wrapped in parafilm and incubated at 52 °C with constant agitation, to break down proteins. After 18 hours, a phenol/chloroform/isoamyl alcohol (25:24:1) extraction, followed by chloroform/isoamyl alcohol (24:1) extraction was performed to get rid of protein products. The RNA (and DNA) were precipitated out of solution using two volumes of cold absolute ethanol and a 1/10 volume of 3 M sodium acetate (pH 5.2), at - 80 °C for a period of 30 mins. The RNA and DNA were pelleted by centrifuging

and the supernatant discarded. To get rid of the DNA, the pellet was treated with an RNase free-DNase I enzyme at 37 °C, for 30 mins. RNA was phenol/chloroform/isoamyl alcohol extracted and precipitated out of solution, using ethanol and sodium acetate, as above. After centrifuging at 4 °C, the pellet was resuspended in DEPC-H_2O and quantified spectrophotometrically (as described in 2.10.3).

Initial attempts to extract RNA and amplify the resulting cDNA formed using oligo (dT) primers in the RT-reaction (as in 2.10.4) resulted in amplification of β-actin cDNA only (in many, but not all cases). Using random primers independently, a band was frequently produced for β-actin, plus a lot of non-specific bands (Fig.3.9.3). Similar results were achieved when using gene-specific primers and random primers, independently. Various combinations of these three primer types were used; the most successful combination being a 1 : 1 ratio of oligo (dT) primers : random primers. Results of this analysis are exemplified in Figs. 3.9.4 - 3.9.6. Successful amplification of the internal control, β-actin, and many of the other mRNA transcripts studied, to a greater or lesser extent, (with the exception of CYPIA1) was achieved. A "smearing" effect was seen in most lanes. This was probably due to RNA degradation products.

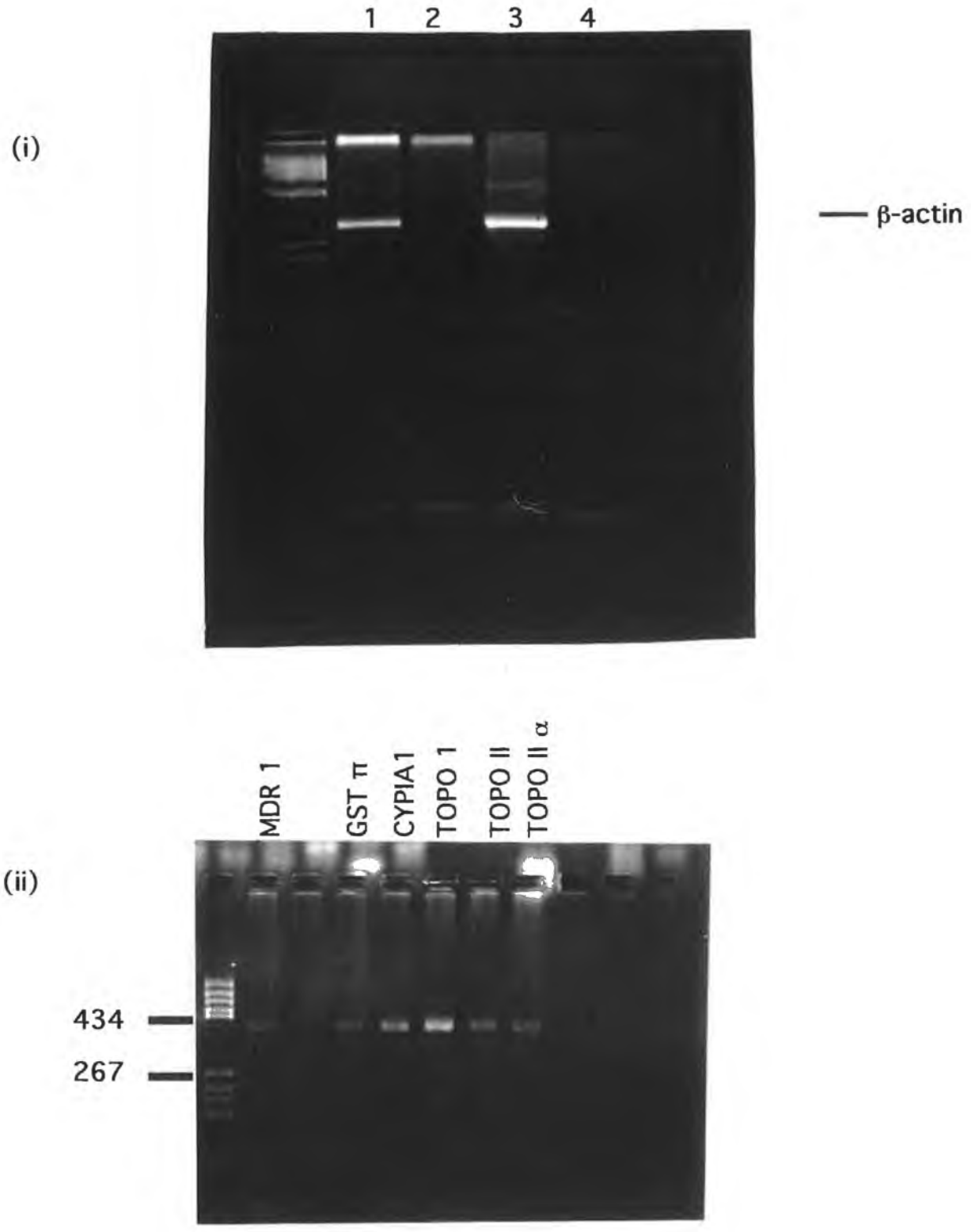


Fig. 3.9.2 (i) Gel electrophoresis of products of RT-PCR analysis using multiplex primers to amplify β-actin (383 bp), GST π (270 bp), Topoisomerase II (216 bp), MRP (203 bp) and MDR 1 (157 bp) from cDNA formed on mRNA templates, extracted from four paraffin-embedded breast tumour biopsies. An RNA extraction procedure, detailed by Jackson *et al.* (1991), was used. cDNA was formed during the RT reaction, using oligo dT primers. Successful amplification of β-actin, only, (in 2/4 cases) was achieved. (Left-hand lane contains "Phi X" molecular weight marker).
(ii) Attempts to individually amplify MDR 1, GST π, CYPIA1, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and β-actin (endogenous control) cDNA formed on RNA templates extracted from paraffin-embedded ovarian biopsy, resulted in amplification, only, of β-actin. The RNA extraction and RT procedures were as for (i) above. (Left-hand lane contains molecular weight marker "V").

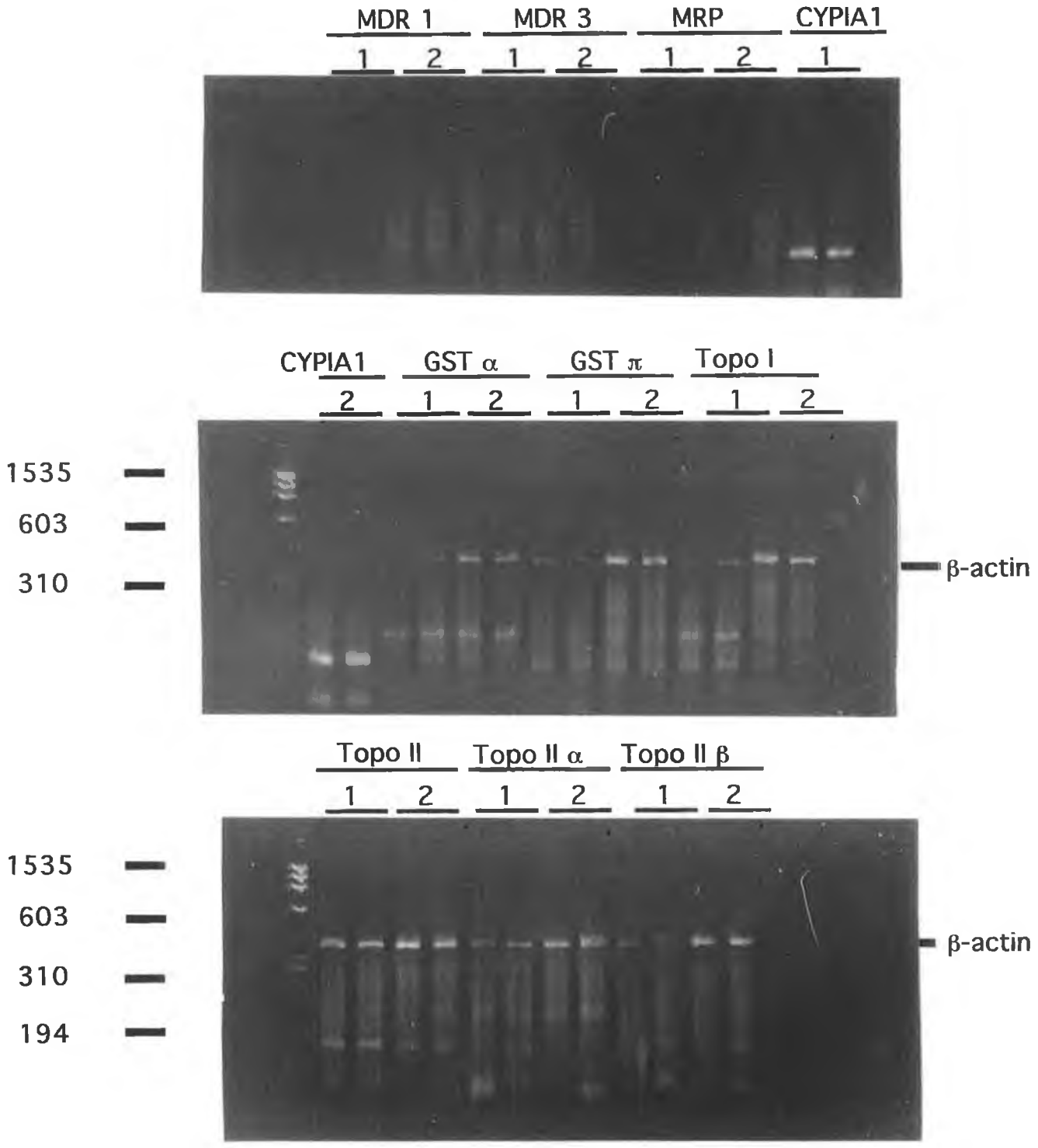


Fig. 3.9.3 Gel electrophoresis of products formed after RT-PCR analysis of mRNA extracted from two paraffin-embedded breast tumour biopsies, designated specimens (1) and (2). RNA was extracted as detailed in Section 3.9.2 and cDNA was formed using Oligo dT primers in the RT reaction. All reactions were performed in duplicate. A band representing β-actin was produced in many cases. No β-actin band was detected, however, after co-amplification with MDR 1, MDR 3, MRP or CYPIA1 primers. "Non-specific" bands resulted in many samples.

Figs. 3.9.4 - 3.9.6 Gel electrophoresis of products resulting from RT-PCR analysis of RNA extracted from six paraffin-embedded breast tumour biopsies. RNA was extracted as detailed in 3.9.2. cDNA was formed on the resulting mRNA templates using a 1 : 1 ratio of oligo dT : random primers, in the RT reaction. Co-amplification of MDR 1 (157 bp), MRP (203 bp), GST π (270 bp), CYPIA1 (327 bp), Topoisomerase I (180 bp), Topoisomerase II (216 bp) Topoisomerase IIα (139 bp) and Topoisomerase IIβ (118 bp) cDNA, respectively, with β-actin (endogenous control), was attempted. The resulting bands were sized by comparison to the molecular weight markers in the left-hand (and sometimes, right-hand) lanes of each gel. Bands representing β-actin resulted in all cases where β-actin primers were included. A number of the target gene products also amplified successfully. Arrows indicate the positions where bands were expected, but successful amplification apparently did not occur.

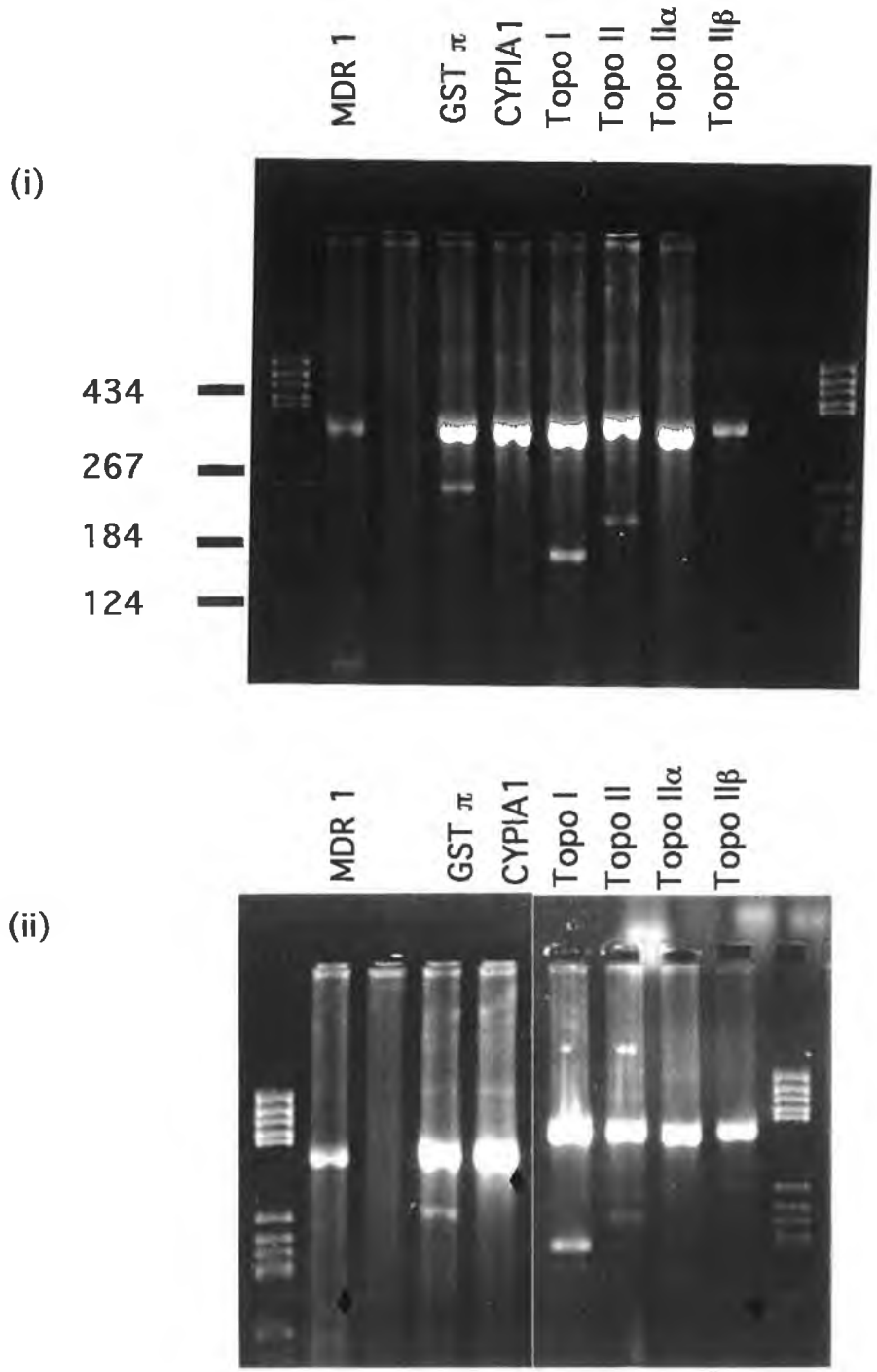


Fig 3.9.4. Gel electrophoresis of products of RT-PCR analysis of mRNA extracted from two paraffin-embedded breast tumour biopsies, designated (i) (Hospital No. 17211/89) and (ii) (Hospital No. 14536/85). These specimens had been stored for 5 yrs. and 9 yrs. respectively, before this analysis was performed. (Arrows indicate the position where bands were expected, but amplification did not occur).

