METHOTREXATE RESISTANCE AND GENE AMPLIFICATION IN CHORIOCARCINOMA CELLS IN VITRO.

by

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ABSTRACT.

The aim of this project was to identify mechanisms of resistance to the antimetabolite methotrexate in human choriocarcinoma and colon tumour cells in vitro. In vitro exposure of human tumour cell lines to intermittent and continuous exposure to methotrexate was studied with reference to protein, DNA and RNA levels of the enzyme dihydrofolate reductase. Colon tumours were analysed as an in vivo model, with reference to dihydrofolate reductase activity and P-glycoprotein DNA and RNA levels.

Drug resistant human choriocarcinoma cell lines were produced, to a range of methotrexate concentrations, by continuous exposure with stepwise increases in drug concentration. Locus specific hybridisation confirmed that the resistant cell lines were sublines of the parent cell line. Studies of the cellular uptake of labelled methotrexate revealed that the resistance was not attributable to defective transport mechanisms. Growth rates indicated no difference between the sensitive and resistant cells. When DNA and RNA extracts from the cells were studied DHFR gene amplification and elevated mRNA expression were identified as the probable mechanism of cellular methotrexate resistance. Protein levels were also found to correlate with the elevations in gene copy number and mRNA expression in the methotrexate resistant cell lines. There was a significant correlation between elevated dihydrofolate reductase activity, mRNA expression and gene

amplification in the resistant cell lines which was proportional to the degree of resistance. The level of amplification seen did not approach those reported in drug resistant mouse leukemia or Chinese hamster ovary cells.

Tumour biopsy extracts from human colonic tumours were found to possess no amplification of the P-glycoprotein gene. There was, however, elevation of P-glycoprotein mRNA expression in a small number of the samples. These samples, with elevated mRNA expression, were found to possess a significantly higher proportion of Dukes' grade C tumours, although sample sizes were small. This may be of some prognostic value in determining treatment schedules. Both DNA and RNA were measured relative to that found in the corresponding non-neoplastic adjacent colonic mucosa.

Studies indicated that in vitro gene amplification and elevated mRNA and protein expression may be responsible for methotrexate resistance, with the control occurring at the gene level as no further increase in elevation was recorded. In vivo, however, multidrug resistance, as monitored by P-glycoprotein levels, seemed to be controlled by increased RNA expression which occurred without gene amplification being present.

To my parents - thank-you,

and the memory of Auntie Helene.

Experience is the name every one
gives to their mistakes.
(Oscar Wilde, 1854-1900)

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LIST OF ABBREVIATIONS.

ADR	Adriamycin.
Ara-c	Cytarabine.
ATP	Adenosine ribonucleotide triphosphate.
bp	Base pairs.
BSA	Bovine serum albumin.
°C	Degrees Centigrade.
cDNA	Complementary DNA.
CEA	Carcinoembryonic antigen.
CHL	Chinese hamster lung.
CHO	Chinese hamster ovary.
Ci, mCi, μCi	Curie, milli-Curie, micro-Curie.
CIAP	Calf intestinal alkaline phosphatase.
CTP	Cytosine ribonucleotide triphosphate.
dATP	2'-deoxyadenosine 5'-triphosphate.
DAU	Daunorubicin.
dCTP	2'-deoxycytidine 5'-triphosphate.
DEPC	Diethyl pyrocarbonate.
dGTP	2'-deoxyguanosine 5'-triphosphate.
DHFR	Dihydrofolate reductase.
DM	Double minute.
DMEM	Dulbecco's modified Eagles medium.
DMSO	Dimethylsulphoxide.
DNA	Deoxyribonucleic acid.
DNase	Deoxyribonuclease.
DNP	2,4-dinitrophenol.
DTT	Dithiothreitol.
dTTP	2' deoxythymidine 5'-triphosphate.
dUMP	Deoxyuridylic acid.
E.coli.	Escherichia coli.
EDTA	Ethylenediaminotetra-acetic acid.
EtBr	Ethidium bromide.
5FdUMP	5-fluoro-2'deoxyuridine 5'monophosphate.
FH_2	Dihydrofolate.
FH_4	Tetrahydrofolate.
F-MTX	Fluoresceinated methotrexate.

5-FU	5-fluorouracil.
g, mg, μg, ng, pg	Gram, milligram, microgram, nanogram, picogram.
g_{av}	Average gravitational force.
GTP	Guanidine tribonucleotide triphosphate.
^{3}H	Tritium.
HEPES	N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid.
HSR	Homogeneously staining region.
HU	Hydroxyurea.
IC_{50}	Drug concentration required to cause 50% cell survival compared to untreated control cells.
IPTG	Isopropyl-β-D-thiogalactoside.
kb	Kilobase pairs.
kDa	Kilo-daltons.
LMP	Low melting point.
MDR/mdr	Multidrug resistant.
mins	Minutes.
mm, μm, nm	Millimetre, micrometer, nanometer.
mRNA	Messenger RNA.
MTT	3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
MTX	Methotrexate.
M, mM, μM, nM	Molar, milli-molar, micro-molar,nano-molar.
NADP	Nicotinamide adenine dinucleotide phosphate (oxidised form).
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form).
O.D.	Optical density.
OLB	Oligolabelling buffer.
$p[dN]_6$	Hexadeoxyribonucleotide primer.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
RNA	Ribonucleic acid.
RNase	Ribonuclease.
RNasin	Ribonuclease inhibitor.
rpm	Revolutions per minute.

RPMI	Roswell Park Memorial Institute medium.
rRNA	Ribosomal RNA.
RT	Room temperature.
SDS	Sodium dodecyl sulphate.
sec	Seconds.
SSC	Saline sodium citrate.
TAE	Tris-acetate electrophoresis buffer.
TBE	Tris-borate electrophoresis buffer.
TFB	Standard transformation buffer.
TMP	Thymidine monophosphate. triphosphate.
Tris	Tris (hydroxymethyl) amino methane.
tRNA	Transfer RNA.
U.V.	Ultraviolet.
UTP	Uridine ribocleotide triphosphate.
V	Volts.
v/v	Volume per volume
VCR	Vincristine.
w/v	Weight per volume.
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase.

CHAPTER 1. INTRODUCTION.

1.1 CHORIOCARCINOMA.

Choriocarcinomas originate in the foetal trophoblastic epithelium of the chorionic villi. They may be preceded by hydatidiform mole (50% of cases), abortion (30% of cases), or a normal pregnancy (20% of cases). The incidence of hydatidiform moles is approximately one in 1500 births (Lewis, 1976). The neoplasm synthesises hormones and like the trophoblast of normal placenta infiltrates host tissue and blood vessels. The neoplasm metastasises via the bloodstream to the brain, liver, lung and pelvic organs. The disease was generally fatal when treatment was restricted to surgery since the tumour is notable for its vascularity and it is rarely localised to the pelvis by the time diagnosis is made (Patillo et al., 1971). Since 1956 chemotherapy has become the main arm of therapy.