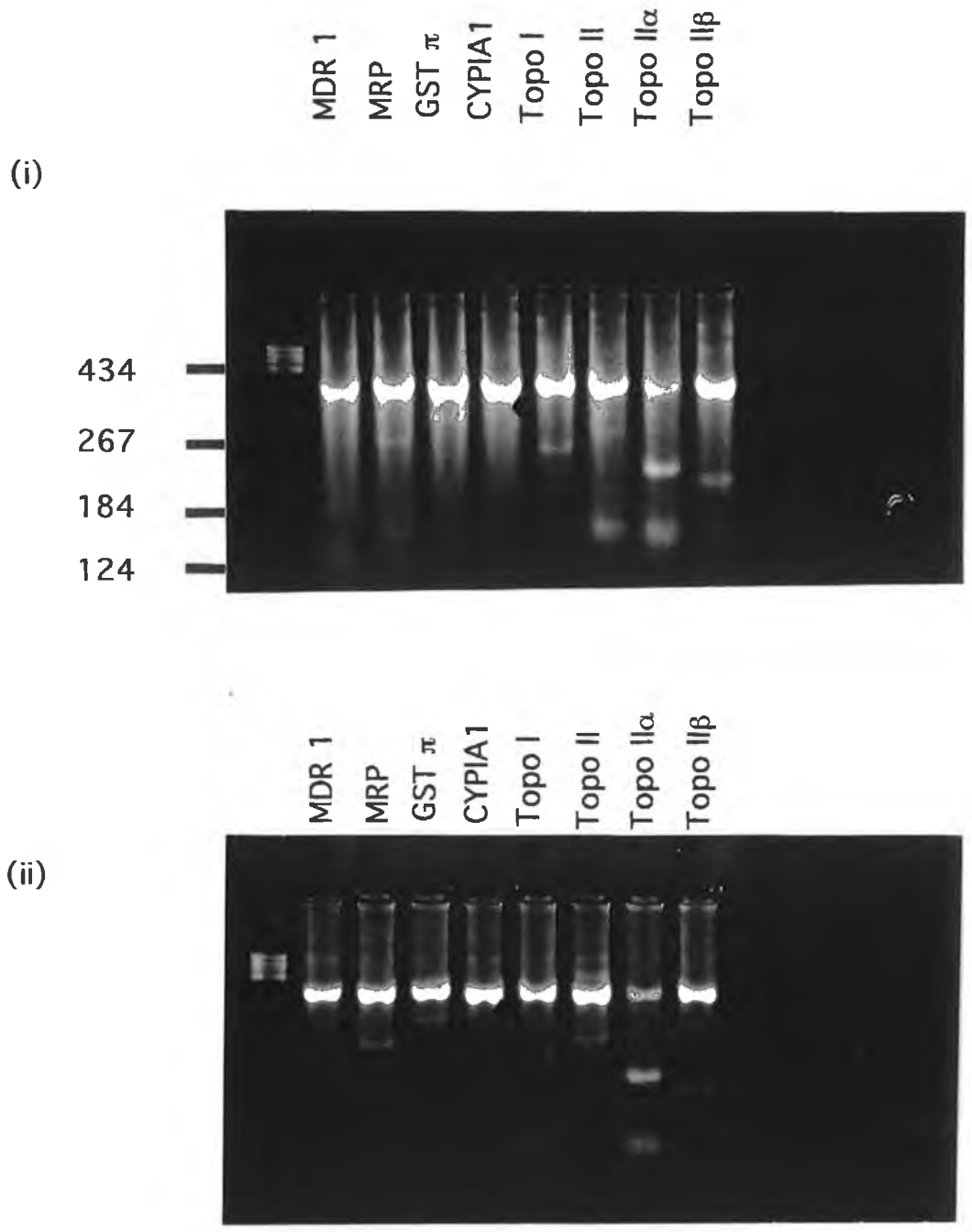


Fig 3.9.5. Gel electrophoresis of products of RT-PCR analysis of mRNA extracted from two paraffin-embedded breast tumour biopsies, designated (i) (Hospital No. 8538/92) and (ii) (Hospital No. 13535/87). These specimens had been stored for 2 yrs. and 7 yrs. respectively, before this analysis was performed. (Arrows indicate the position where bands were expected, but amplification did not occur).

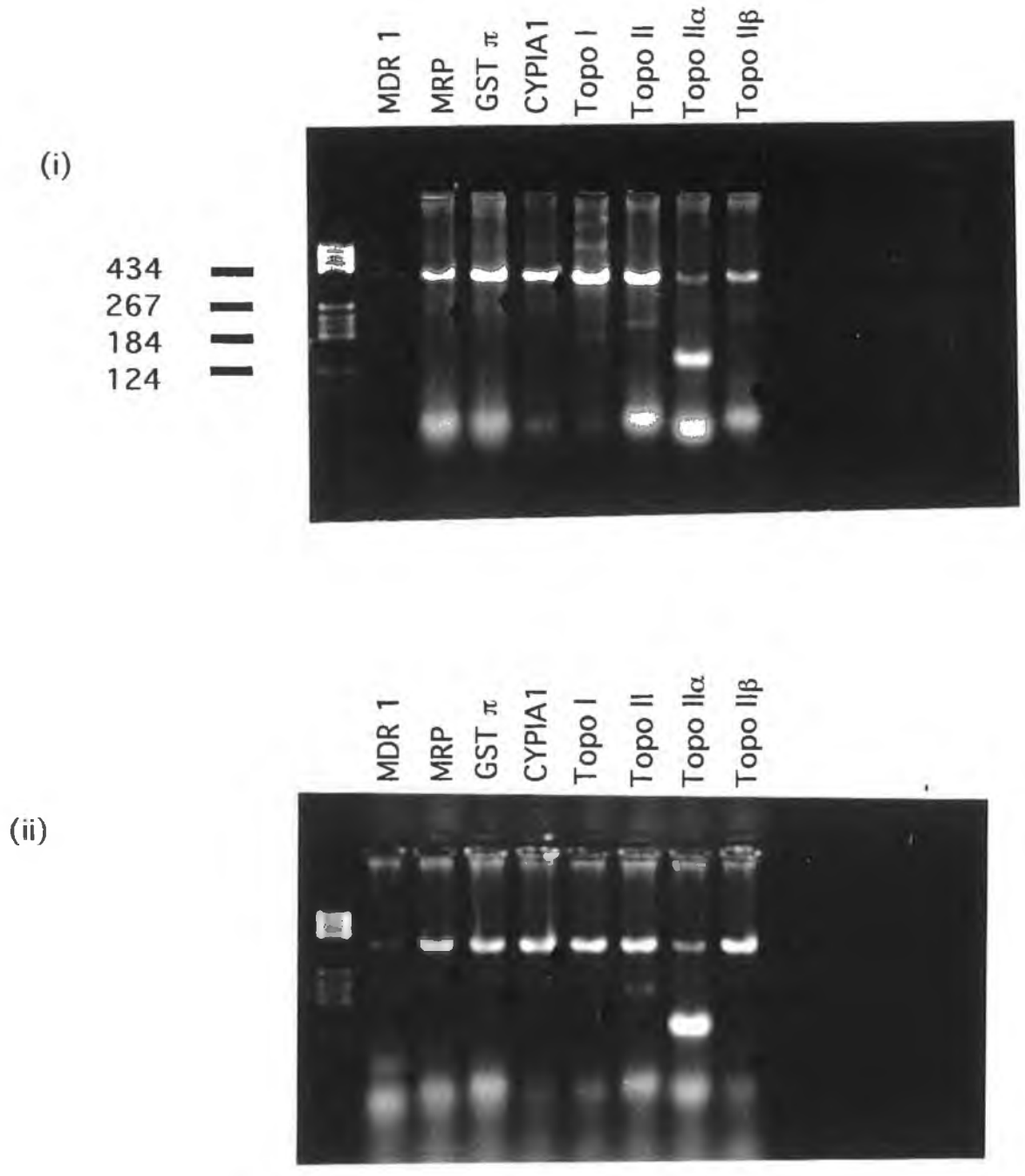


Fig 3.9.6. Gel electrophoresis of products of RT-PCR analysis of mRNA extracted from two paraffin-embedded breast tumour biopsies, designated (i) (Hospital No. 17998/86) and (ii) (Hospital No. 16897/89). These specimens had been stored for 8 yrs. and 5 yrs. respectively, before this analysis was performed. (Arrows indicate the position where bands were expected, but amplification did not occur).

3.10 MDR 1 Ribozyme Transfection and Assessment of its Expression.

An MDR 1-specific ribozyme was transfected into, DLKPA-5F, the most resistant clone of DLKPA. The ribozyme was also transfected into a clone of the parent cell line, DLKP-4 (cloned out of a heterogenous population by Shirley Mc Bride), as a negative control. The transfection technique (Section 2.8) involved calcium-phosphate precipitation of the ribozyme onto the cell monolayer of the cultured cells, followed by glycerol shocking the cells to increase the efficiency of ribozyme uptake. Five colonies of each cell line, arising from single, well-dispersed cells, and known to contain the ribozyme due to their ability to grow in the selective agent, geneticin, were cloned out and maintained as individual cell lines. These were designated DLKP4-RZ1, DLKP4-RZ2, DLKP4-RZ3, DLKP4-RZ4, DLKP4-RZ5, DLKPA5F-RZ1, DLKPA5F-RZ2, DLKPA5F-RZ3, DLKPA5F-RZ4 and DLKPA5F-RZ5.

3.10.1 Ribozyme DNA Analysis by PCR.

Successful transfection of the MDR 1 ribozyme into both DLKP-4 and DLKPA-5F cells was confirmed by performing 20 cycles of PCR amplification on DNA extracted from each cell line (and comparing it to that from cells not transfected) (Fig. 3.10.1). The PCR primers, one specific for a sequence upstream of the polylinker in the plasmid acting as vector and the other for the ribozyme DNA, amplified a 221 bp product. This was clearly distinguishable from the 380 bp GAPDH product (amplified as a control). Densitometry analysis of the bands resulting from PCR amplification of the ribozyme DNA (Table 3.10.1.1) after background subtraction and normalising with GAPDH suggest that the levels of ribozyme successfully transfected into both the sensitive and MDR cell lines was similar in each of the resulting transfectants.

3.10.2 Ribozyme RNA Analysis by RT-PCR.

Expression of the ribozyme mRNA in all cell lines into which the ribozyme was transfected was realised by performing 20 cycles of PCR amplification on RNA extracted from these cell lines (in comparison to DLKP-4 and DLKPA-5F untreated cells). As for the PCR analysis of the ribozyme DNA, the primers for the RT-PCR study were selected so that they did not amplify any endogenous cellular mRNA products (Scanlon and co-workers, City of Hope Medical Center). As indicated in Fig. 3.10.2, successful transcription of the ribozyme occurred in both the sensitive and multiple drug resistant transfected variants, resulting in a 118 bp product. In both the DLKP-4 and DLKPA-5F non-transfected cells, only the GAPDH primers produced a band. This confirms that the 118 bp product is a result of the transfection, and not an inherent cellular component. Contamination of the extracted RNA by DNA would have resulted in an extra band of 880 bp. The fact that the RNA is free from any such DNA is verified by the lack of such an extra band (Fig. 3.10.2).

The levels of expression of the ribozyme appeared to be similar in both the DLKP-4 and DLKPA-5F transfectants, when the intensity of the corresponding GAPDH band was taken into consideration, by visible analysis. This was confirmed by densitometry analysis (Table 3.10.1.1).

3.10.3 MDR 1 mRNA Analysis in Transfectants, by RT-PCR.

To establish if the ribozyme transfected into the cells had an effect on the MDR 1 mRNA levels, RT-PCR analysis of a 157 bp MDR 1 mRNA region was performed using MDR 1 primers detailed in Table 3.1, which flank the MDR 1 mRNA region to which the ribozyme was targeted. The results from this suggest that the DLKP clone, DLKP-4, does not express this gene transcript. As expected, therefore, MDR 1 mRNA expression was not observed in the DLKP-4 transfected cell lines. It has previously been established in this study (see 3.5) that DLKPA-5F cells express

MDR 1 mRNA. This was confirmed in this analysis. Likewise, the presence of MDR 1 gene transcripts were also realised in all of the DLKPA-5F transfected cell lines (Fig. 3.10.3).

Although the ribozyme has been successfully transfected into DLKPA-5F clones (Fig. 3.10.1) and is being expressed in these cell lines (Fig. 3.10.2), MDR 1 mRNA levels in the transfectants appeared to be unaltered, when compared to the transcript levels in DLKPA-5F, the untransfected cell line. This was confirmed by densitometry analysis (Table 3.10.1.1). These results suggest that either the ribozyme is not meeting and cleaving its target MDR 1 mRNA, or that the effect is so small that it is not obvious when analysing the MDR 1 mRNA levels.