Patients are stratified into three main categories according to the propensity of the tumour to become resistant to cytotoxic drugs: "low risk", "medium risk" and "high risk". "Low risk" patients may have metastases limited to the pelvis or lungs, with initial human chorionic gonadotrophin (HCG) serum level excretion less than 100,000 IU/24 hours. Patients in "low risk" group generally start treatment within four months of the apparent onset of the disease as estimated from the end

of the preceding pregnancy which is usually hydatidiform mole. "High risk" patients generally present with metastases in the lungs or other sites, including the brain and liver, and HCG serum levels may be greater than 100,000 IU/24 hours. The interval between antecedent pregnancy and diagnosis and treatment for choriocarcinoma ranges from a few weeks to many years. Further definition of choriocarcinoma grading and the scoring system involved is shown in Table 1.1. In the present thesis no distinction is made between choriocarcinoma and invasive mole.

1.1.1 Choriocarcinoma and Methotrexate.

Chemotherapy yields excellent results even in most patients whose tumour has metastasised. Hysterectomy is now restricted to a few patients with evidence of resistant tumour in the uterus. In low risk disease localised to the pelvis, single agent chemotherapy using methotrexate in intermittent 5-day courses with a week between courses results in a complete remission rate of over 90% (Hammond et al., 1973).

The initial breakthrough using methotrexate in the treatment of metastatic choriocarcinoma came in 1956, based on the observation that tissues of the female genital tract had a high requirement for folic acid (Li et al., 1956).

POINTS	0	1	2	6
Age	<39	>39		
Antecedent pregnancy(AP)	Mole	Miscarriage or unknown	Term	
Interval* (months)	<4	4 - 7	7 - 12	>12
hCG (iu/l)	10^3 - 10^4	<10^3	10^4 - 10^5	>10^5
ABO blood group (woman x man)		A x O O x A O or A x unknown	B x A or O AB x A or O	
No. of metastases	Nil	1 - 4	4 - 8	>8
Site of metastases	Not detected Lungs Vagina	Spleen Kidney	GI tract Liver	Brain
Largest tumour mass (cm)	<3	3 - 5	>5	
Previous chemotherapy	Nil		Single drug	Two or more drugs

* End of Antecedent Pregnancy to chemotherapy.
GI Gastrointestinal tract.

LOW RISK= 0 - 5;	MEDIUM RISK = 6 - 9;	HIGH RISK = >9

TABLE 1.1 Scoring System For Gestational Trophoblastic Tumours. (Bagshawe et al., 1989)

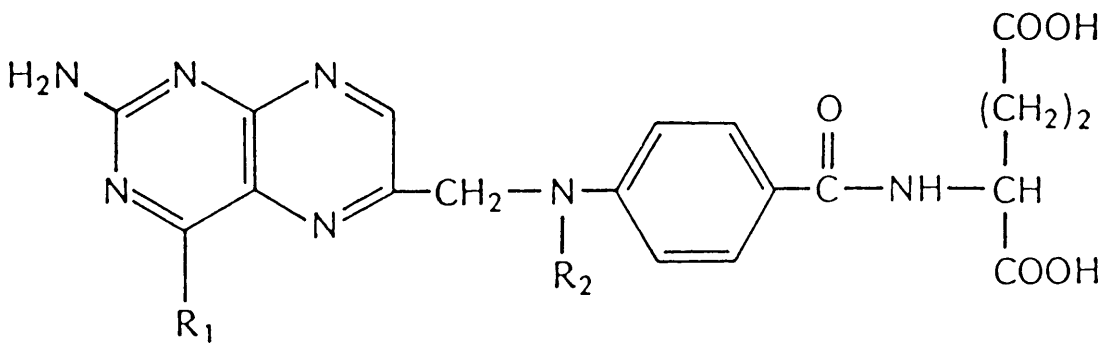
Between 1964 and 1974 the cure rate in "low risk" patients with single agent methotrexate chemotherapy was about 95% (Bagshawe, 1976). Between 1974 and 1986 a further 361 "low risk" patients were treated and a 99% survival rate was reported over a minimum follow-up period of 16 months. 20% of patients in the low risk category required other cytotoxic drugs to achieve sustained remission, either because of incorrect scoring, hypersensitivity to methotrexate or resistance of the tumour to methotrexate (Bagshawe et al., 1989).

Patients with "medium" or "high risk" choriocarcinoma start treatment with combination chemotherapy in an attempt to avoid the problems of drug resistance. These patients are treated with a combination of etoposide, methotrexate, actinomycin D (EMA) and an alkylating agent, frequently chlorambucil or cyclophosphamide (Rustin et al., 1989). Patients are treated with etoposide, actinomycin and methotrexate on day one, followed by actinomycin, etoposide and calcium leucovorin on day two. On day eight the patient is treated with cyclophosphamide and vincristine; treatments on days one and two are repeated on days 15 and 16. The whole procedure is repeated after a six day interval until biochemical remission is achieved (serum HCG $\leq$ 2 IU/ml) and then for a further 12 weeks (Newlands et al., 1986).

1.2 FOLATE ANTAGONISTS.

The initial impetus to produce folate antagonists began when Richard Lewisohn attempted to establish why primary tumours of the spleen were rarely encountered (reviewed by Wiley et al., 1986). Concentrated beef spleen extract was injected into mice bearing transplanted S180 tumours. Complete regression was seen in 60% of the animals. More plentiful supplies of spleen extract were required and vitamins of the B group that were present in the liver were considered. Yeast extracts were used as a cheap, convenient source of these vitamins, the active part of which was considered to be folic acid. From these experiments researchers investigated the possibility that folic acid had antineoplastic activity (Leuchtenberger et al., 1945). However, the disease was accelerated by exposure to folic acid (Heinle and Welch, 1948). The first clinical attempt to use folic acid as a antineoplastic agent was performed by Faber, who found that folic acid accelerated the disease in children suffering from acute leukemia (Farber et al., 1947). These studies led to the development of drugs which would cause a folate deficiency.

The early folate antagonists were analogues of the natural folates. The first agent used clinically was aminopterin, Figure 1.1 (Farber et al., 1948). Remissions in acute leukemia were one of the first demonstration of an antimetabolite acting as an antineoplastic agent.



	R_1	R_2
Folic acid	OH	H
Aminopterin	NH_2	H
Methotrexate	NH_2	CH_3

Figure 1.1 The Structure of Folic Acid and its Analogues.

Aminopterin was replaced by an analogue which is slightly less potent but has equal clinical activity and more predictable clinical toxicity, namely methotrexate (see Figure 1.1).