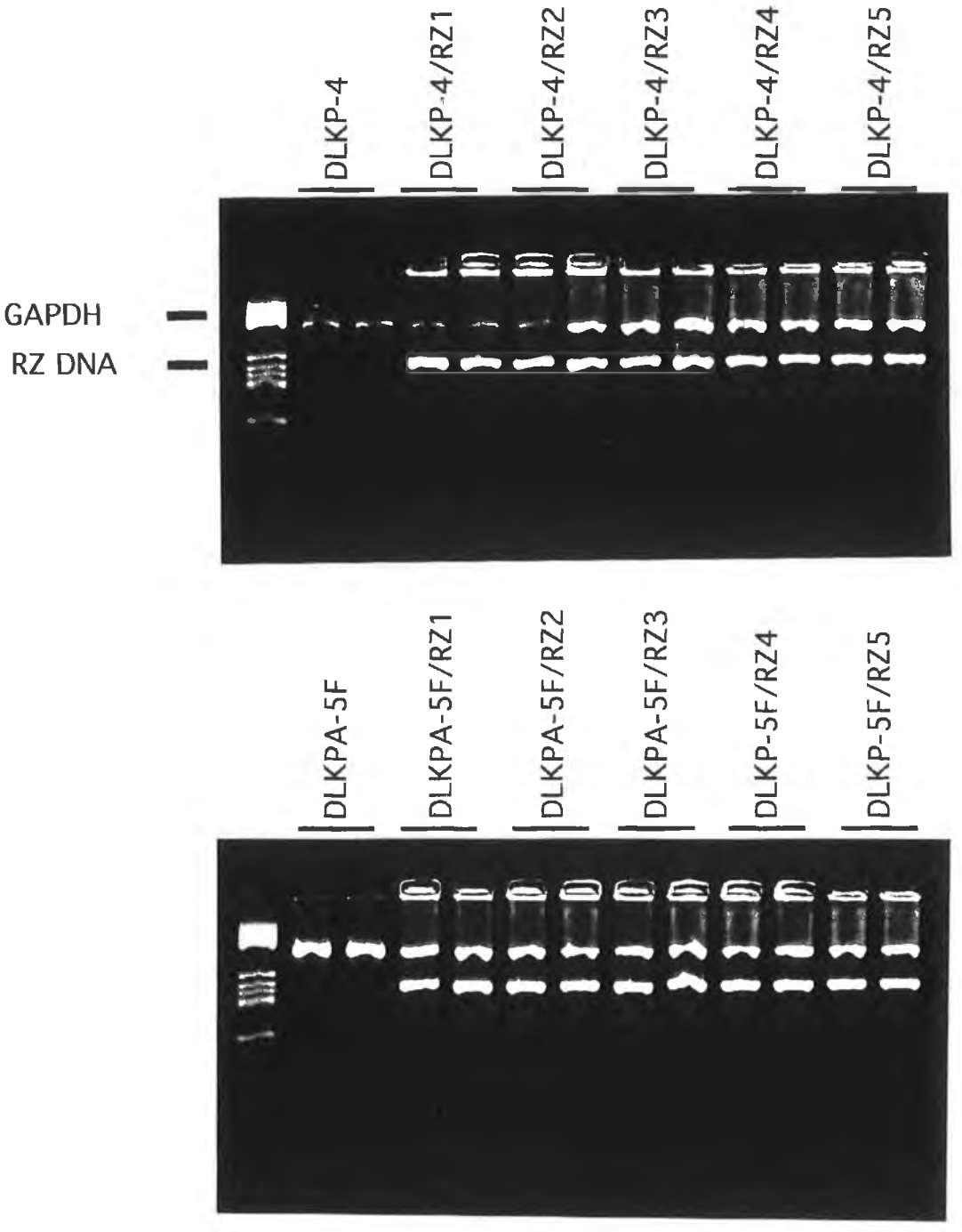


Fig. 3.10.1 Gel electrophoresis of PCR products resulting from amplification of DNA extracted from ribozyme transfectants - DLKP-4/RZ1 - RZ5 and DLKPA-5F/RZ1 - RZ5, and their respective untransfected cell lines. All reactions were performed in duplicate. Two bands were produced in all cases with DNA extracted from the ribozyme transfectants. The upper band (380 bp) was produced by the GAPDH primers, and the lower band (221 bp) by the primers selected for ribozyme DNA amplification, verifying successful transfection of the ribozyme into the cells. Only GAPDH bands were produced for the parent cell lines.

Note: The first few GAPDH bands on the DLKP-4 gel appeared faint due to inconsistant staining of the agarose gel in this region. An average densitometry reading for GAPDH bands in the remaining lanes was used when normalising their corresponding DNA bands.

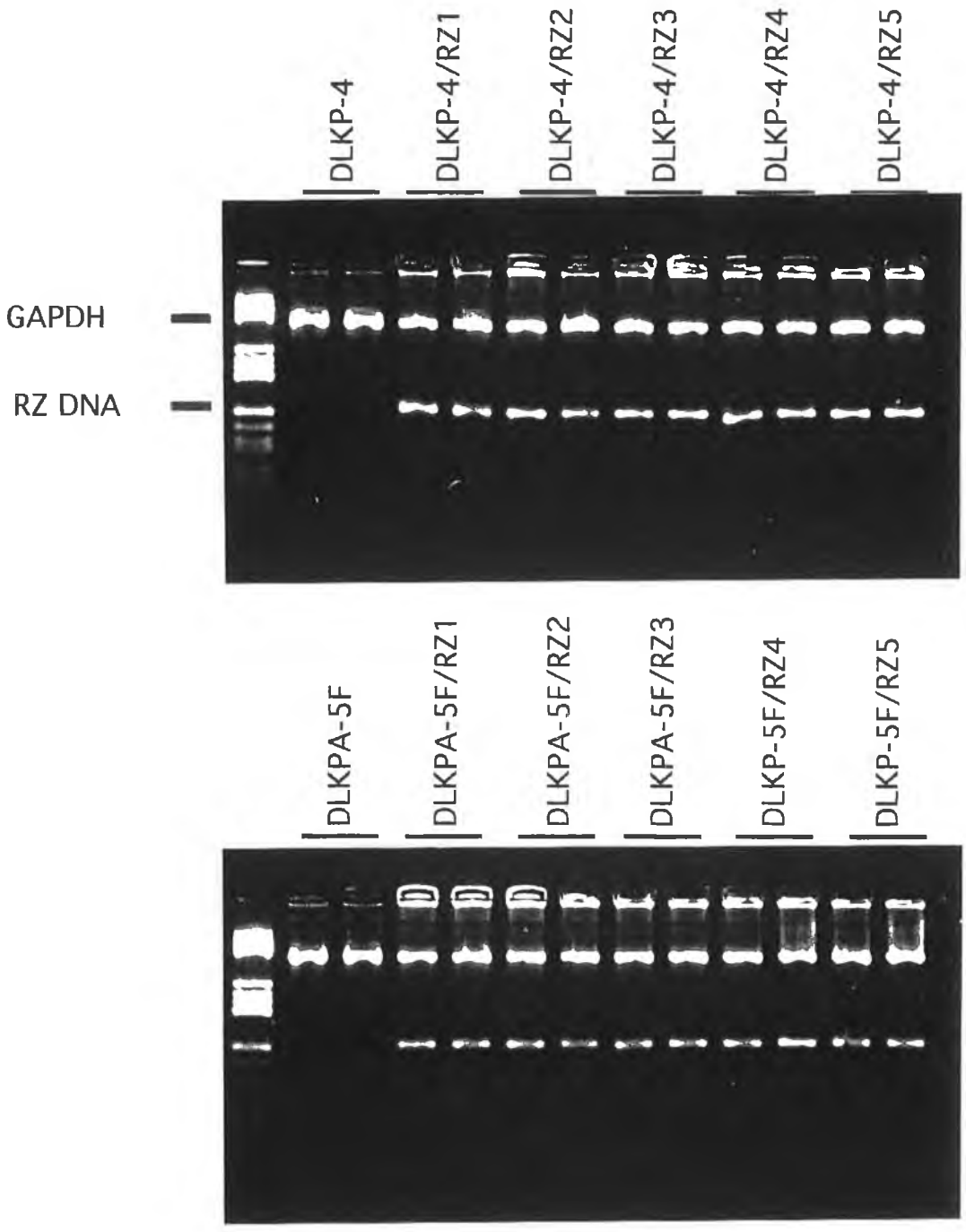


Fig. 3.10.2 Gel electrophoresis of RT-PCR products resulting from analysis of RNA extracted from ribozyme transfectants - DLKP-4/RZ1 - RZ5 and DLKPA-5F/RZ1 - RZ5, and their respective untransfected cell lines. All reactions were performed in duplicate. Two bands were produced in all cases with RNA extracted from the ribozyme transfectants. The upper band (380 bp) was produced by the GAPDH primers, and the lower band (118 bp) by the primers selected for ribozyme RNA analysis, verifying successful expression of the ribozyme in the transfectants. Only GAPDH bands were produced for the parent cell lines.

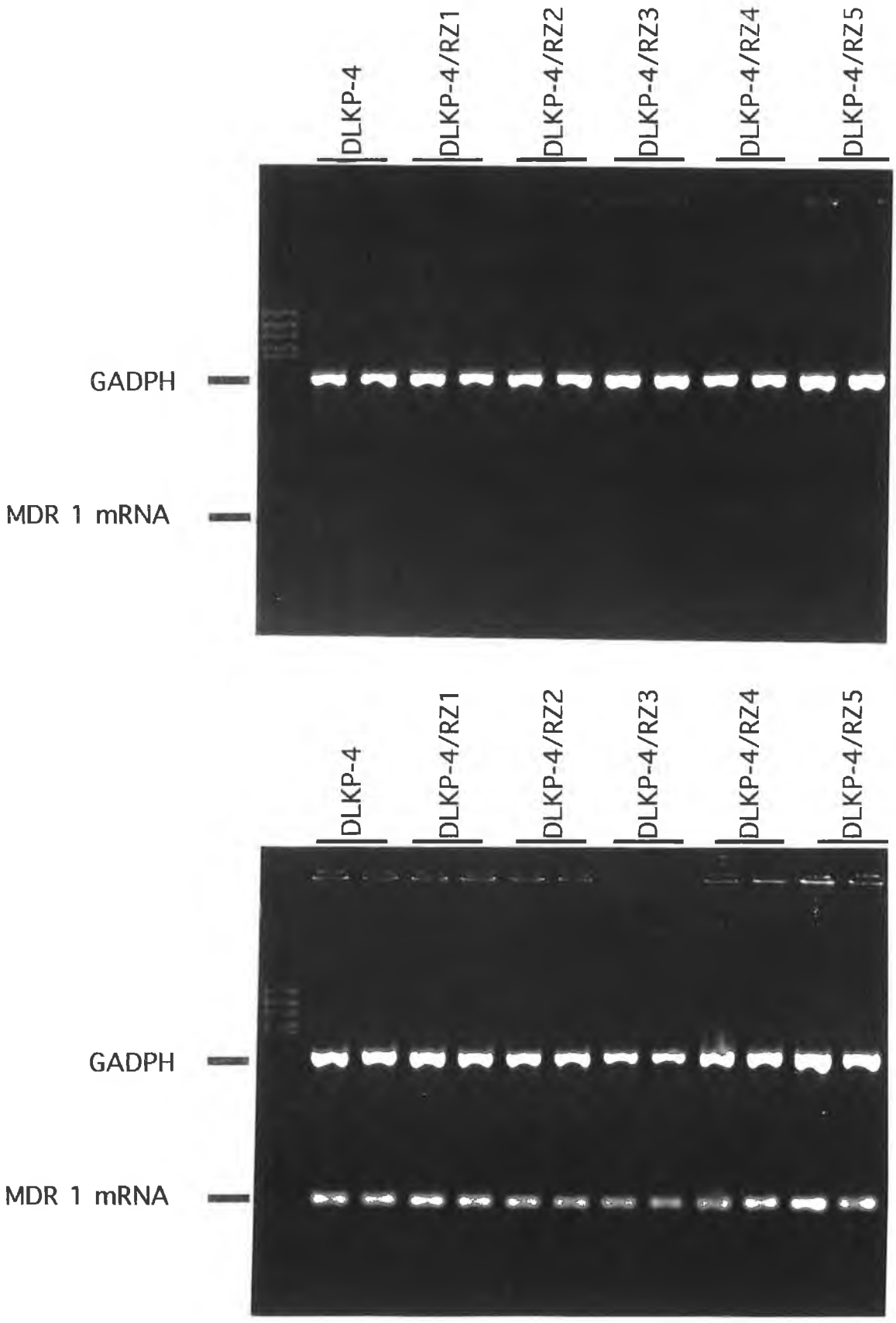


Fig. 3.10.3 Gel electrophoresis of RT-PCR products resulting from amplification of MDR 1 mRNA extracted from ribozyme transfectants - DLKP-4/RZ1 - RZ5 and DLKPA-5F/RZ1 - RZ5, and their respective untransfected cell lines. All reactions were performed in duplicate. One band was produced after amplification of cDNA derived from DLKP-4 and its transfected variants, representing the endogenous control, GAPDH. No detectable MDR 1 mRNA levels were apparent in these cell lines. Two bands, indicative of GAPDH (upper 380 bp band) and MDR 1 (lower 157 bp band) mRNA resulted, on analysis of mRNA extracted from DLKPA-5F and its five transfectants.

Cell Line/Clone	Ribozyme DNA (Fig. 3.10.1)	Ribozyme RNA (Fig. 3.10.2)	MDR 1 mRNA (Fig. 3.10.3
DLKP-4	0.000*	0.000	0.000
DLKP-4/RZ1	0.509*	0.460	0.000
DLKP-4/RZ2	0.493*	0.451	0.000
DLKP-4/RZ3	0.500	0.457	0.000
DLKP-4/RZ4	0.496	0.461	0.000
DLKP-4/RZ5	0.500	0.465	0.000
DLKPA-5F	0.000	0.000	0.610
DLKPA-5F/RZ1	0.499	0.452	0.600
DLKPA-5F/RZ2	0.502	0.450	0.595
DLKPA-5F/RZ3	0.507	0.456	0.608
DLKPA-5F/RZ4	0.500	0.459	0.609
DLKPA-5F/RZ5	0.496	0.448	0.596

Table 3.10.1.1 Analysis, by densitometry, of bands resulting after PCR analysis of (i) transfected ribozyme DNA, and RT-PCR analysis of (ii) RNA resulting from ribozyme expression and (iii) MDR 1 mRNA levels, corresponding to Figs. 3.10.1, 3.10.2 and 3.10.3, respectively. (The data presented is the mean of each of the duplicate results after correction by background subtraction and normalising with GAPDH).

3.10.4 Multiple Drug Resistance Profile of Transfectants.

To determine if expression of the MDR 1 ribozyme had an effect on the cross-resistance profile of the DLKPA-5F cells, toxicity assays were performed in which the sensitivities of all the ribozyme-transfected cell lines (and the DLKP-4 and DLKPA-5F cell lines) to a range of chemotherapeutic drugs, were assessed. The compounds tested included adriamycin, vincristine and VP-16 as these are the chemotherapeutic compounds to which DLKPA-5F has previously been found to be highly resistant (by Dr. Mary Heenan). The sensitivities of all the cell lines to the different chemotherapeutic agents was tested a minimum of three times, with eight replicas performed in each assay. The results are presented in Table 3.10.4.1.

No significant increases (or decreases) in sensitivity to any of the three drugs in the DLKP-4 variants, by compared to their sensitive parent cell was observed. This is as expected as no MDR 1 mRNA has been detected in this cell line.

Results from analysis of the DLKPA-5F transfectants suggested a slight increase in drug sensitivity to adriamycin, vincristine in the DLKPA-5F/RZ2 transfectants, by comparison to DLKPA-5F. Reduced levels of resistance to adriamycin and VP-16 were noted in DLKPA-5F/RZ5. No changes in sensitivity to VCR was observed with this transfected cell line. Reduced levels of drug resistance were not observed in any of the other DLKPA-5F transfectants.