Methotrexate inhibits the normal pathway of the folate co-enzymes. Many of the clinically-relevant folate antagonists, including methotrexate, share a common mode of action: competitive inhibition of the enzyme dihydrofolate reductase (DHFR). Methotrexate is a weak acid with a molecular weight of 454 and is only slightly soluble in water and alcohol. The sodium salt of methotrexate is soluble and it is this form that is used for injections as a solution with a pH of 8.5. It may also be given by the oral, intramuscular, intravenous, intraarterial or intrathecal routes (Dorr and Fritz, 1980).

1.2.1 Membrane transport.

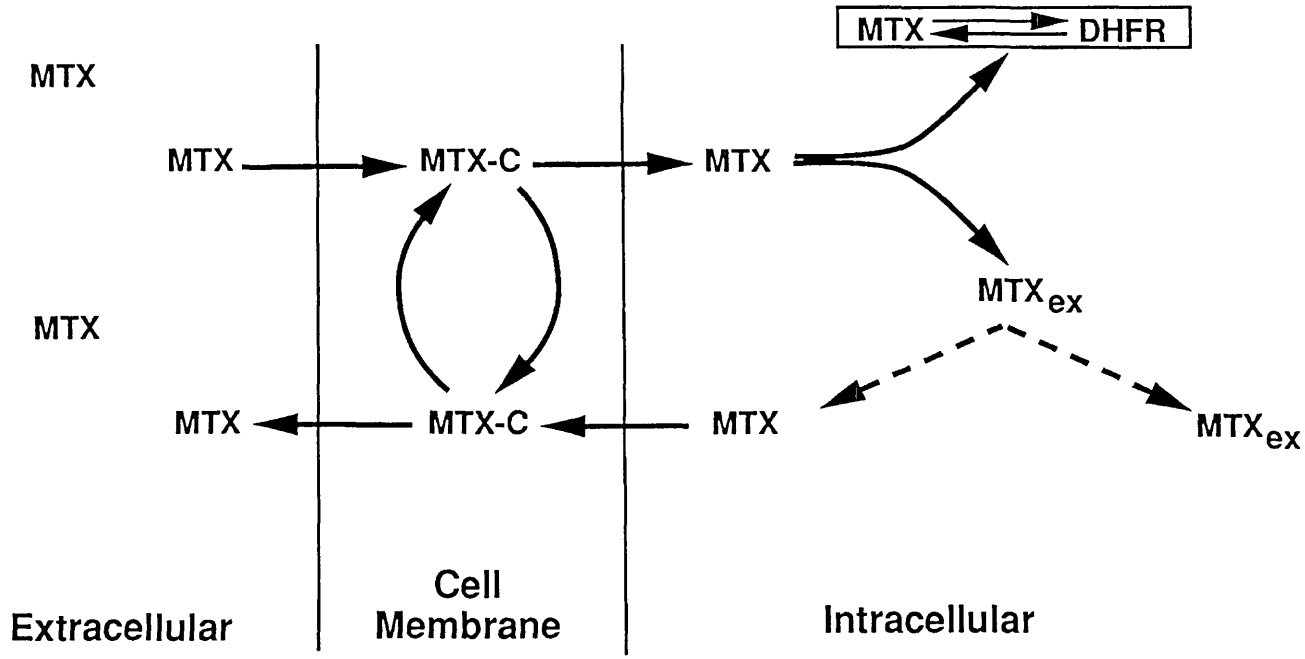
Methotrexate must cross the cell membrane before it can function. Transmembrane movement of methotrexate was first studied in detail by Goldman et al., (1968), and found to be a carrier mediated process. The drug's affinity for the carrier protein is high (approximately 10^{-6}M), but once inside the cell the affinity for its target enzyme, dihydrofolate reductase, is much higher (approximately 10^{-10}M). Therefore, the drug dissociates

from the carrier and binds to the target enzyme. Once intracellular dihydrofolate reductase binding sites are saturated methotrexate exists as free drug in several forms (Figure 1.2) (Bender, 1975).

The K_m values for the rate of drug uptake varied in a murine lymphoblastic leukemia model depending whether "high" or "low" dose methotrexate was administered; Km = $75 \pm 29 \times 10^{-3}$ M and $0.39 \pm 0.03 \times 10^{-3}$ M respectively (Goldenberg et al., 1974). This implies that there may be two routes for drug uptake: a "low" dose route with high affinity and low capacity, and a "high" dose route with low affinity and high capacity. This could help to explain the important, but different, clinical applications of "low" and "high" dose methotrexate therapy (Bender, 1978).

Low dose methotrexate influx is not significantly altered by the addition of metabolic inhibitors such as sodium azide. The addition of folic acid inhibited low dose methotrexate uptake producing data consistent with competitive inhibition of a common carrier (Warren et al., 1977, 1978). The low-dose route for methotrexate uptake was not, however, inhibited by metabolic poisons whilst inhibition occurs at ATP-requiring high-dose methotrexate uptake (Warren et al., 1978).

The inference from the many studies on methotrexate transport is that both carrier-mediated and passive



MTX-C: Carrier-bound drug

MTX_{ex}: Exchangeable (free) drug

Figure 1.2 Schematic Representation of Membrane Transport of Methotrexate.

(Redrawn from Bender, 1978)

diffusion are effective mechanisms of methotrexate transport across the cell membrane; the former predominating at low drug dose levels and the latter at higher drug dose levels (Bender, 1978).

1.2.2 Biologic Activity.

Mammalian cells require tetrahydrofolate for replication, usually from folate or 5-methyl-tetrahydrofolate (see Figure 1.3). Folate is reduced to tetrahydrofolate by the NADPH-dependent dihydrofolate reductase. Inhibition of this enzyme by methotrexate results in inhibition of DNA synthesis.

Methotrexate should be in molar excess to dihydrofolate reductase to inhibit the enzyme (Goldman, 1975). The binding of methotrexate to dihydrofolate reductase is extremely tight and at pH 6.0 virtually no dissociation occurs, encouraging a stoichiometric relationship (Km = 1.5×10^{-6}; Ki = 6.7×10^{-10}) (Bertino et al., 1964). In tumour cells, the addition of methotrexate blocks some of the dihydrofolate reductase, causing an internal rise in dihydrofolate levels. This causes a decrease in binding between enzyme and inhibitor (Nixon et al., 1973). The enzyme dihydrofolate reductase is in excess in most cells and only a small excess is needed to maintain its reduced folate pool.