DLKPA-5F							
IC_{50} value (μM)		Parent	RZ1	RZ2	RZ3	RZ4	RZ5
Adriamycin	1	14.988	9.215	11.177	8.873	17.363	8.751
	2		14.504	8.998		14.904	5.027
	3	10.924	14.420	6.278	10.018	7.773	10.092
	X	12.958 (2.872)	12.519 (2.869)	8.820 (2.452)	9.447 (0.812)	13.347 (4.982)	7.958 (2.625)
Vincristine	1	1.608	1.504	1.256	1.589	2.804	1.375
	2		1.869	2.084	4.321	5.114	1.864
	3	3.327 2.483	1.876 3.106	2.089 1.588	3.060 3.024	3.482	3.193 2.199
	X	2.472 (0.859)	2.089 (0.700)	1.754 (0.406)	2.999 (1.115)	3.800 (1.188)	2.109 (0.768)
VP-16	1	13.575	3.840	4.759	9.162	10.141	8.755
	2		5.369	5.260	16.281	10.737	7.543 4.490
	3		4.273	5.292	9.570	9.676	11.582
	X	13.575	4.494 (0.788)	5.104 (0.299)	11.672 (3.998)	10.185 (0.532)	8.092 (2.937)
DLKP-4							
IC_{50} value (ηM)		Parent	RZ1	RZ2	RZ3	RZ4	RZ5
Adriamycin	1	16.311	35.862		15.880		14.052
	2	18.621	25.862	25.862	16.156	19.655	17.087
	X	17.414 (1.552)	30.862 (7.069)	25.862	16.017 (3.145)	19.655	15.568 (2.069)
Vincristine	1	1.809 1.896	2.026		1.690 1.603	1.972 1.744	1.972 2.037
	2		2.015	2.167	1.560	1.831	
	X	1.852 (0.108)	2.015 (0.006)	2.167	1.749 (0.108)	1.852 (0.108)	2.005 (0.108)
VP-16	1	168.026	169.725			181.787	180.088 139.314
	2	161.570	227.659	280.326			181.787
	X	164.798 (10.703)	198.777 (40.775)	280.326		181.787	166.497 (23.785)

Table 3.10.4.1 : The toxicity of adriamycin, vincristine and VP-16 to DLKP-4 and DLKPA-5F cell lines and to their ribozyme-transfected sublines (RZ1, RZ2, RZ3, RZ4 and RZ5). The toxicities of the chemotherapeutic drugs are represented by the IC_{50} values for each drug (in μM and ηM quantities for DLKPA-5F and DLKP-4 cell lines, respectively), as determined by 96-well plate assays and with cellular acid phosphatase levels as an indication of cell growth. Numbers 1-3 refer to repeat assays. X is the mean IC_{50} value, as determined from these assays and the value in brackets are the standard deviations on the mean values.

Chapter 4

Discussion

DISCUSSION

The phenomenon of multiple drug resistance was first discovered a quarter of a century ago. Because of its complex nature, involving many mechanisms (some of which yet remain to be discovered, this major obstacle in the pathway of cancer treatment has yet to be overcome. To aid in the research on MDR, a method for routine analysis of specific gene transcripts in cultured MDR cells and especially in tumours, where very limited amounts of tissue are available, is needed. This method could then, ideally, be used to measure determinants of response to anti-cancer drugs in tumour biopsies from individual patients.

4.1 mRNA Analysis in MDR

A number of established methods for mRNA analysis have been documented to date. As mentioned in Section 1.2, each of these methods has its advantages and disadvantages. The main disadvantages of many of these techniques are their lack of sensitivity (which would prevent them being developed as quantitative techniques), their frequent requirement of radioactive labels as a detection method, and the fact that many of these techniques are laborious and time-consuming, hence limiting the number of samples that can be studied in a given period of time.

It has been long established that even very low levels of MDR 1 gene expression can confer a several-fold increase in the level of drug resistance which may be clinically significant (Shen *et al.*, 1986a; Chin *et al.*, 1989). MDR 1 expression has been analysed by conventional RNA slot/dot blotting techniques (Goldstein *et al.*, 1989; Fojo *et al.*, 1987; Bourhis *et al.*, 1989) and Northern blots (Shen *et al.*, 1986b; Roninson *et al.*, 1986; Fojo *et al.*, 1987; Kramer *et al.*, 1993). However, in cells with a several fold increase in cellular drug resistance, MDR 1 expression levels are close to the limits of sensitivity of

conventional methods even when large amounts of RNA are used for the assay (Shen *et al.*, 1986b; Fojo *et al.*, 1987; Noonan *et al.*, 1990). RNA blotting techniques may not be sensitive enough to detect low levels of MDR 1 gene expression which may be sufficient for clinically relevant multiple drug resistance (Shen *et al.*, 1986b; Noonan *et al.*, 1990; Noonan and Roninson 1991; Holzmayer *et al.*, 1992).

There are a number of additional problems in the study of gene expression in multidrug resistance in clinical samples. The amount of available tissue is often limited; analysis of RNA from these samples is often problematic because RNA may have suffered significant degradation. Detection of MDR 1 is often complicated by the existence of a homologous gene, MDR 3, which is apparently not associated with resistance to chemotherapeutic drugs; MDR 3 mRNA shows cross reactivity with some MDR 1 nucleic acid probes. When using MDR 1 and MDR 3 DNA probes high stringency hybridisation conditions are required to avoid cross hybridisation of MDR 3 probes with MDR 1 mRNA. This limits the sensitivity of the assay and as a result, cells with a low degree of resistance may escape notice. MDR expression in tumours is often heterogeneous with only a subpopulation of tumour cells expressing the gene (Weinstein *et al.*, 1990). Even if some cells in the tumour express the gene at a relatively high level, heterogeneity may lead to a very low signal when total RNA extracted from the tumour is analysed. Subpopulations of p-glycoprotein expressing cells within the tumour may have a growth advantage in the course of chemotherapy (Noonan *et al.*, 1990). These problems were all faced when selecting a method of gene transcript analysis for MDR 1 and a number of other drug related genes.

The development of PCR-based technology has provided a sensitive, specific and quantitative protocol for measuring the levels of MDR 1 mRNA in clinical samples which overcomes many of these drawbacks. PCR methods require much less tissue than traditional methods and so are applicable to the detection of MDR 1 in clinical samples, even from a heterogeneous population

of cells. In addition, by careful choice of sequence specific primers, it is possible to amplify selectively even such highly related sequences as MDR 1 and MDR 3. Therefore, although the RT-PCR method has one main disadvantage (*i.e.* it does not render information on transcript size), this method was chosen for the work in this thesis due to its sensitivity, specificity, speed and ease.

4.2 Selection of an Endogenous Control to use in routine RT-PCR Analysis

The comparison of biochemical quantities requires normalisation so that the data can be compared to a common denominator. Although when studying cultured cells this may be based on cell number, the number of cells that are present in a tissue specimen cannot usually be established with certainty. One main advantage of using the expression of an endogenous gene in this capacity as an internal standard is that the reference mRNA and target share the same circumstances throughout preparation of the sample (*e.g.* fixation and embedding of tissue) and mRNA isolation and storage.

Considerable differences in efficiencies of PCR amplification between tubes has been reported (Gilliland *et al.*, 1990a,b; Wiesner, 1992), reasons for which are suggested in Section 1.3. Because this study involved analysis, by RT-PCR, of many gene transcripts in many cell lines and biopsies, an effort was made to select an endogenous control (or controls) that could be used routinely. Some of the main considerations when choosing such an endogenous control were that its levels of expression should be the same in each of the samples to be compared, not varying appreciably during the cell cycle or among different tissues, and not affected by experimental conditions. The products resulting from its amplification had to be easily distinguished from the resulting target products and the primers chosen for its study should not cross-react with primers amplifying any of the target cDNAs. From a list of at least nine

potential endogenous controls documented in the literature (see Section 1.3), β-actin, β_2-microglobulin, Esterase D and Glyceraldehyde-6-phosphate dehydrogenase (GAPDH) were considered for this role. Previously published primers for the amplification of β_2-microglobulin, Esterase D and GAPDH were considered, after assessing how they met with the criteria suggested for choosing primers and checking that they did not cross-react with any of the other primer pairs included in this study, with which they would potentially be used.

β-actin: Suitable primers for β-actin cDNA amplification were very difficult to select. Actin is the most abundant structural protein in eukaryotic cells (Nakajima-Iijima *et al.*, 1985; Pollack, 1980; Firtel, 1981; Fulton, 1981) with at least six isoforms in vertebrates. β-actin, a cytoplasmic actin, co-exists with γ-actin in many cell types and is involved in a variety of functions. Multiple gene copies related to the cytoplasmic actins have been found to exist, most of which seem to be pseudogenes (Engel *et al.*, 1981; Moos and Gallwitz, 1982; Moos and Gallwitz, 1983; Ponte *et al.*, 1983). Furthermore, over-expression of cytoplasmic actin has been found to be associated with neoplastic transformation of human fibroblasts (Leavitt and Bushar, 1992; Nakajima-Iijima *et al.*, 1985). β-actin, however, is generally favoured as a standard by many researchers including Horikoshi *et al.* (1992), Avraham *et al.* (1992) and Lönn *et al.* (1992).

The primers selected for β-actin (Table 3.1) in this thesis successfully amplified a region of 383 bp (and 590 bp had there been any contaminating DNA present). Care was taken to ensure that neither of these primers would cross-react with any of the other primer pairs selected to either amplify another cDNA region or form "primer-dimers". Examples of the bands resulting after amplification with these primers can be seen in many of the figures included in the results (Section 3.2). When levels of β-actin were evaluated in drug resistant and sensitive cell lines, equal levels of this mRNA were found to exist, proving that the experimental conditions did not effect its expression.

This is exemplified by results from analysis of HEp-2 and HEp-2A (Fig. 3.2(i), Table 3.2.1).

β_2-Microglobulin: β_2-microglobulin has also been used as an internal control (Noonan *et al.*, 1990). β_2-microglobulin is a small (12 kDa) polypeptide found in serum and associated with the major histocompatibility complex class 1 heavy chain on the surface of nearly all cells (Güssow *et al.*, 1987). It has been claimed by others as a gene that is ubiquitously expressed and its expression correlates with the cell surface area in most cell types (Noonan *et al.*, 1990). Noonan *et al.*, (1990) suggested that as the amount of β_2-microglobulin is not constant in different cell types, but is proportional to the cell surface area, it may not be the best control for genes encoding for intracellular proteins.

In tumour types, including small cell lung cancer, the amount of β_2-microglobulin and another HLA-related gene may be decreased or eliminated (Doyle *et al.*, 1985). Horikoshi *et al.* (1992) studied the ratios of β_2-microglobulin : β-actin and found a considerable variation among the tissues tested. This suggested that either one or both are not expressed at similar levels among different tissues. β-actin was found to be more constantly expressed and so was favoured by them as the internal standard. In their studies of bladder cancer, Clifford *et al.* (1994) observed a 140-fold variation in β_2-microglobulin levels between individual tumours, rendering it unacceptable as a control. Noonan et al. (1990), however, considered this as an acceptable control for their studies of MDR 1 expression in a range of cell lines and human normal and tumour tissues.

Although the levels of β_2-microglobulin were generally slightly reduced (as assessed by densitometry analysis) in the MDR cell lines included in this study (Fig. 3.2(ii), Table 3.2.1), the levels of transcript reduction were probably insignificant.

Esterase D: Esterase D has also been chosen as an internal control by Cole *et al.*, (1991) who claimed it to be less dependent on cell cycle than other genes *e.g.* β and γ actins. Because of the nature of their study they favoured Esterase D over β_2-microglobulin as they believed β_2-microglobulin to be often undetected in small cell lung cancer. However, the polymorphic enzyme Esterase D (13q14) has been used as a marker for loss of heterozygosity in linkage analysis studies of the retinoblastoma gene (RB1). Mutations of the RB1 gene have been found in many tumour types and in particular, in small cell lung cancer. In 77% of small cell lung cancers studied, absence or trace levels of RB1 mRNA have been reported (Macdonald and Ford, 1991). It is possible therefore that Esterase D may also be affected in this way. This would make it an unreliable control if its presence in all tumour types studied was uncertain.

In this study, levels of Esterase D were frequently found to be slightly increased in MDR cells, by comparison to their corresponding sensitive cell lines. An example of this is seen in duplicate studies of HEp-2 and HEp-2A (Fig. 3.2(iii), Table 3.2.1). Based on these studies, Esterase D was not considered as an appropriate control for further studies.

GAPDH: GAPDH, like β-actin, is frequently used as an endogenous control in Northern blot analysis (Nakajima *et al.*, 1993). In their evaluation of a number of "house-keeping" genes as potential endogenous controls, Dukas *et al.* (1993) suggested GAPDH to be a very reliable endogenous control for studies of gene expression. Based on this, GAPDH levels were assessed in this study, but were generally found to be slightly increased with MDR (Fig. 3.2(iv), Table 3.2.1).

Although β-actin, β_2-microglobulin and GAPDH may be acceptable as endogenous controls, β-actin was favoured for several reasons. The main reason for the choice of β-actin as control for routine RT-PCR analysis was that the levels of this gene product appeared to be practically the same in all of the cell lines studied, when compared to their parent cell lines.

The bands resulting from amplification with these primers was generally strong and so easily detected (compared, for example, with that produced when analysing Esterase D). This product length was slightly greater than all of the other target products assessed in this study. Therefore, if RNA degradation occurred, based on the size of the amplified regions, β-actin would probably be at least as likely to be affected as any of the target amplified products. In a few cases throughout this thesis, however, β-actin was not suitable and so another endogenous control was amplified.

For example, when amplified products of Topoisomerase IIα or Topoisomerase IIβ were to be digested with a restriction enzyme to confirm amplification of the expected region, β_2-microglobulin was used as a control instead of β-actin. This is because the region amplified with the primers chosen for β-actin also contain a recognition site for the restriction enzymes used to cleave Topoisomerase IIα (Alu 1) and Topoisomerase IIβ (Hph 1) products. It therefore simplified matters to use a control region not affected in this way.

β_2-microglobulin (114 bp) was also chosen as endogenous control in quantitative PCR studies (Section 3.6) where the MDR 1 exogenous control was used. The reason for this is that the exogenous control amplified products is 331 bp in length, and those of β-actin and GAPDH are 383 bp and 380 bp, respectively. Because of the relatively close proximity of the exogenous control and β-actin bands on the resulting agarose gels, the results were more clear and more easily (and so possibly more accurately) evaluated by densitometry if they were well separated.

GAPDH was favoured as control when analysis of the ribozyme transfectants was performed. This is because the vector which contains the ribozyme DNA at its polylinker site and which is transfected into the cells, also contains β-actin sequences upstream from this region (which are used for other purposes in other studies). To prevent the possibility of the primers

selected for β-actin binding to this sequence and possibly resulting in amplification of "non-specific" bands, β-actin primers were not used.

4.3 Primers used in RT-PCR Analysis.

The primers used in this RT-PCR analysis of MDR-related gene transcripts were designed and selected as part of this thesis, with the exception of MDR 1 and β_2-microglobulin primers, which had previously been published by Noonan *et al.* (1990). However, as with all the other primer pairs the ability of these MDR 1 and β_2-microglobulin primers to fit the pre-determined criteria for primer selection was assessed. As detailed in Section 3.1, it was not possible to conform to all the guide-lines when choosing primers, so compromises had to be made between differing requirements. It was ensured, however, that "primer-dimers" would not occur between any of the selected primer pairs and those chosen to amplify the endogenous controls. The primers were also designed in such a way that cross-reactivity would not occur between any of the primers chosen to amplify the target cDNA regions, so that many of these primer could be used simultaneously as multiplex primers. To confirm amplification of the expected molecules, cleavage with a restriction endonuclease enzyme whose site of recognition in the amplified region is known, was included.