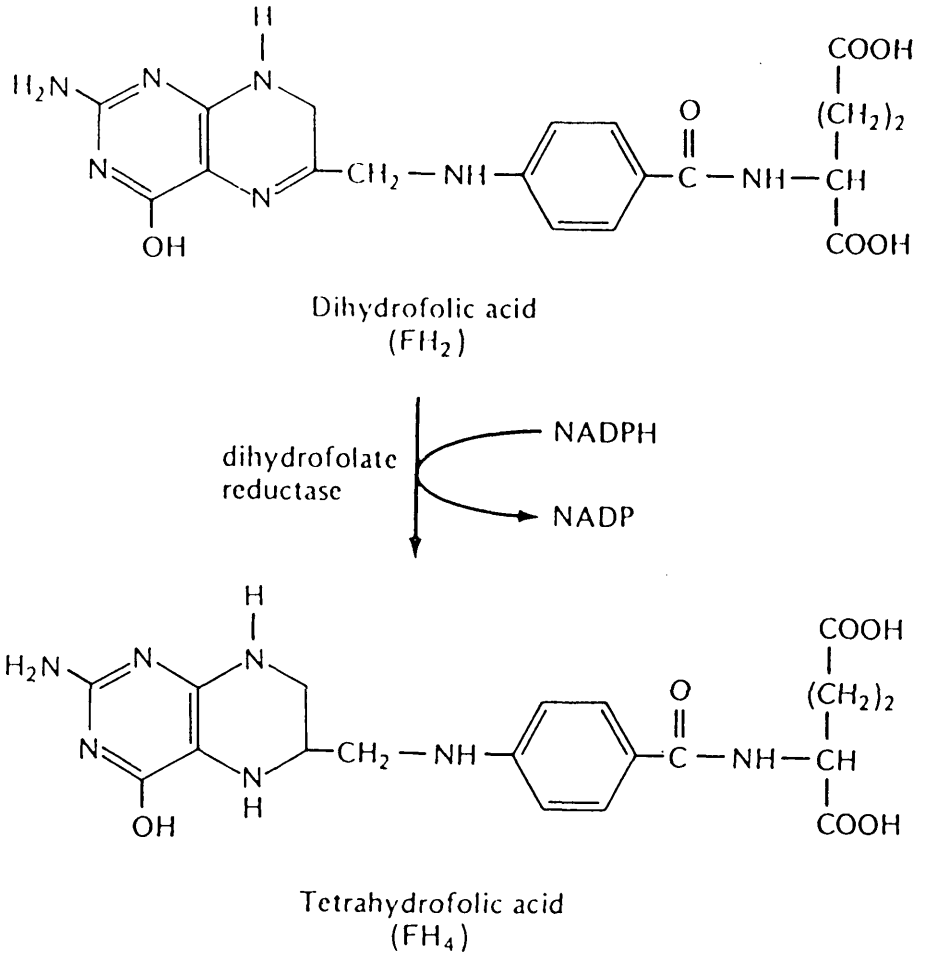


Figure 1.3 **The Dihydrofolate Reaction, Conversion of Dihydrofolic Acid to Tetrahydrofolic Acid.**

(Pratt and Ruddon, 1979)

Methotrexate inhibits DNA synthesis if the cells are exposed during growth phase. Thus methotrexate is most effective against rapidly dividing populations where only a low percentage of the population is in G_0 or resting phase (Johns and Bertino, 1982). Inhibition of DNA synthesis leads to inhibition of RNA and protein synthesis which, in turn, slows the entry of cells into S phase, thus making methotrexate a "self-limiting" S phase specific drug (Pratt and Ruddon, 1979). Clearly, cell populations with a small number of G_0 cells will be more affected by exposure to methotrexate than would a population with a high number of G_0 cells exposed to the same drug concentration for the same length of time. Consequently, where possible, methotrexate is administered over a period exceeding the generation time of the cell population, on the basis that sufficient drug reaches the target site in sufficient concentrations to inactivate the target enzyme, ideally in a rapidly growing population with a low percentage of cells in G_0 (Hryniuk and Bertino, 1969).

Other important factors involved in the antimetabolite effects of methotrexate include cellular transport, rate of turnover of dihydrofolate reductase and drug/enzyme interactions (Johns and Bertino, 1982). Cells which have both a long generation time and a rapid turnover of dihydrofolate reductase may be seen to be "naturally resistant" to methotrexate (as discussed later).

1.2.3 Mechanism of action.

Tetrahydrofolate is converted to many coenzymes, all of which are necessary for the one-carbon transfer reactions shown in Figure 1.4. Many studies have shown that it is the depletion of thymidine which is the critical event leading to cell death (Figure 1.5) (Rueckert and Mueller 1960). Thymidylate synthetase is involved in the production of thymidine monophosphate (TMP) from deoxyuridylic acid (dUMP). This reaction differs from most of those involved in one-carbon transfer reactions. Normally, a simple transfer of the one-carbon unit occurs allowing the regeneration of the tetrahydrofolate. The thymidylate synthetase reaction, however, forms dihydrofolate with the concomitant oxidation of the coenzyme. Maintenance of the cycle requires that dihydrofolate (FH_2) is reduced to tetrahydrofolate (FH_4), a reaction controlled by dihydrofolate reductase. Thymidylate synthetase can also be repressed by methotrexate competing for and occupying the folate coenzyme site (Borsa and Whitmore, 1969). This second inhibitory method is less important, but may contribute to methotrexate cytotoxicity when high concentrations of the drug are used.