The successful choice of all primers was, therefore, verified by bands of the predicted size being produced following amplification with each primer pair (Fig. 3.1.2.1) and, again, following restriction of the resulting products (Fig. 3.1.2.2) with appropriate restriction enzymes (Table 3.1). The predicted lengths of bands that would result from DNA amplification with these primers are also detailed in Table 3.1, when ever the gene sequence information was available. Successful isolation of RNA, free of contaminating DNA was shown by lack of amplified bands, corresponding to DNA. Information on the corresponding DNA sequences is not available for all gene products studied.

However, as analysis of all gene transcripts was performed on the same stocks of cDNA from each RNA extract (which was shown to contain no contaminating DNA when amplified with other target regions) it can be assumed that the bands resulting in all cases are representative of cellular RNA.

4.4 Precautions taken when Analysing RNA Levels by RT-PCR

As with all PCR studies, general precautions were taken and good laboratory procedures exercised. These included pre-aliquoting all solutions in small amounts and discarding the remains after each use; physically separating the reaction preparation from the amplified products; using separate pipettes *etc.* for setting-up the reaction and analysing the product; and optimising the conditions for each set of templates and primers.

More obvious precautions included autoclaving all necessary solutions, eppendorfs, pipette tips *etc.* and wearing gloves. Extra precaution were taken *e.g.* baking glassware, DEPC-treating solutions *etc.* because of the labile nature of the RNA under study. Although great care was taken when extracting RNA after sedimenting through the cesium chloride cushion, the possibility of DNA contamination (from the cesium chloride : guanidium thiocyanate interface) could not be ruled out. Therefore, because it must be possible to discriminate between the RNA product and that of contaminating DNA, primers from different exons were used whenever possible. Different product sizes would then result if mRNA or DNA were used as the template. Reaction components were added on ice and the accuracy of the pipettes was monitored regularly. This last point, although somewhat obvious, is extremely important for these procedures as the volumes involved are quite small. Due to the sensitivity of this technique a very small inaccuracy in setting up the reaction components could result in a very large inaccuracy in the final product.

Good experimental design was considered very important. This involved including negative controls to detect contamination and positive controls, whenever possible, to ensure the reaction was conducted successfully. All reactions were set up at least in duplicate. To standardise the procedure, it was generally considered better to prepare a master-mix of all the ingredients including the enzyme and aliquoting (on ice) just prior to the template addition. The minimum number of PCR cycles required were performed in order to minimize the chances of a rare contaminating template being amplified.

4.5 Method of Analysis of RT-PCR Products

Products of RT-PCR may be detected in a number of ways. For quantitative studies, incorporation of a radio-label during PCR amplification is generally the method of choice (Rappolee *et al.*, 1989; Wang *et al.*, 1989b; Gilliland *et al.*, 1990a,b; Murphy *et al.*, 1990; Noonan *et al.*, 1990; Diviacco *et al.*, 1992; Horikoshi *et al.*, 1992; Siebert and Larrick, 1992; Wiesner, 1992; Futscher *et al.*, 1993; Phillips *et al.*, 1993; Clifford *et al.*, 1994). This generally involves the use of either end-labelled primers or the inclusion of a radio-labelled deoxynucleotide in the deoxynucleotide mix to be incorporated into the newly synthesised strands of DNA. The resulting radio-labelled products are then quantified by either excising bands from the gel, indicated by ethidium bromide staining and subsequently performing scintillation counts, or scanning the bands after autoradiography. The use of fluorescent-labelled primers, followed by analysis using a fluorescent automated DNA analyser, has also been reported (Cottrez *et al.*, 1994).

The method of product analysis chosen in this study was the more simple method of densitometry scanning ethidium bromide stained gels (Nakayama *et al.*, 1992; Babu *et al.*, 1993; Deng *et al.*, 1993; Dukas *et al.*, 1993). Although it has been claimed that at larger cycle numbers there may be variability in the strength of the ethidium bromide signal for individual samples

and so incorporation of a radio-label into the PCR generated fragments may be the preferred method for quantitative analysis (Rappolee *et al.*, 1989), rapid saturation of radioactivity incorporated into amplified products has also been claimed (Phillips *et al.* 1993). This suggests that one method is possibly as valid as the other. The method of densitometry scanning ethidium bromide-stained gels was favoured, therefore, because of its speed and ease, which subsequently meant that many more samples could be analysed and many more repeat experiments performed.

Using this procedure it was realised, however, that the densitometry readings did not always correspond proportionally with (visibly) obvious differing band intensities. There are many examples of this seen generally throughout the results, including Fig. 3.5.1.1, E1/R1. For each respective initial RNA concentration, the resulting bands seem, by eye, to be at least double in intensity between 15 and 20 cycles of PCR amplification. However, according to the densitometry data, the greatest increase between 15 and 20 cycles was when analysing the 0.5 $\mu g/\mu l$ RNA samples, where only a 1.4-fold increase was detected. The differences according to the densitometry data for the 1.0 $\mu g/\mu l$ and 2.0 $\mu g/\mu l$ RNA concentrations between 15 and 20 cycles was even less than this (Fig. 3.5.1.1, Table 3.5.1.1.1). This effect was more pronounced when the band intensities were relatively weak or relatively strong, suggesting that the densitometer is not sensitive enough to detect faint bands and that the range of linearity of the machine is quite limited. To establish if this was so, or if differences suggested by visible analysis were not real, serial dilutions of DNAs of known concentrations were ran on an agarose gel and analysed by densitometry (Fig. 3.5.2.1). Although the two-fold differences in intensities of subsequent bands were clearly visible by eye after ethidium bromide staining, this was not apparent from the densitometry data. For this reason, the limitations of the densitometer had to be taken into consideration when analysing all results.

4.6 Analysis of MDR 1 Gene Products: MDR 1 mRNA and P-glycoprotein.

MDR 1 mediated multiple drug resistance is an important and often acquired mechanism of drug resistance in many tumours, identified by an over-expression of the MDR 1 gene at the mRNA and protein levels (p-glycoprotein) (Bradley *et al.*, 1989; Reeve *et al.*, 1989; Baas *et al.*, 1990). Over-expression of the MDR 1 gene transcripts (determined by RT-PCR) were observed in all of the cell lines included in this study *i.e.* DLKPA (Figs. 3.3.1 and 3.5.1.15) and its cloned variants (DLKPA-2B, DLKPA-6B and DLKPA-5F (Fig. 3.5.1.15)), DLKP/VP-3 and DLKP/VP-8 (Fig. 3.5.1.15), OAW42A, HEp-2A, SKMES-1A (Fig. 3.3.2) and SKMES-1ADR, when compared to their common parent, DLKP. It is known, however, that cells can modulate gene expression, even after transcription and RNA processing has occurred, by controlling mRNA transport from the nucleus to the cytoplasm, cytoplasmic mRNA accumulation and degradation, translation, protein processing and localisation (Carter and Malter, 1991). The levels of p-glycoprotein (the product of the MDR 1 gene) was also analysed, therefore, to establish if the levels of this protein reflect the MDR 1 mRNA levels.

Overexpression of p-glycoprotein, when analysed in the DLKP MDR variants, was observed (by Western blot analysis using C219 antibody). However, neither the levels of mRNA nor protein detected in the DLKP variants correlated precisely with the resistance profiles of these cells, as detailed in Table 4.1. Simplifying the details from this Table, DLKP/VP-8, DLKPA-5F and DLKPA seem to be the most resistant cell lines, overall; followed by DLKP/VP-3, DLKPA-6B and then, DLKPA-2B. Analysis of mRNA levels by RT-PCR, during exponential product accumulation, suggests that the MDR 1 mRNA levels in the DLKPA and its cloned variants could generally be correlated with their respective drug resistance profiles (Fig. 3.5.1.15, Table 3.5.1.15.1) *i.e.* with greatest levels of MDR 1 mRNA observed in DLKPA and DLKPA-5F. Greater levels of MDR 1 gene transcripts were detected in

DLKPA-6B compared to DLKPA-2B, which is the least resistant variant (with the exception of E1/R2). The apparent increased levels in DLKPA-2B cells in this case, may be due to inconsistent printing of the photograph (Fig. 3.5.1.15, Table 3.5.1.15.1).

FOLD RESISTANCE	Adriamycin	Cisplatin	Vincristine	VP-16	5-Fluorouracil
DLKPA	254.14 +/- 119.43	1.46 +/- 0.42	1504.26 +/- 707.36	60.67 +/- 8.00	1.75 +/- 0.74
DLKPA-2B	36.8 +/- 15.97	0.57 +/- 0.07	228.32 +/- 70.74	18.17 +/- 8.33	1.28 +/- 0.77
DLKPA-6B	94.59 +/- 32.64	0.71 +/- 0.08	367.11 +/- 163.86	29.77 +/- 12.71	0.77 +/- 0.33
DLKPA-5F	330.59 +/- 68.53	1.39 +/- 0.03	1275.04 +/- 624.98	50.60 +/- 8.07	0.98 +/- 0.47
DLKP/VP-3	90.10 +/- 13.24	0.32 +/- 0.17	1088.98 +/- 161.08	58.23 +/- 2.31	0.47 +/- 0.38
DLKP/VP-8	272.42 +/- 38.07	0.22 +/- 0.06	1737.42 +/- 347.60	101.24 +/- 6.54	0.90 +/- 0.43

Table 4.1 Fold increase in resistance of DLKP MDR variants to chemotherapeutic drugs, with respect to DLKP. (This analysis was performed by Dr. Mary Heenan).

The results from p-glycoprotein analysis by Western blotting indicate that the VP-16 MDR cell lines express significantly higher levels of this protein, by comparison with the adriamycin-selected cell lines (Fig. 3.7.1, Table 3.7.1.1). In these cell lines, greatest levels of p-glycoprotein expression seemed to correlate with the least resistant cell line (DLKP/VP-3), with slightly lower levels apparently occurring in the DLKP/VP-8 cells. This correlated with slight differences in MDR 1 mRNA levels in DLKP/VP-3 and DLKP/VP-8 cells, as detected by densitometry. However, no visible difference in mRNA levels was apparent (Fig. 3.5.1.15).

Although the levels of p-glycoprotein appeared greater in DLKP/VP-3 by comparison to DLKP/VP-8, by visual analysis of the Western blots, a two-fold difference, as suggested by densitometry (Table 3.7.1.1), was not apparent. These overall analyses of the VP-16-selected cell lines seemed to correlate, inversely, with the levels of adriamycin accumulation (as determined by fluorescence spectrophotometry, by Dr. Mary Heenan). DLKP/VP-3, the least resistant variant, expressed higher levels of MDR 1 mRNA and p-glycoprotein, and accumulated less adriamycin than DLKP/VP-8, the more resistant cell line, which apparently expressed lower levels of both MDR 1 mRNA and its product, p-glycoprotein. Discrepancies between the suggested p-glycoprotein levels (as determined by mRNA analysis) and the detected p-glycoprotein levels (as determined by Western blot analysis) may be as a result of inadequate protein translation due to mRNA instability. It is known that mRNAs coding for certain proteins, including transport molecules, tend to be rapidly degraded with half-times from 10 to 60 minutes (Carter and Malter, 1991). Alternatively, they may be the result of post-translational modifications, alterations in the location of the protein and/or partial protein degradation.

From the analysis of MDR 1 mRNA, p-glycoprotein expression and MDR fold-resistance it seems that, although the over-expression of this gene may contribute greatly to the phenomenon

of MDR in DLKPA and its cloned variants, other mechanisms of resistance are also involved.

4.7 Ribozyme Transfectants

Due to the ability of tumour cells to develop multiple drug resistance, the use of chemotherapy as a treatment is very limited in certain tumour types. The potential role of ribozymes in the treatment of cancer is, at present, undergoing investigation *in vitro* in many laboratories. For a ribozyme to be effective as an anti-cancer agent, it must enter specific cells and then locate and cleave its specific target mRNA.

Increased expression of the MDR 1 gene has been associated with MDR in many cancer types (Clynes *et al.*, 1993). Ribozymes specific for this mRNA have recently been formed and transfected into human tumour cells (Holm *et al.*, 1994; Kobayashi *et al.*, 1994). To date, however, the numbers of studies done with MDR 1 ribozymes have been limited to only a few MDR cell line types.

Non-small cell lung carcinoma (NSCLC) is generally considered to be an intrinsically resistant tumour type, unresponsive to chemotherapy. Although certain studies have indicated that at least some NSCLCs may show response to chemotherapy (Volm *et al.*, 1992; Wilbur *et al.*, 1992), this form of treatment alone is not adequate for routine treatments of NSCLCs.

The ability of an MDR-directed ribozyme to enter, be expressed and function in DLKPA-5F, an MDR variant of a human lung squamous cell carcinoma, DLKP, which has been shown in this thesis (Section 3.5) to significantly overexpress MDR 1 mRNA, was assessed in this study. Simultaneously, the MDR 1 ribozyme was also transfected into a cloned variant (DLKP-4) of the sensitive parent cell line which does not express detectable levels of MDR 1 mRNA. This was done as a control *i.e.* if the

ribozyme had any adverse toxic effects on the cell, this should occur with DLKP-4 cells, as well as the DLKPA-5F cells.

Successful transfection of the ribozyme into both cell lines was achieved and, in turn, the ribozyme was successfully expressed. This was verified by PCR (Fig. 3.10.1, Table 3.10.1.1) and RT-PCR analysis (Fig. 3.10.2, Table 3.10.1.1) of DNA and RNA extracted from all the transfectants. However, no significant effects were apparent in any of the MDR variants studied *i.e.* the MDR 1 mRNA levels were not notably reduced when analysed by RT-PCR using primers which amplify the region to which the ribozyme was targeted (Fig. 3.10.3, Table 3.10.1.1). The drug resistance profiles of the transfectants were analysed by performing toxicity assays with chemotherapeutic drugs to which DLKPA-5F cells have previously been found to be highly resistant, including adriamycin, VP-16 and vincristine. No significant alterations in the drug resistance levels of any of these transfectants, compared to DLKPA-5F, were noted.

A number of possible explanations for this lack of effect may be that, although the ribozyme entered the cell and was transcribed, the transcript was unable to reach its target mRNA. Results of this nature have been referenced by Barinaga (1993) in which a hammer-head ribozyme to target HIV RNA was successfully transfected into cells, but was ineffective against the HIV RNA. This was suggested to result from "traffic-control" within the cell, preventing the ribozyme mRNA from getting close to its target. Alternatively, because of the relatively high levels of MDR 1 mRNA expression in these cells, the effect of the ribozyme may be insignificant to the overall MDR 1 transcript levels. This suggestion is supported by studies by Holm *et al.* (1994) and Kobayashi *et al.* (1994) who transfected MDR 1 ribozyme into drug resistant human hepatic carcinoma and acute leukemia cultured cell lines, respectively. Although significant reductions in MDR 1 mRNA levels were reported in both these studies, complete resistance reversal was not achieved. This was suggested to result from insufficient

accumulation of ribozyme to cleave the constantly over-produced MDR 1 mRNA.

Although further studies may be necessary to establish why the expressed ribozyme did not exert its effects on MDR 1 mRNA in this lung cell line, it must be considered that the levels of MDR 1 expression in this cell line are possibly not clinically relevant to lung tumours, where generally only low levels of MDR 1 mRNA, when detected, have been reported (Lai et al., 1989; Holzmayer *et al.*, 1992). Transfection of this ribozyme into cell lines *e.g.* DLKPA-2B and DLKPA-6B which are less resistant and which possess lower levels of MDR 1 mRNA, may be of more significance now that it has been established that successful transfection and expression of this ribozyme in DLKPA variants can be achieved.

4.8 MDR 1 Gene Induction

Transcriptional control mechanisms have been suggested to play a significant role in the regulation of MDR 1 mRNA levels (Goldstein *et al.*, 1989; Zastawny *et al.*, 1993). Induction of MDR 1 expression in a number of subpopulations of leukemia cell lines, following transient (12 hrs. - 4.5 days) exposure to a range of p-glycoprotein substrates including adriamycin, daunorubicin, vinblastine and VP-16 and non-p-glycoprotein transported drugs including cisplatin, methotrexate, fluorouracil and hydroxyurea, was reported by Chaudhary and Roninson (1993). Activators of protein kinase C were also shown to increase MDR 1 gene expression, which was blocked by protein kinase C inhibitors. Such increases in MDR 1 levels after short-term exposure to such agents, which may be due to mRNA stabilisation or increased transcription, have also been associated with exposure to heat shock, arsenite (Chin *et al.*, 1990; Kioka *et al.*, 1992) and certain differentiation agents (Mickley *et al.*, 1989; Bates *et al.*, 1989). In experiments where the MDR 1 promoter has been isolated and used to drive the CAT gene, DNA Topoisomerase I (Camptothecin) and Topoisomerase II

(VP-16 and VM-26) inhibitors have been found to induce CAT activity, by activation of the MDR 1 promoter. Similar effects have been observed with the mutant form of p53, whilst the wild-type represses p-glycoprotein promoter activity. Although this is apparently associated with the natural physiological role of p-glycoprotein as a protector of the cell against foreign stimuli, this may also be of major significance in the development of MDR in cancer cells.

To establish if such a phenomenon would occur with tumour cell lines from other origins, DLKP and OAW42 (lung and ovarian drug sensitive cell lines, respectively), were exposed to adriamycin for 72 hours *i.e.* until physical signs of cell distress were seen, after which transcript levels of a number of MDR-related genes including MDR 1, MRP, GST π and Topoisomerase II were evaluated. β-actin mRNA levels were also analysed. Exposure to adriamycin caused a number of visible signs characteristic of cell damage including increased granularity, the development of extensions forming contacts between cells, cell swelling, altered cell shape and growth inhibition in both DLKP (Fig. 3.8.1) and OAW42 (Fig. 3.8.2) cell lines. Induction of only MDR 1 gene expression was observed only in the ovarian cell line (Fig. 3.8.3). No changes in MRP, GST π, Topoisomerase II or β-actin levels were observed.

The short-term drug exposure involved in this induction study may be more closely related to clinical MDR than other *in vitro* MDR models where cells are selected following continuous (up to 18 months) exposure to drug. Damage-induced activation in tumour cells may occur during chemotherapy treatment and account, at least in part, for the increased incidence of MDR 1 expression in drug-treated human tumours (Chaudbary and Roninson, 1993).

Why induction of MDR 1 occurred in the ovarian and not the lung cell line is unclear as adriamycin has been used in the treatment, although not generally the primary treatment, of both tumour types. Over-expression of the MDR 1 gene with no DNA

amplification has been associated with MDR in both types of tumour cell lines. Furthermore, the presence of MDR 1-expressing tumour cells has been suggested as a useful predictive marker for clinical resistance to combination chemotherapy in ovarian and small cell lung carcinoma (Holzmayer *et al.*, 1992). The differential MDR 1 gene induction in these cell lines may, in some way, be related to the fact that because of their location and physiological role, lung cells are routinely exposed to foreign particles in the environment, including cigarette smoke, smog, chemical waste, *etc.*, whereas ovarian cells are in a much more protected environment. Therefore, the response by ovarian cells may be much more rapid and extreme than in lung cells, which are more accustomed to insult by foreign matter.

4.9 Topoisomerase II mRNA and Protein Levels.

"Atypical" MDR in cells may occur due to alterations in the nuclear enzyme DNA Topoisomerase II. The cytotoxicity of most chemotherapeutic Topoisomerase II poisons is directly related to the number of active Topoisomerase II molecules. Therefore, a reduction in this number - generally involving the α subunit of Topoisomerase II - may lead to drug resistance (Per *et al.*, 1987; de Jong *et al.*, 1990; Mirski *et al.*, 1993). Slight reductions in Topoisomerase II mRNA expression were observed in each of the adriamycin-selected MDR cell lines included in this study *i.e.* DLKPA (Fig. 3.3.1), OAW42A, HEp-2A, SKMES-1A (Fig. 3.3.2) and SKMES-1ADR, when compared to their respective parent cell lines. This trend was also seen when Topoisomerase IIα transcripts were studied independently. Topoisomerase IIβ levels, on the other hand, seemed to be slightly increased. However, these initial studies were performed prior to assessing the range of exponential product accumulation for each primer pair and so can not be considered quantitative in any way.

The gene transcript levels of both Topoisomerase II (Figs. 3.5.1.7 - 3.5.1.10) and its α subunit (Figs. 3.5.1.11 - 3.5.1.14) were assessed throughout the PCR exponential phase of

product accumulation in DLKP and its drug resistant variants DLKPA, DLKP/VP-3 and DLKP/VP-8. However, as with similar studies performed to assess the relative differences in MDR 1 mRNA levels, because the samples from each cell line were run out on separate gels and the densitometry readings from the resulting bands could not be directly compared, amplification of the Topoisomerase IIα region in each of the four cDNA stocks from each cell line was repeated at 25 cycles. The products of amplification of each extract were then ran together on a single gel (Fig. 3.5.1.16) and were analysed together by densitometry (Table 3.5.1.16.1). This allowed a direct comparison be made between the Topoisomerase IIα levels in each of these cell lines.

From the RT-PCR analysis of Topoisomerase IIα mRNA, it appears that the levels of this gene transcript are reduced in the drug resistant variants - DLKPA, DLKP/VP-3 and DLKP/VP-8, by comparison to the sensitive parent cell line. However, the reductions in band intensities resulting (when analysed by densitometry) were very small and were not obvious, when analysed visually.

The levels of Topoisomerase II enzyme studied, using a polyclonal antibody, which detects a nuclear protein of 170-180 kDa, were reduced in the DLKP MDR variants, DLKPA and DLKP/VP-3, when compared to the levels in the sensitive cell line. DLKP/VP-8 cells, however, apparently possessed slightly elevated levels of this protein. From results obtained using this polyclonal antibody, it cannot be established, however, whether it is the Topoisomerase IIα or Topoisomerase IIβ subunit which is affected in this way. When the Topoisomerase IIα levels of this enzyme were analysed independently (using a specific monoclonal antibody), reduced levels were observed in each of the MDR variants compared to DLKP. This suggests that the increase in Topoisomerase II observed in the DLKP/VP-8 nuclear extracts is indicative of alterations in the Topoisomerase IIβ subunit. This may also explain the more significant changes in Topoisomerase

IIα levels in the other cell lines, which were not so obvious when studying Topoisomerase II, as a whole.

By densitometry (Table 3.7.2.1) and visual analysis of the Topoisomerase IIα Western blot, the greatest reductions in gene transcript levels were observed in the adriamycin selected MDR cell line, DLKPA, (when compared to the VP-16 selected cell lines) although its fold-resistance profile is slightly less than that of DLKP/VP-8 (Table 4.1). In the case of the VP-16 selected cell lines, the levels of Topoisomerase IIα mRNA seemed to correlate with the resistance profiles of these cell lines *i.e.* the more resistant cell line, DLKP/VP-8, apparently possess lower levels of this gene transcript than the less resistant line, DLKP/VP-3.

From these studies of Topoisomerase IIα it appears that this enzyme may be of importance in the development of MDR in variants of DLKP. Although the Topoisomerase IIα protein levels were found to correlate well with the resistance profiles of the cell lines, this was not observed at the mRNA level. This suggests that regulation of this enzyme may be at the translational, rather than the transcriptional level.

When comparing the two most resistant cell lines, although both DLKPA and DLKPA/VP-8 exhibited similar levels of resistance to adriamycin, vincristine and VP-16, DLKPA possessed the lowest levels of Topoisomerase IIα, while DLKP/VP-8 had the highest levels of p-glycoprotein. This suggests that although both mechanisms of resistance are of significance in both the adriamycin and VP-16 selected cell lines, alterations in Topoisomerase IIα may be a dominant mechanism in the adriamycin-selected cells and p-glycoprotein, in the VP-16 cell lines. This is based on the assumption that the results from p-glycoprotein analysis are real, and not a consequence of protein degradation, prior to Western analysis. However, the fact that the membrane preparations for p-glycoprotein analysis by Western blotting were prepared together, during the exponential growth phase of each of the six cell line, that the isolation was performed in

the presence of a proteolytic inhibitor (PMSF), that the lyophilised samples were stored at -80 °C until required and were all quantified simultaneously against a common standard curve, suggests that if protein degradation occurred, it would probably be equally destructive for all cell lines.

4.10 Analysis of MDR-related Gene Transcripts in Clinical Specimens

Cell culture systems, because of their extensive availability and immortality, are useful models for analysis of MDR in which the direct role of a particular mechanism in determination of drug resistance can be evaluated (for review see Clynes *et al.*, 1993). Although many important studies can be performed on cultured cells *e.g.* establishing how subpopulations of cells exist in a heterogenous population of cells, *etc.*, it is obviously of great importance for all analysis to be performed on clinical biopsies. This is to ensure that results obtained from studies on cultured cells and on whole tumours correspond, if they are to contribute in any way to the design of chemotherapeutic stratagies, acting as a predictor of response before therapy or an indicator of acquired resistance during therapy.

The extraction of un-degraded template RNA from tissues is particularly difficult because of its labile nature and the presence of active RNase enzymes in the tissue itself (Jackson et al., 1991). Although many of the RNA studies reported to date involve using cultured cells, successful extraction and analysis of RNA from tumour biopsies has been reported (Horikoshi *et al.*, 1992; Kan-Mitchell, 1993; Vergier *et al.*, 1993). In these situations, the tissue fragments obtained at surgery were frozen in liquid nitrogen within 60 seconds to 30 minutes of their removal, to prevent RNA degradation.

In this study, the possibility of extracting intact RNA from normal and tumour biopsies kept for up to four hours (1.5 -

4.0 hours) after surgery was assessed. As illustrated in Figs. 3.9.1.1 - 3.9.1.4, successful extraction of RNA, reverse transcription of this RNA into cDNA, and amplification of the desired regions of MDR 1, MDR 3, MRP, GST π, Topoisomerase I, Topoisomerase II, Topoisomerase IIα, Topoisomerase IIβ and β-actin were achieved in 8/8 normal and tumour lung biopsies studied. Amplification of the CYPIA1 region did not occur. However, this was frequently found to be the case when studying cultured cells. Differences in transcript levels, between normal and tumour tissues, of a number of genes were observed in this study, the most significant effects being on MDR 1 mRNA levels. Because 30 cycles of PCR amplification were performed when analysing each of the transcripts, this analysis cannot be considered quantitative. However, these preliminary studies indicate that in situations where it is not possible to immediately start processing biopsies after their removal, studies on a broad range of MDR-related RNAs can be successfully performed. This may help broaden the range of material in which gene transcripts can be studied.

The ability to predictably recover sufficient undegraded RNA from paraffin-embedded tissue to act as a template for RT-PCR analysis has broad implications for cancer research, because of the almost unlimited amounts of material available for retrospective analysis.

In this thesis, a technique was developed which enabled RNA to be extracted from formalin-fixed paraffin-embedded breast and ovarian tissues in 24 hours (see Section 3.9.2) and subsequently used to study a range of MDR-related gene products. Initial attempts to amplify cDNA formed on these RNA templates, using oligo (dT) primers, resulted in the successful amplification of only one message, namely β-actin, the endogenous control. A number of, apparently, "non-specific" products also resulted. Using a combination of oligo (dT) and random primers, however, resulted in amplification of most of the gene transcripts under analysis in many of the specimens studied (Fig. 3.9.4 - 3.9.6).

The reason for the success of this combination, in these circumstances, unlike oligo (dT) alone, may be as follows: Reverse transcription of RNA extracted from cultured cells and normal and tumour lung biopsies was performed successfully using oligo (dT) primers which bind to the poly $(A)^+$ tail of mRNA. This mRNA, however, was extracted under ideal conditions and stored at -80 °C, until required for analysis. With polyadenylated mRNAs, however, removal of the poly $(A)^+$ tail appears to be an early stage in mRNA degradation. Therefore, in at least some of the paraffin-embedded specimens, which had been stored for between 2 and 10 years, degradation of the poly $(A)^+$ tail may have occurred. Lack of this tail, or nicks in the mRNA in the 3' region would prevent reverse transcription occurring. Random primers, as suggested by their name, bind randomly to the mRNA templates. Although full-length cDNAs (which may be obtained with oligo dT primers under ideal conditions) do not result using random primers, their application is obviously necessary for "sub-optimal" studies. Recent reports of procedures for RNA isolation, from paraffin-embedded tissues, for RT-PCR analysis have been published (Mies, 1994; Foss *et al.*, 1994). Studies of estrogen receptor mRNA in seven human breast cancers, which had been stored for 1-3 years, were reported by Mies (1994). The procedure involved using a commercial available solution, RNAzol, which contained guanidinium thiocyanate, phenol and an unspecified lysis agent. Foss *et al.* (1994) studied the effects of fixation and fixation time on amplification of two "house-keeping" gene transcripts, GAPDH and HPRT, extracted from paraffin-embedded mouse spleen tissue. Most successful amplification was achieved after fixing in "Omnifix II" with no amplification after formalin fixation. However, no such application of these techniques to the study of an extensive range of gene products, as described in this thesis, has been documented.

Although further work may be required to optimise the procedures developed in this thesis for RNA extraction for formalin-fixed paraffin-embedded tissues, these preliminary studies of both "fresh" and paraffin-embedded tumours indicate

the potential to study RNA levels of an unlimited number of genes, in an unlimited number of clinical specimens.