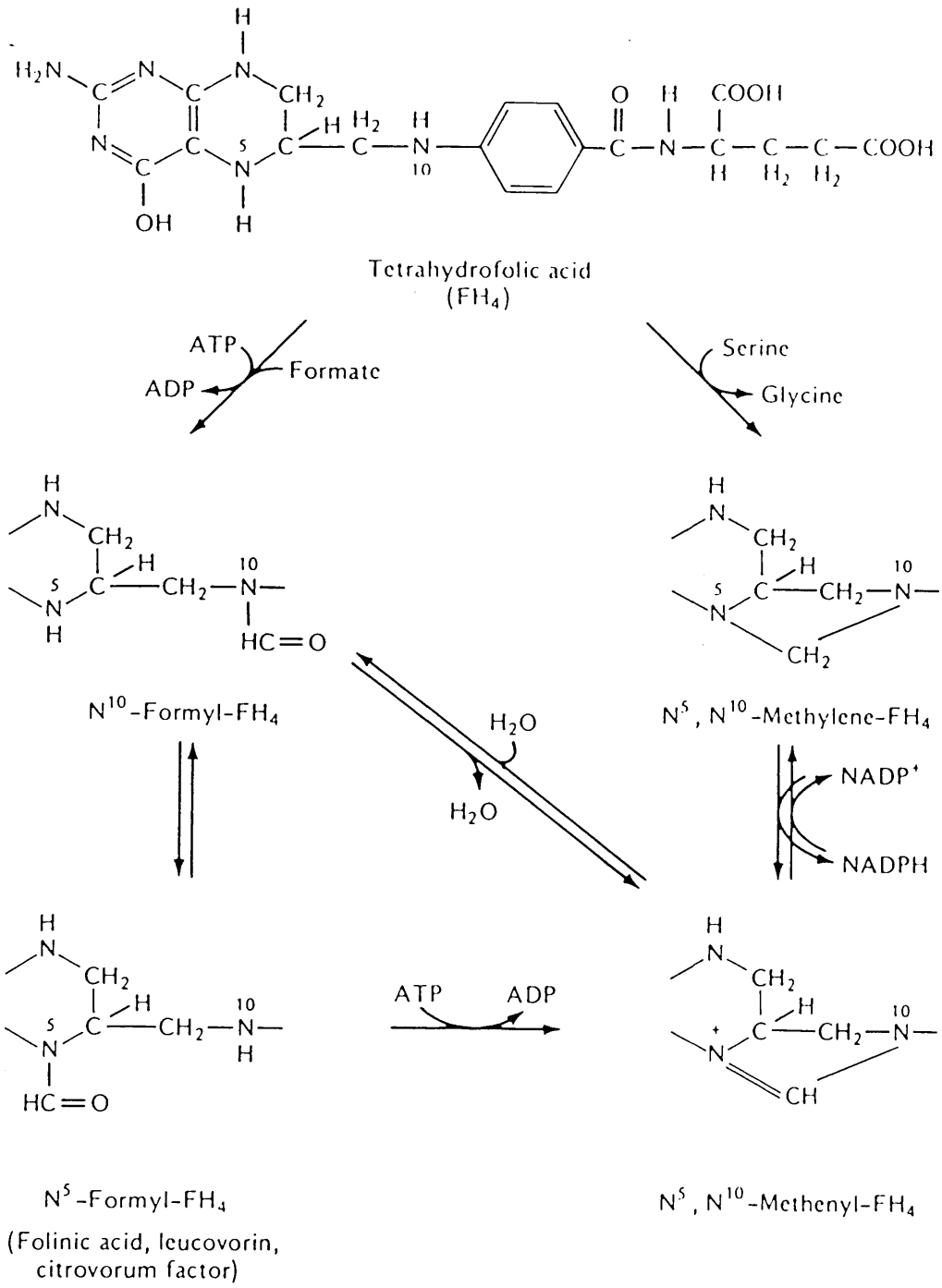


Figure 1.4 **Interconversion of the Tetrahydrofolate Coenzymes Neccessary for the Synthesis of Purines and Thymidine Monophosphate.**

(Pratt and Ruddon, 1979)

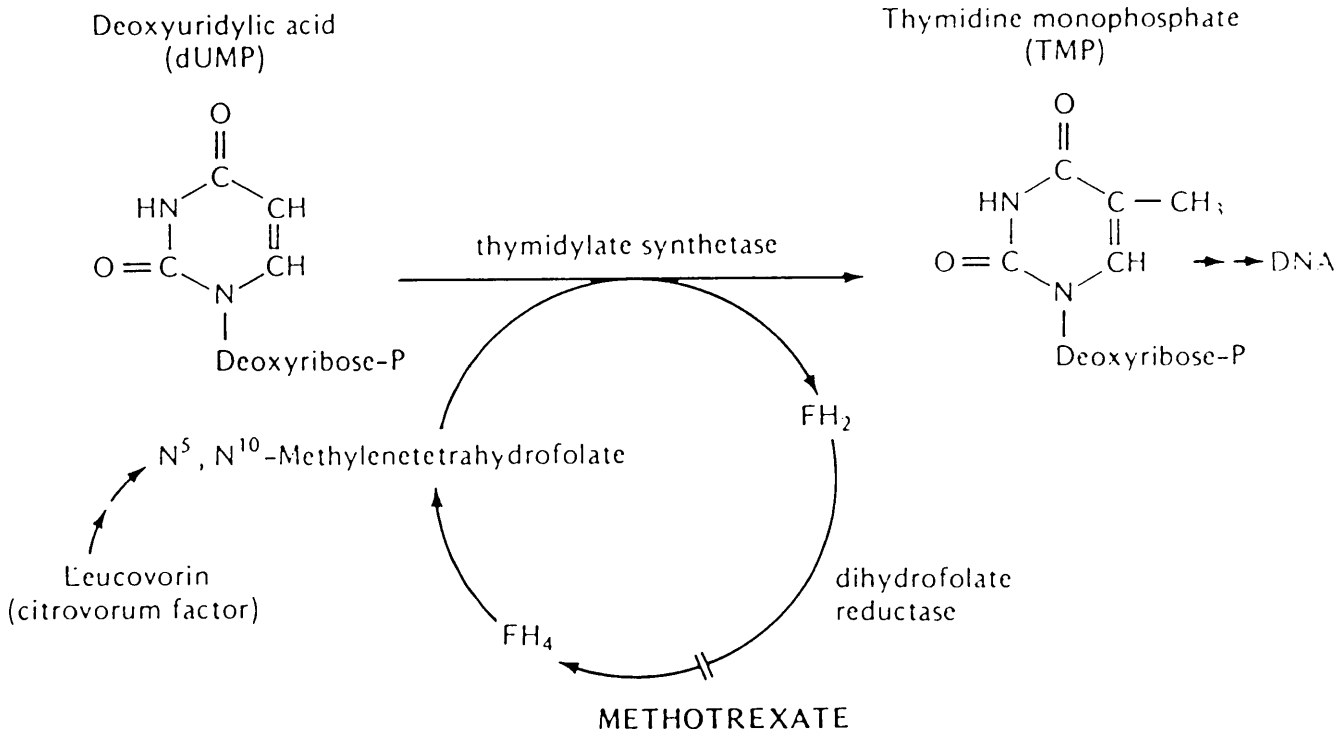


Figure 1.5 **Synthesis of Thymidine Monophosphate from Deoxyuridine Monophosphate. Methotrexate prevents the regeneration of Tetrahydrofolate.**

(Pratt and Ruddon, 1979)

1.3 DRUG RESISTANCE.

Drug resistance is either intrinsic or acquired following exposure to a cytotoxic agent. Drug resistant clones can arise in initially sensitive tumours (Curt et al., 1984), so the appearance of a single drug-resistant mutant could result in treatment failure.

There are many biochemical mechanisms of methotrexate resistance in cancer cells including:

Defective transport.
Defective drug metabolism.
Altered target protein.
Atypical multidrug resistance.
Gene amplification.

Methotrexate may induce more than one mechanism of resistance as shown in Table 1.2. An additional and more serious complication is that resistance may arise as a result of exposure to a single agent but which confers cross resistance to structurally dissimilar drugs with different modes of action. Examples of each mechanism relating to methotrexate are given below.

1.3.1 Methotrexate resistance by defective transport.

It was shown in 1965 that there was a clear correlation between MTX uptake in leukemic cell lines in vitro and

Mechanism	Alteration	Reference
Defective transport	Decreased carrier mediated transport	Kessel et al.,1965 Harrap et al.,1971 Hill et al.,1979 Jansen et al.,1989 Underhill and Flintoff,1989
Defective drug metabolism to active species	Defective polyglutamation	Jolivet et al., 1982 Cowan and Jolivet,1983 Curt et al.,1983
Altered target protein	Altered dihydrofolate reductase	Jackson and Neithammer,1977 Flintoff and Essani,1980 Melera et al.,1980
Pleiotropic drug resistance	Atypical multi-drug resistance	Haber et al.,1989 Norris et al.,1989
Gene amplification	Increased DHFR gene copy number	Alt et al.,1976 Alt et al.,1978 Schimke et al., 1981 Warr and Atkinson, 1988

TABLE 1.2 Mechanisms of methotrexate resistance

survival of methotrexate-treated mice bearing tumours originating from these cell lines (Kessel et al., 1965).