4.11 Quantitative RT-PCR

RT-PCR is an extremely sensitive method for mRNA analysis, and for a number of reasons may be favoured over the more conventional methods of RNA analysis including Northern blotting, RNase protection, slot blotting, *in situ* hybridisation, *etc.* Because of the sensitivity and relative simplicity of this technique, it can be used in many circumstances, including analysis of gene expression in cultured cells and especially in clinical biopsies where tissue amounts are very limited. This method has now been developed for retrospective studies of paraffin-embedded tissues, increasing its worth as a medical research tool many fold. RT-PCR may also be very important in virological diagnosis (Goswami *et al.*, 1994) *e.g.* viruses that do not grow easily in culture or when results are required more quickly than the time required for cell culture or electron microscopy. Such techniques also have potential for automation, which is important from a diagnostics point of view. Because of its extreme sensitivity, RT-PCR shows great potential for development as a quantitative method. However, obtaining quantitative information with this technique can be difficult, due primarily to the fact that there are two sequential enzymatic steps involved *i.e.* the synthesis of cDNA on the mRNA template by reverse transcriptase enzyme and the polymerase chain reaction catalysed by a DNA polymerase enzyme. Although PCR and RT-PCR have recently been developed to a quantitative level for the analysis of expression of specific genes (pioneered by Wang *et al.*, 1989b), as detailed in Section 1.4, conflicting ideas seem to exist as to what extent this quantitative analysis can be achieved.

Basically, these are two types of quantitative PCR/RT-PCR, both of which were examined in the work described in this thesis. These include a semi-quantitative method where relative differences in gene expression can be compared and which, theoretically, offers a basis on which to obtain absolute data.

The method of choice depends on the information required and the time and effort available for developing the technique. For many purposes, for example to establish whether a drug causes amplification of levels of a particular mRNA in tissues from a patient, after certain periods of treatment, relative ratios between gene expression in tumour samples are as useful as absolute values and are more easily obtained experimentally. Because of this, many more samples may be studied in a given time period. The potential use of RT-PCR is not without problems, however, and there are conflicting opinions as to whether or not this application of PCR technology can provide a reliable and reproducible measure of gene expression (Phillips *et al.*, 1993). In this thesis, attempts were made to develop the RT-PCR analysis of MDR-related gene expression to a semi-quantitative and quantitative level.

Quantitation of mRNA levels or of changes in mRNA levels can be problematic, due to the exponential nature of PCR, where small variations in amplification efficiency can lead to dramatic changes in product levels. This has the effect of obscuring differences in levels of the target mRNA. In addition due to the consumption of the reaction components and generation of inhibitors, the amounts of product generated plateaus during later stages of the reaction (Cottrez *et al.*, 1994). Therefore, two of the most fundamental requirements for any form of quantitative PCR or RT-PCR are that (a) internal controls are used in all reactions to act as standards with which each specific amplified product can be normalised and so compared from sample to sample, and (b) all analysis be performed during the PCR exponential phase of product accumulation, as PCR product accumulation is proportional to that of the starting target DNA only as long as the product accumulation remains exponential (Singer-Sam *et al.*, 1990).

The choice of internal control is very important. This must be expressed at a constant level in all cell types studied. This control product must differ in size from the target amplified product so that they can be easily distinguished from

each other, yet it must be close enough in size so that they amplify with similar efficiencies. As previously discussed (Section 4.2), in this study of MDR-related genes, β-actin was chosen as an internal control for use throughout this thesis (with few exceptions), as its expression was found to be constant in both the drug sensitive and MDR cells analysed.

The exponential range of product accumulation can be determined in two ways *i.e.* either performing the PCR for a fixed number of cycles on serial dilutions of cDNA, or by performing the reaction with a varying number of cycles on a fixed quantity of cDNA, both of which produce similar results (Murphy *et al.*, 1990). In this study, the latter method was favoured as it requires less input RNA and so more repeats of the analysis can be performed to determine its reproducibility. The exponential phase for each primer pair was estimated from plots of PCR cycle number *versus* product yield (as determined by densitometry). This is the range of PCR cycles from which initial exponential product accumulation is observed, to the point at which continued cycles do not produce significantly increased product yields. As this involved a visual estimation of the resulting plots, mid-range cycle numbers were considered suitable for routine studies, to ensure that they were within the exponential phase.

The PCR exponential phase was found to vary from one primer pair to another and from one cell type to another (as detailed in Section 3.4), although no significant differences in this range was observed between each sensitive cell line and its respective MDR variant. Because the ranges of exponential accumulation for each primer pair overlapped when evaluated for RNA from different cell types, a mid-range cycle number which was common to all cell types was considered ideal (as summarised in Table 3.4.3). The cycle ranges for which product accumulation appeared to be exponential were similar in experiments performed with batches of RNA extracted from different stocks of each cell line and between batches of cDNA formed on these mRNA templates (Table 3.4.1 and 3.4.2). This supported the use of these

particular cycle numbers of PCR amplification in subsequent quantitative experiments.

Many genes considered as endogenous controls are expressed at higher levels than most messages under study and so reach a plateau more quickly, so that a false appearance of comparable loading may occur. When co-amplifying the target and reference gene (*i.e.* endogenous control) in the same tube, the exponential amplification range of both must coincide for the quantitation to be valid. Murphy *et al.* (1990) reported studies where the internal control (β_2-microglobulin) had entered the plateau phase even before the target mRNA was detectable. To overcome this, independent amplification of the target and control, with which it was to be normalised, were performed in separate tubes within their respective exponential ranges. This was not considered ideal in this study, however, for a number of reasons. Firstly, it is generally considered necessary for the standard and target to be co-amplified in the same tube, in order to overcome poor reproducibility of the PCR step (Horikoshi *et al.*, 1992). (Variability in the yield of PCR products have been reported to be as much as six-fold among duplicate amplifications (Gilliland *et al.*, 1990a)). Secondly, using densitometry analysis, in this study, it was found that although the relative ratios were comparable from one gel to another, the actual densitometry data could not be directly compared. Unless the target and control were run on the same gel, the target could not be normalised by the control. In this study, therefore, the exponential and plateau ranges for β-actin (the endogenous control) were also evaluated. As indicated in Figs. 3.1.10.1 & 3.1.10.2, Table 3.4.1 & 3.4.2, the exponential range of product accumulation with these primers is between approximately 16 - 27 cycles. As this range includes the proposed "optimum" cycle numbers for amplification with each of the other primer pairs, this also supports the suitability of β-actin as control in these studies.

4.11.1 Semi-Quantitative RT-PCR

Using β-actin as endogenous control and the proposed "optimum" cycle numbers, attempts were made to develop this study to a semi-quantitative level for the analysis of MDR 1 (Figs. 3.5.1.1 - 3.5.1.6), Topoisomerase II (Figs. 3.5.1.7 - 3.5.1.10) and Topoisomerase IIα (Figs. 3.5.1.11 - 3.5.1.14) gene transcript levels, as these were the most significant differences observed (in preliminary experiments) between the sensitive and drug resistant cell lines. As a linear relationship between the quantity of mRNA and the final PCR product requires both efficient cDNA formation and PCR product accumulation, studies were initially performed to establish if both the RT and PCR reactions were 100 % efficient. This involved forming cDNA copies from RNA at a range of concentrations, from each cell line to be analysed; amplifying the resulting cDNA by PCR; and analysing the results visually and by densitometry. With few exceptions, the densitometry results implied that a linear relationship existed between the band intensities representing product and the initial RNA concentrations. However, this data was not indicative of a directly proportional relationship. Similar results were produced from each repeat of the experiment. These results suggested that either (or both of) the RT and PCR reactions might be less than 100 % efficient. To further investigate this, MDR 1 mRNA levels in a range of RNA concentrations, and a range of resulting cDNA amounts, were analysed by RT-PCR (Fig. 3.5.3.1 - 3.5.3.8). RNA and cDNA derived from cells previously shown to contain relatively low (DLKPA-2B) and high (DLKPA-5F) levels of MDR 1 mRNA were analysed in this way. These cell lines were chosen to establish if, in the case of DLKPA-5F where the mRNA levels under study were relatively high, possibly the RT and/or PCR reactions were saturated with excess substrate. However, the results from this study, as with the previous studies, showed that a non-proportional relationship existed between the densitometry analysis of the product and the initial RNA and cDNA concentrations. This was found even for DLKPA-2B, where the

levels of MDR 1 mRNA were relatively low and also for DLKPA-5F, when a range of lower concentrations of RNA were evaluated.

From visual analysis of the photographs resulting from both these studies, however, it was seemed that in many cases, although the densitometry data suggested otherwise, the intensities of the bands seemed to indicate proportionality with both the starting RNA and starting cDNA concentrations (*e.g.* Fig. 3.5.3.1(i)), particularly at low RNA and cDNA concentrations, suggesting that both the RT and PCR reactions were efficient. This observation was further supported by evaluating the sensitivity and linearity of the densitometer with known DNA concentrations (Fig. 3.5.2.2 & Fig. 3.5.2.3) (as previously discussed) and realising that the resulting data did not correlate proportionally with the corresponding DNA concentrations.

Overall from these results it can be concluded that although this method (of mRNA and cDNA dilutions) is suitable and necessary for semi-quantitative analysis, a more accurate means, than densitometry (as performed here), of quantifying the products is necessary. In many cases throughout this study, visual analysis was apparently more accurate than densitometry analysis (at low RNA concentrations a two-fold difference in intensity could often be detected). Obviously by visual analysis there is the possibility of bias unless all analyses are done as a blind-study. Furthermore, the precision of visual analysis is, of course, not great enough for absolute quantitative PCR/RT-PCR. HPLC may be more suitable for this analysis. It must, however, be realised that the number of samples that could be analysed in a given time period would be very much reduced if using HPLC instead of gel electrophoresis and densitometry analysis.

4.11.2 Absolute Quantitative RT-PCR

Quantitation of mRNA by RT-PCR is performed using an exogenous RNA or DNA standard of known concentration in the RT or PCR reactions, respectively. With both RNA and DNA exogenous controls, a dilution series of either the control or target are made and are titrated against a constant amount of the other component. When the PCR products resulting from the control and target are equal, the amount of mRNA in the original sample is assumed to equal the amount of template added.

The differences between these two types of controls is as follows: In these systems, the exogenous RNA control is added with the target gene mRNA template in the RT reaction and so they are simultaneously reverse transcribed. The products of this exogenous control and target mRNA then compete for primers in the PCR reaction. Exogenous DNA controls, in contrast, are added after the RT reaction, with the resulting cDNA for competitive amplification by PCR. In both methods, the target and control standards are co-amplified with the same primers and the target mRNA is quantified in its cDNA form from a known amount of the standard (Babu *et al.*, 1993).

Exogenous RNA controls, theoretically, are more useful and have frequently been favoured over DNA controls (Wang *et al.*, 1989b; Futscher *et al.*, 1993) because they control for variations in both the efficiency of the RT and PCR reactions. However, they also have certain disadvantages, including the fact that secondary structure formations may cause inefficiency of RT reactions. Because of the possibility of secondary structure formations the control and target must be as close as possible in size and structure so that comparisons can be made. Furthermore, RNA controls are more laborious than DNA controls to form.

It has been suggested that these problems may be overcome by using a DNA control and taking into account the RT reaction efficiency (Babu *et al.*, 1993). In this study, therefore, a DNA

control was used for the quantitative analysis of MDR 1 mRNA in DLKPA-2B and DLKPA-5F cells. DNA controls compete for amplification with the target cDNA primers during the PCR reaction. However, as described in Section 1.4.2.1 (Fig. 6 and Fig. 7) the intervening sequence may be homologous to that of the target cDNA or slightly longer or shorter as a result of an intron insertion or deletion or an internal region. Alternatively, the intervening sequence may be non-homologous to that of the target (Section 1.4.2.1, Fig. 8). In this study the latter form of control was used (constructed by Dr. Carmel Daly). This control was amplified by the same primers as the endogenous MDR 1 mRNA product and so competed with it for amplification.

In the original attempts of this analysis, the concentrations of exogenous controls used were too high and so did not correlate with any of the corresponding DLKPA-5F bands (Fig. 3.6.1). DLKPA-5F MDR 1 mRNA levels could not be quantified with this range of exogenous control concentrations. A lower concentration range of exogenous control was, therefore, titrated against a constant amount of cDNA formed on a 1.0 $\mu g/\mu l$ RNA template (initial total RNA concentration) from DLKPA-2B cells and on a 0.25 $\mu g/\mu l$ RNA template from DLKPA-5F cells (Fig. 3.6.2). From this analysis, the concentrations of MDR 1 mRNA levels in the DLKPA-5F cell line seems to be approximately 8 times that in the DLKPA-2B cells. Similar results were obtained by both visual and densitometry analysis in this case. By semi-quantitative analysis, this difference had been estimated as approximately 2 - 3-fold. Although there seems to be a conflict between the results obtained using semi-quamtitative and quantitative methods, it must be considered that the semi-quantitative studies on DLKPA-5F were performed prior to the range finding experiments. Greater differences in MDR 1 mRNA levels in DLKPA-2B and DLKPA-5F are probably obscured by this. The semi-quantitative and quantitative techniques are probably more in agreement than suggested by these results.

Although use of this exogenous control has proven quite successful in these studies, bands resulting from endogenous MDR 1 were relatively weak and so not very easily correlated with those of the exogenous control, by visual analysis. This may be a reflection of the size difference between the exogenous control (331 bp) and the endogenous MDR 1 cDNA region amplified (157 bp), rather than a difference in gene transcript copy number *i.e.* less ethidium bromide may intercalate with the MDR 1 product and so its intensity would seem less. (This obviously would also be a problem if endogenous controls were used in semi-quantitative studies which differed greatly in size from the target product). It may be easier for comparisons to be made if controls (either endogenous or exogenous) were close in size to the target that they quantified.

4.12 Summary and Conclusions

1. This thesis investigated mechanisms of multiple drug resistance in a range of human tumour cell types. This involved analysis by RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) of gene expression at the mRNA level in both clinically derived lung and breast tumour specimens, and MDR variants of cell lines derived from human tumours. Investigations on both adriamycin- and VP-16-selected variants of DLKP (a human squamous cell carcinoma line) were performed. Homogenous subpopulations with varying drug resistance profiles, previously cloned from a heterogenous cell population (the adriamycin-selected MDR variant of DLKP), were also studied. Qualitative analysis was also performed on other adriamycin-selected MDR variants of cell lines including OAW42 (an ovarian carcinoma), HEp-2 (an epidermoid carcinoma of the larynx) and SKMES-1 (a human squamous cell carcinoma).

2. A list of criteria for PCR primer design was prepared and primers for RT-PCR analysis of MDR-related genes were selected for MDR 3, MRP, GST α, GST π, CYPIA1, Topoisomerase I, Topoisomerase II, Topoisomerase IIα and Topoisomerase IIβ. Primers for β-actin amplification were also selected. Care was taken (by sequence search analysis in the EMBL data base) to ensure that none of the ten pairs of primers selected in this thesis amplified any "non-specific" cDNA regions. Others primers pairs previously reported in the literature, including those for amplification of MDR 1, β_2-microglobulin, GAPDH and Esterase D were also evaluated. Before applying to routine analysis, each set of primers was tested to ensure that it produced a single band of the correct size. The identity of the product was further verified by restricting with an endonuclease enzyme, the recognition site of which was previously identified as being in the amplified region.

3. An endogenous control which would act as a "common denominator" by which all target genes from normal, tumour (drug sensitive) and MDR cells could be normalised, was selected. A

number of so-called ubiquitously expressed genes were considered for this role, including β-actin, β_2-microglobulin, GAPDH and Esterase D. β-actin was favoured as control because its gene transcript levels were found to be consistent between sensitive tumour cells and MDR cells. The region amplified, when studying β-actin was slightly larger than that of any of the target genes. Therefore, it was clearly distinguished, after gel electrophoresis, from the target gene products. Furthermore, because of its slightly larger size, the β-actin product may be considered to be a relatively good marker for RNA degradation as its size may increase its probability of degradation.

4. Although slight alterations in β_2-microglobulin (decreased) and GAPDH (increased) mRNA levels (assessed by RT-PCR) were observed in MDR cells, the most significant effects were seen for Esterase D mRNA (increased), which was therefore considered inappropriate as a control.

5. Using this range of primers for amplifying MDR-related gene products, with β-actin as a control, it was found that after 30 cycles of amplification (qualitative RT-PCR), the most significant general effects in MDR cell lines were over-expression of the MDR 1 gene and apparent reduced expression of Topoisomerase IIα mRNA. The importance of working within the exponential range was obvious from many of these results.

6. To develop this technique to a semi-quantitative level, the range of exponential product accumulation, when amplifying with each primer pair, was assessed in four cell lines (two sensitive cell lines and their sensitive resistant variants) to act as representatives for subsequent studies. The exponential ranges were found to be similar in sensitive and MDR cells. Although the range varied slightly in the cell lines from different origins and when amplifying with different primers, a significant overlap in their respective ranges was found to exist. The ranges of exponential accumulation were found to be the same in duplicate cDNA stocks formed on RNA templates extracted from two or three independent stocks of each cell

line. A mid-range cycle number (which varied from one primer pair to another) was selected as suitable for use in the subsequent semi-quantitative and quantitative studies.

7. Semi-quantitative analysis of MDR 1 gene expression was performed on the adriamycin-selected variants of DLKP, namely, DLKPA and its cloned variants DLKPA-2B, DLKPA-6B and DLKPA-5F; and the VP-16 selected cell lines, DLKP/VP-3 and DLKP/VP-8. Similar analysis of Topoisomerase II and Topoisomerase IIα transcript levels in DLKP, DLKPA, DLKP/VP-3 and DLKP/VP-8 were conducted. A range of three initial concentrations of RNA were studied in each cell line. These studies were performed on duplicate stocks of cDNA formed on two independent RNA extractions. Analysis of the results by densitometry alone was found to be unreliable *i.e.* although increased readings resulted with increased band intensities, this relationship was not proportional to the band intensities (or the starting RNA concentrations). With few exceptions, a two-fold difference in band intensities was detected by visual analysis in any given cell line, within the exponential phase of product accumulation. Obviously from visual analysis it could not be proven that the differences were, in fact, precisely two-fold. However, it could confidently be said that, in the majority of cases, if two-fold serial dilutions of RNA were analysed, the rank order of their concentrations could be determined. Beyond the PCR exponential phase, obviously no such claims could be made.

This observation was generally found to be very consistent on repeat analysis from any given RNA extract. The reproducibility of results from one RNA extract to another was also generally good. In a few cases, however, both β-actin and the target gene products were apparently less in one extract, compared to the other. This was possibly due to partial RNA degradation. In a few cases where such an observation was made, however, inconsistent printing of the negative was found to be responsible. This was realised by the fact that the molecular weight markers also appeared faint on the photographs.

Because of the obvious limitations of the densitometry analysis, it was not possible to confidently compare results from one gel to another. One concentration of RNA from each batch of all cell line included in this analysis, were therefore studied simultaneously. These semi-quantitative results suggested that the levels of MDR 1 expression in the DLKP MDR variants were in the following rank order: DLKPA > DLKPA-5F > DLKP/VP-3 > DLKP/VP-8 > DLKPA-6B > DLKPA-2B, although the differences in densitometry readings between DLKP/VP-3 and DLKP-VP-8 were quite small. The greatest differences between the cell lines expressing highest (DLKPA) and lowest (DLKPA-2B) levels of this gene transcript was 1.6-fold. By visual analysis, the MDR 1 transcript levels were apparently as follows: DLKPA = DLKPA-5F = DLKP/VP-3 = DLKP/VP-8 > DLKPA-6B > DLKPA-2B, estimated to be between a 2-3 fold difference between the cell lines expressing highest and lowest levels. This relationship was more obviously detected in the analysis on the second extract of each cell line where the resulting bands were, in general, printed more faintly. No overall differences in Topoisomerase IIα mRNA levels were noted between DLKP, DLKPA, DLKP/VP-3 and DLKP-VP-8 by visual analysis, although slightly reduced levels of expression were apparent in the in the MDR variants by densitometry analysis. Generally, relatively bright bands were more difficult to analyse visually than weaker bands. This emphasised the importance of performing densitometry analysis on negatives, as inconsistent printing (identified by the molecular weight marker intensities) may apparently bias the results.

8. Further semi-quantitative studies using a range of RNA concentrations and cDNA dilutions from the DLKPA clones expressing the lowest levels (DLKPA-2B) and the greatest levels (DLKPA-5F) of the MDR 1 gene were performed. Differences could be detected (i) between the RNA concentrations, and (ii) between cDNA amounts for DLKPA-2B. When different concentrations of RNA and cDNA were analysed, fold increases with RNA concentrations could only be confidently detected in the lowest concentrations of cDNA. At higher concentrations differences were seen, but not

proportionally. This may be indicative of saturation of the ethidium bromide detection method. A range of lower concentrations of RNA extracted from DLKPA-5F cells were, therefore, analysed in this way. The bands produced, although of slightly greater intensity, were similar to those of DLKPA-2B i.e. visually detectable increases in band intensities, more or less in proportion to both the RNA and cDNA concentrations. Two very significant conclusions can be made from this: (i) if such a procedure is to be used to study expression of a gene in a number of cell lines, an initial range-finding experiment should be performed, for each cell line, to determine the range of serial RNA and cDNA dilutions over which PCR amplification is linear for each target species; (ii) since bands of proportional intensities were produced with both RNA and cDNA concentrations within the ranges determined by (i), it can be assumed that at this level both the RT and PCR reactions are of a constant levels of efficient between samples.

9. For quantitative analysis, using known concentrations of an exogenous competitor, it was assumed that a DNA control, rather than an RNA control (which is generally believed to be more problematic due to the labile nature of RNA, possibility of secondary structure formations, more laborious preparation technique *etc.*), is sufficient. By visual analysis of the quantitative studies, the levels of MDR 1 mRNA in DLKPA-5F cells was found to be slightly less than eight-fold greater than in DLKPA-2B cells (and slightly more than eight-fold when analysed by densitometry). There may be a number of reasons for the varying fold-differences detected by quantitative analysis (approximately 8-fold) and semi-quantitative analysis (2-3 fold) between DLKPA-2B and DLKPA-5F, including the inaccuracy of visual detection. The most likely reason is, however, that these semi-quantitative analyses were performed on a common RNA template concentration for each cell line, prior to observing that range-finding experiments should initially be performed for all cell lines.

10. A direct correlation between mRNA levels and protein levels (detected by Western blotting) was generally not observed with either the MDR 1 or Topoisomerase IIα gene products. In the case of the MDR 1 gene, lower levels of p-glycoprotein were apparent in DLKPA and DLKPA-5F, although compared to DLKPA-2B and DLKPA-6B, these cells express greater levels of MDR 1 mRNA and their levels of resistance are greater. This suggests that p-glycoprotein may be a less significant mechanism of MDR in DLKPA and DLKPA-5F than in DLKPA-2B and DLKPA-6B cells. Alternatively this result may be due to altered protein degradation in the different clones during extraction. In the VP-16-selected cell lines, over-expression of both MDR 1 mRNA and p-glycoprotein were detected.

Although possibly slight reductions in Topoisomerase IIα mRNA levels may occur with resistance in both the adriamycin- or VP-16-selected cell lines; much more significant reductions in Topoisomerase IIα protein levels were found, which seemed to correlate with drug resistance levels. This suggests that this gene is involved in the MDR phenomenon in these resistant variants and that it may be translationally, rather than transcriptionally controlled.

11. Induction of MDR 1 mRNA expression was observed after short-term exposure of OAW42 cells to adriamycin. (This analysis was performed using multiple primers on porated whole cells ("*in situ*"), rather than extracted RNA). Induction was associated with physical signs of stress in the cells, and was proportional to the drug concentration to which the cells were exposed. Although similar signs of stress were apparent after exposing DLKP cells to these concentrations of adriamycin for the same time period, no changes in MDR 1 transcript levels were observed. This supports the physiological role of p-glycoprotein as a cell protector and may be of clinical relevance.

12. An MDR 1-specific ribozyme was transfected into DLKPA-5F cells (and DLKP-4 cells, as a control) to evaluate its potential to reduce or reverse MDR. This ribozyme was

successfully transfected and transcribed, but no significant effects on MDR 1 mRNA or resistance to adriamycin, vincristine or VP-16 were observed. This may be due to the fact that the expressed ribozyme is not locating and cleaving it's substrate (the MDR 1 transcripts). Alternatively, it may be due to the fact that because MDR 1 mRNA is very highly over-expressed in DLKPA-5F cells, a small, but significant reduction in its levels may not be easily detected.

13. Successful analysis of a number of MDR-related gene transcript levels were performed on normal and tumour lung biopsies, which had been removed at surgery for up to 4 hours before extracting RNA. Although this analysis was only qualitative, it shows potential for extensive studies of gene transcripts in clinical material previously not considered suitable.

14. A method was developed by which RNA could be extracted from paraffin-embedded tissues, which would allow retrospective studies be performed on an extensive range of archival tissue. Preliminary attempts at this resulted in amplification of only one gene product, namely β-actin (and the production of non-specific bands). As this was probably indicative of degraded RNA, a combination of different primers were included in the RT reaction. Oligo (dT) primers (which bind to the poly $(A)^+$ tail), when used alone resulted in reverse transcription of only β-actin. This suggests that many of the RNAs under analysis were partially degraded. This was supported by the fact that random primers, when used independently only resulted in β-actin amplification and the production of "non-specific" bands. However, using a 1 : 1 ratio of oligo (dT) : random primers, successful amplification of cDNA, representing many of the genes under study, was observed. Results from this study prove that successful analysis of mRNA from tissues embedded in paraffin for at least ten years, can be achieved.

Overall, the aims (as described in Section 1.6) were fulfilled in so far as RT-PCR was proven to be a suitable technique for analysis of MDR-related gene expression at the RNA level. Suitable primers for the study of a number of such gene transcripts were selected and an appropriate endogenous control for these studies was also chosen. Methods were developed to allow quantitative analysis of specific mRNAs, but a number of limitations and potential problems were identified.

Techniques were developed which enabled RNA, suitable for RT-PCR analysis, be extracted from tissue biopsies which had been removed at surgery for up to 4 hours. RNA was also extracted from tissues embedded in paraffin for up to 10 years.

A ribozyme was successfully transfected into MDR cells which seemed to slightly reverse the MDR phenomenon in some, but not in all, transfectants.

Results from Western blot analysis, taken in conjunction with RT-PCR analysis, suggest that translational control, rather than transcriptional control, may be important in determining multiple drug resistance in some of the cell lines studied in this thesis.

4.13 Future Work arising from this Thesis

Overall, from the results of this research, it seems that RT-PCR is a necessary technique for studies of mRNAs from a range of origins. By comparison to results published from other methods of mRNA analysis, including Northern blotting, it seems that, in many cases, due to its sensitivity, specificity and potential as a quantitative method, RT-PCR is a more superior technique. Because of its sensitivity, mRNA levels in clinical specimens, which could not have been detected by other methods available, can now be analysed using RT-PCR. The realisation that this technique can be applied to studies of many gene transcripts from very small amounts of tissue embedded in paraffin for a decade, adds a new dimension to MDR studies (and mRNA studies, in general), which frequently are performed on cultured cells. Furthermore, using this technique, qualitative, semi-quantitative and absolute quantitative details may be obtained, depending on the information required.

From these studies, however, it is also apparent that RT-PCR as a quantitative method is not completely without problems. During the course of this work, a number of important observations were made which help to define the capabilities and limitations of this technique. The results from this thesis also give rise to a number of potential areas of further study.

From studies using the exogenous competitive control, it seems that the accuracy of results comparing products detected by intercalation of ethidium bromide may possibly be dependent on size differences of the products and not just on their copy number. It would, therefore, be interesting to evaluate other detection methods *e.g.* incorporation of fluorescent dNTP's during PCR amplification, and compare them with results obtained using ethidium bromide. Smaller exogenous controls (closer to the size of the endogenous target cDNA) could be constructed to establish if size differences is, in fact, a problem.

Semi-quantitative and absolute quantitative analyses of endogenous MDR 1 gene transcripts were performed in this study. It may be interesting to develop exogenous controls for the analysis of a number of other gene products, in a similar way.

In many cases throughout this research, it was apparent that visual analysis was more accurate than densitometry analysis. Alternative methods of quantitation *e.g.* HPLC could therefore be evaluated.

Using the method which has been developed for RNA extraction from paraffin-embedded tissues, extensive retrospective studies of paired normal and tumour biopsies from a number of organ types could be performed. The results could be correlated with histological studies and the patients medical records.

It may be interesting to study the effects of the MDR 1 ribozyme in MDR cell lines expressing low levels of MDR 1 mRNA, to establish if a more significant effect would result than that observed in DLKPA-5F cells.

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Abbreviations

A	Adenine
ATCC	American Tissue Culture Collection
β_2-m	β_2-microglobulin
bp	Base pair
BSA	Bovine Serum Albumin
C	Cytosine
$CaCl_2$	Calcium Chloride
CYPIA1	Cytochrome P_{450IA1}
DEPC	Diethyl Procarbonate
DMEM	Dulbeccos Minimum Essential Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
FCS	Fetal Calf Serum
Fig.	Figure
G	Guanine
GAPDH	Glyceraldehyde-6-phosphate Dehydrogenase
GST	Glutathione-S-Transferase
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory Concentration 50 percent
MDR	Multiple Drug Resistance/Resistant
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium Chloride

MMLV-RT	Moloney Murine Leukemia Virus-Reverse Transcriptase
mRNA	Messenger RNA
MRP	Multiple Drug Resistance Associated Protein
	Multi-drug Resistance/Resistant
NaCl	Sodium Chloride
$NaHCO_2$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NSCLC	Non-Small Cell Lung Cancer
O.D.	Optical Density
p.s.i	Pounds per square inch
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride
RFLP	Restriction Fragment Length Polymorphism
r.p.m.	Revolutions per minute
	Reverse Transcriptase
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RZ	Ribozyme
SCLC	Small Cell Lung Cancer
SDS	Sodium Dodecyl Sulphate
T	Thymine
TBS	Tris Buffered Saline
TEMED	N,N,N',N'-Tetramethyl-Ethylenediamine
TOPO	Topoisomerase
Topoisomerase I	DNA Topoisomerase I enzyme
Topoisomerase II	DNA Topoisomerase II enzyme

Tris	Tris(hydroxymethyl)aminomethane
U	Uracil
UV	Ultraviolet