Resistance to methotrexate was considered to be intrinsic or acquired (Harrap et al., 1971). Acquired resistance was studied by comparing a drug-sensitive lymphoma line (L5178Y) with a drug-resistant line. The resistant cell line was 100-fold less sensitive and was selected by stepwise increments to sublethal methotrexate concentrations in long-term exposure. On exposure to tritiated methotrexate, the sensitive cells took up the drug 80 times faster than the resistant cells. Drug uptake was compared after 30 minutes, by which time the sensitive cells contained 10 times as much MTX. If the two cell lines were exposed to cytotoxic concentrations of methotrexate for 4 hours similar uptake figures were observed for them both. The affinity of methotrexate for the DHFR of both cell lines was also the same, indicating that the resistance was due to decreased methotrexate uptake (Harrap et al., 1971).

Stepwise increases in methotrexate concentration produced resistant lymphoblastic tumour cell lines and these were shown to have defective transport mechanisms (Ohnoshi et al., 1982; Hill et al., 1979; Underhill and Flintoff, 1989; Jansen et al., 1989;). A human acute lymphoblastic T-cell line, MOLT-3, became resistant to 10^{-7}M methotrexate following continuous exposure to increasing concentrations over a period of 10 months (Ohnoshi et

al., 1982). The cell line was 30-fold resistant to methotrexate on a 50% inhibitory concentration basis (IC_{50}). Kinetic analysis of MTX uptake showed a 20% decrease in the V_{max} in the resistant cells but no change in K_m values. This implies that either the turnover rate of the folate transport sites had been reduced or there are fewer functional active sites present.

In the lymphoblast cell line L5178Y methotrexate resistance was attributed to a permeability defect (Hill et al., 1979). These cells also show a marked decrease in the membrane transport of aminopterin and 5-methyltetrahydrofolate. In comparison to the parent cell line, the influx of each drug was reduced by more than 90%. The L5178Y MTX resistant cells survived an extracellular drug concentration of approximately 10^{-6}M. Further work has shown that these cell lines are not resistant to high (10^{-4} M) extracellular drug concentrations (Hill et al., 1982). This indicates that at high levels of methotrexate, defective transport may not be the determining factor for drug sensitivity.

DNA-mediated gene transfer has been used to study methotrexate uptake using Chinese hamster ovary cells containing a methotrexate uptake defect (Underhill and Flintoff, 1989). A MTX-resistant CHO cell line was produced, Mtx RII Oua^R 2-4, which was deficient in methotrexate transport and was 50-fold resistant compared to the parent cell line (Flintoff et al., 1976). These

cells have a requirement for 100- to 200-fold the level of folinic acid to support growth at a similar level to the parental cell line. Transfection with DNA, isolated from wild type CHO cells, produced revertant cells which had regained the ability to take up methotrexate. This successful transfection allows the possible isolation at a future date of the gene responsible for methotrexate transport to aid understanding of the MTX uptake system and identification of the mutational basis for the resistant phenotype (Underhill and Flintoff, 1989a).

A folate binding protein has recently been identified in leukemic CCRF-CEM cells resistant to methotrexate by virtue of impaired methotrexate transport, CEM/MTX (Jansen et al., 1989). The resistant cells were grown in medium with physiological levels of folates (folic acid); typical commercial culture medium contains 2-10 x 10^{-6} M folic acid whereas physiological folate concentrations are 5-50 x 10^{-9} M. Over 7 months the resistant cells were grown in culture media containing folic acid concentrations gradually lowered from 2 x 10^{-6} M to 2 x 10^{-9} M. No changes were evident in the transport deficient phenotype. Expression of the folate binding protein was down regulated in cells exposed to standard folate-containing medium but up regulated in folate-free medium. The implication from this study is that although the cells may be resistant by defective transport, this is controlled by a defective reduced folate/MTX carrier, but under physiological conditions the cells can supply

their folate requirements via the membrane folate binding protein. This indicated that MTX-resistance was associated with the protein having a low affinity for MTX but a high affinity for natural folates.

1.3.2 Methotrexate resistance by altered target protein.

Drug resistant tumour cells have been isolated containing dihydrofolate reductase with decreased affinity for the drug in rodent (Flintoff and Essani, 1980) and human cells (Jackson and Niethammer, 1977). MTX resistant CHO cells contained an altered DHFR with a 2.5- to 6-fold decrease in affinity for the drug compared to the wild type enzyme (Flintoff and Essani, 1980). Resistance is the result of DNA mutation causing amino acid substitutions at the enzymatic active site. The molecular weight of the mutant enzyme is unaltered when assayed by gel filtration techniques (Jackson and Niethammer, 1977). However, other research groups have found a slight alteration in molecular weight in other systems, such as 16 independently derived methotrexate resistant clones from Chinese hamster lung fibroblasts (Melera et al., 1980).

Methotrexate resistant Chinese hamster lung cells (DC-3F) over produce two dihydrofolate reductases with different molecular weights as defined by electrophoretic mobility

(M_r = 20,000) and (M_r = 21,000) (Melera et al., 1980). The higher molecular weight protein is the same size as that found in the parental cell line, whereas the lower molecular weight protein is unique to the resistant cell line. Each protein is encoded by separate mRNAs present in different amounts in the sensitive and resistant cells. Nucleotide sequence analysis has indicated that with respect to the proteins encoded by the different mRNA species, the only difference is at amino acid position 95: a conversion from aspartic acid (Asp) to asparagine (Asn). This is responsible for the different electrophoretic mobility of the two proteins even though they do not differ in size. Sequence analysis of DHFR from resistant CHL cells has also indicated a leucine (Leu) to phenylalanine (Phe) change at position 22 in the M_r 21,000 protein ('normal' protein weight) and it is this change which is responsible for methotrexate resistance. Cells containing the mutant M_r 21,000 protein (phenylalanine at position 22) have an IC_{50} of 8.5×10^{-5} M for methotrexate whereas sensitive cells with the normal M_r 21,000 protein (leucine at position 22) have an IC_{50} of 4.0×10^{-6} M.

1.3.3 Methotrexate Resistance by Defective Polyglutamation.

Like the physiological folates, methotrexate can be metabolised to polyglutamate derivatives which accumulate in large quantities intracellularly, and which, in the absence of extracellular drug, are preferentially retained. These derivatives are capable of causing delayed cytotoxicity (Jolivet et al., 1982).

Methotrexate resistant human breast cancer cells were isolated which were 100-fold less sensitive to methotrexate than the sensitive parental cell line (Cowan and Jolivet, 1983). Both cell lines possessed similar levels of DHFR, with the same binding affinity for MTX. Differences were noted, however, between the two cell lines' ability to form polyglutamate derivatives of methotrexate. The cells were exposed to 2.0×10^{-6} M MTX for 24 hours and the levels of intracellular drug were monitored. The sensitive cells, ZR-75-1, contained 85% of the intracellular methotrexate as polyglutamate derivatives, with only 12% of the intracellular drug bound to protein (DHFR) and 88% free. In the resistant cells, MTX^R-ZR, 89% of the intracellular drug was present as parent drug, with 66% bound to DHFR and 34% free. Thus, it was deduced that resistance was due to defective drug metabolism and it was suggested that MTX-polyglutamate derivatives played an internal role in the

cytotoxicity of methotrexate and did not merely exist as stable intracellular storage forms of MTX.

Similar results were obtained by Curt and coworkers studying cultured cell lines derived from relapsed patients with small cell lung carcinomas (SCLC's). The SCLC lines were 100-fold more resistant to MTX, as defined by clonogenic assay, compared to untreated SCLC cultured cells. No alteration was seen in transport, DHFR levels or binding. A 24 hour exposure to 1.0×10^{-6} M MTX resulted in similar total cellular MTX, but the sensitive cells contained 66% polyglutamate derivatives, whereas two resistant cell lines contained only 16% and 43% polyglutamates. Drug efflux studies indicated that it was the polyglutamate forms that were retained after 24 hours in drug free media and that no free drug remained in the drug resistant cells (Curt et al., 1983).

1.3.4 Methotrexate Resistance and Atypical Multidrug Resistance.

Methotrexate is not one of the drugs considered to induce the multidrug resistant phenotype and cross resistance resulting from exposure to methotrexate usually only involves other folate antagonists (Haber et al., 1989). A human leukemic cell line, CCRF-CEM, was exposed to high levels of methotrexate and was found to be cross resistant to vincristine, vinblastine and actinomycin D

(Haber et al., 1989). No resistance was seen to doxorubicin, daunorubicin or teniposide. The leukemic cells were exposed to stepwise increases in methotrexate concentrations up to 5 x 10^{-4}M MTX. The levels of resistance to the MDR-associated drugs vincristine, vinblastine and actinomycin D were directly proportional to the level of MTX resistance. The pattern of resistance seen to some, but not all MDR-associated drugs, implies that these cells have an "atypical multidrug resistant" phenotype.

When studied further, the MTX-resistant CCRF-CEM cells had no amplification or rearrangement of the mdr1 gene, and no elevation of the levels of P-glycoprotein as detected by monoclonal antibodies MRK16 and JSB1 (Norris et al., 1989). The antibody C219, however, detected a 170kDA protein at levels proportional to the degree of MTX-resistance. This allows for the possibility that a C219 reactive protein, not the P-glycoprotein, may mediate resistance to high dose methotrexate and certain multidrug resistance associated drugs.

1.4 GENE AMPLIFICATION AND METHOTREXATE RESISTANCE.

1.4.1 Amplification of the Dihydrofolate Reductase Gene.

Resistance to methotrexate resulting from amplified DHFR levels is most often achieved by stepwise selection of cells in progressively increasing concentrations of methotrexate (Schimke et al., 1981). The increased DHFR levels are proportional to the degree of resistance (Alt et al., 1976). These elevated levels of DHFR are directly proportional to the number of DHFR genes present as a result of gene amplification (Alt et al., 1978).

Dihydrofolate reductase levels were compared between a sensitive (S-180) and a resistant mouse sarcoma cell line (AT-3000) which was resistant to 5.0×10^{-5} M MTX (Alt et al., 1976). The level of resistance was determined by relative plating efficiencies in different drug concentrations. The resistant cells, AT-3000, showed a 140-fold increase in DHFR enzyme activity. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis showed that the resistant cells over-produced a protein which migrated with dihydrofolate reductase. This over-produced protein and DHFR were analysed for their activity per methotrexate binding site and the results indicated no difference between the two. Other properties of the enzymes were studied, including rate of inactivation and immunochemistry and these indicated no

difference from the enzymes extracted and purified in sensitive and resistant cells. The conclusion from these data was that in the selected resistant murine cells no structural changes had occurred to produce significant changes in the physical and kinetic properties of the resistant cell enzyme.

Increases in the levels of DHFR in the S-180 cells were due to increased rate of enzyme synthesis and this was correlated with increased cellular levels of translatable DHFR mRNA (Kellems et al., 1976). These levels of mRNA were found to correlate with proportional increases in DHFR gene copy numbers. As was observed previously with AT-3000 cells grown out of methotrexate, the DHFR levels gradually decreased with time (Alt et al., 1976). This finding was confirmed by similar declines in DHFR mRNA levels in these cells when passaged in MTX-free medium.

The selective multiplication of DHFR genes was also studied in AT-3000 methotrexate resistant murine cells (Alt et al., 1978). The AT-3000 cell line had approximately 200- to 250-fold increased DHFR synthesis levels when compared to the sensitive cells and had approximately 200-fold amplified DHFR genes. There was a clear direct correlation between DHFR protein levels, DHFR gene copy number and DHFR mRNA levels.

1.4.2 Stability of Resistance.

The initial amplification event in the generation of drug resistance by gene amplification is usually unstable. Fifty per cent reduction in the resistance of AT-3000 cells was noted over 20 cell doublings when the selecting agent, methotrexate, was removed (Alt et al., 1976). This reversion is associated with an approximately equal reduction in the number of amplified genes.

Stepwise selection of resistance in mammalian cells produces a cell population with variable numbers and stability of amplified genes (Schimke et al., 1985). Unstable drug resistance occurs when removal of methotrexate results in loss of resistance and reversion to sensitivity (Kaufman et al., 1979). Unstable drug resistance is associated with double minutes. The double minute chromosomes were identified by fluorescence and karyotype analysis. Metaphase spreads were separated on sucrose gradients and stained with ethidium bromide. Double minutes (DMs) were seen in high copy numbers (20-60), in unstable methotrexate resistant cell lines and in low copy numbers (0-5) in stable methotrexate resistant cell lines. The number of DMs correlates with the degree of unstable resistance and levels of DHFR produced. Reversion to sensitivity shows a corresponding drop in the number of DMs and enzyme levels (Warr and Atkinson, 1988).

This isolation and characterisation of extrachromosomal elements in unstably resistant cell lines has allowed the following conclusions to be drawn:

(1) DMs are unique to unstably methotrexate resistant cell lines and are not present in the sensitive parent cell lines or are present in insignificant numbers in stably resistant cell lines.

(2) As a cell line is selected for resistance to methotrexate, double minutes appear.

(3) When unstably resistant cells are grown in a methotrexate-free environment, the double minutes and, by definition, any gene amplification, are lost.

(4) The number of double minutes is proportional to the cells' degree of resistance to methotrexate.

(5) Upon continued selection and transfer to stable methotrexate resistance the DMs are lost from the population.

The characterisation of the properties of double minutes helps to explain heterogeneity in resistant cell lines in the number of DHFR genes. One of the properties of a DM is the lack of a centromere, so during mitosis double minutes segregate randomly producing daughter cells with unequal numbers of copies of the DHFR genes.

The continued growth of resistant cell lines with DMs in methotrexate produces, with time, a stably resistant cell line. As few as 100 cell doublings of Chinese hamster

ovary (CHO) cells can be enough to induce stable methotrexate resistance (Kaufman and Schimke, 1981). Stable resistance to MTX is associated with the presence of amplified DHFR genes located intrachromosomally in the form of homogeneously staining regions (HSRs). MTX resistant CHO cells' gene amplification is seen in conjunction with an expanded region on chromosome 2 (Nunberg et al., 1978). This region was not expanded in the corresponding sensitive parent cell line. In situ hybridisation studies of these resistant CHO cells located the DHFR amplified genes to the HSR (Nunberg et al., 1978).

DMs have also been studied in relation to growth rates (Kaufman et al., 1981). The level of DHFR and, therefore, the degree of gene amplification represented by the number of double minutes, was inversely related to the growth rate of the cells when grown out of methotrexate. This could be explained by DM-containing cells having a higher metabolic demand, which may slow down growth out of selective conditions. This may cause selective pressure for cells with lower gene amplification when reversion to sensitivity occurs by virtue of their increased growth rates. Although indicative of stable resistance, there is a slow loss of HSR size when resistant cells are cultured out of selection, but it is much slower than in DM containing cells.

The interconversion of double minutes into homogeneously staining regions is thought to occur with continued drug selection producing a stably resistant cell line from an unstable one (Kaufmen et al., 1981). It has been suggested that DMs may assemble to form chromosome like structures which may integrate into the chromosome to form HSRs (Cowell, 1982). When DMs and HSRs are reported in the same cell population they are mutually exclusive to individual cells (Balaban-Malenbaum and Gilbert, 1977) although rare exceptions to this have been observed (Quinn et al., 1979).

Other theories exist concerning the production of DMs and HSRs. The loss of HSRs from established neuroblastoma cell lines coupled to the concomitant appearance of DMs implies that HSRs may become unstable, break down and give rise to DMs (Balaban-Malenbaum and Gilbert, 1980). It is, however, thought that HSR-containing cells have a growth advantage over DM-containing cells (Biedler et al., 1983).

1.4.3 Chromosomal and Extrachromosomal Locations.

Amplified DNA has been observed in one or more of the following three states (Stark and Wahl, 1984):-

(1) Completely extrachromosomal as DMs.

(2) Integrated into linear DNA as HSRs.

(3) Associated with, but not integrated into chromosomes in a partially polytene (giant chromosome) structure.

The position of the integrated amplification varies. The amplified DNA may appear at or near its origin, at a completely different locus on the same chromosome or be translocated to a different chromosome.

Detailed analysis of HSR regions of methotrexate resistant Chinese hamster lung (CHL) cell lines has produced a variety of observations on the position of amplified DNA. Using cell lines with low level amplification (<5), 12 cases were found where amplification within HSRs was located to the short arm of chromosome 2. This has been shown previously to contain one of the wild type DHFR genes in CHO cells (Lewis et al., 1982; Flintoff et al., 1984). However, in another six cases HSR regions were located on other chromosomes including chromosomes 4, 9 and X (Biedler et al., 1980). It was also noted that even when the abnormally staining region was located on chromosome 2, its exact location varied, interrupting the normal banding pattern in several different locations (Biedler, 1982).

1.4.4 Rate of Amplification.

The rate of gene amplification in response to methotrexate selection has been studied using mouse 3T6 cells (Brown et al., 1983). Resistance was monitored by the ability to form colonies in the presence of MTX, and gene amplification quantified by hybridisation techniques. The frequency of amplification was found to be between 10^{-5} - 10^{-4} events per cell generation (Tlsty et al., 1984).

Fluorescence-activated cell sorting was also used to monitor the rate of gene amplification (Johnston et al., 1983). CHO cells were exposed to high levels (3.0×10^{-5} M), of fluoresceinated methotrexate (F-MTX) for 24 hours and then grown in F-MTX free medium. Amplification frequencies as high as 10^{-3} per cell division were seen. Further studies were performed on these CHO cells to analyse amplification of DHFR genes in a single cell cycle (Mariani and Schimke, 1984). Bromodeoxyuridine was used to label newly replicated DNA. Temporary inhibition of DNA synthesis by hydroxyurea for 6 hours, two hours after the commencement of S phase, indicated that all the DNA replicated prior to hydroxyurea inhibition was rereplicated when DNA synthesis resumed, including the DHFR genes. The CHO cells which already contained amplified DHFR genes were found to emerge from this block highly resistant to methotrexate.

The implication from these studies is that amplification can occur at random and the amount of the genome which is amplified is both large and variable. The appearance of such a high degree of specific DHFR gene amplification is a result of selection by MTX, and it is probable that non-selected amplified genes are lost (Mariani and Schimke, 1984).

1.4.5 Frequency of Amplification.

Amplification frequencies can be enhanced by agents that interfere with DNA synthesis, for example, the anticancer drug hydroxyurea. Hydroxyurea is a potent inhibitor of ribonucleotide reductase and inhibition of this enzyme results in depletion of deoxynucleoside diphosphate pools. Unless this depletion is overcome the cell will suspend DNA synthesis (Timson, 1977). The frequency of gene amplification has been studied using mouse 3T6 cells under methotrexate selection with and without the temporary inhibition of DNA synthesis by hydroxyurea (Brown et al., 1983). 3T6 clones were chosen which already contained a 2- to 3-fold amplification of the DHFR genes. The cells were exposed to various concentrations of hydroxyurea (0 - 0.3 x 10^{-3} M) for the duration of one cell generation and then exposed to 1.2 x 10^{-7} M MTX. The number of surviving colonies increased in a dose dependent manner until hydroxyurea levels became toxic (1.0 x 10^{-4} M). The relative resistance, monitored

by plating efficiency (PE) in MTX, increased up to 50-fold. By varying the time between HU exposure and MTX selection, this increase in PE diminished as the period increased before addition of the selecting agent. After 10 days growth without selection the HU had no effect on the frequency of resistance when methotrexate was added. Up to a 70-fold enhancement of the frequency of DHFR gene amplification was seen in cells exposed temporarily to hydroxyurea and then reselected in methotrexate. The mechanism of increased DHFR gene amplification after pulsing with hydroxyurea was studied using varying lengths of duration of hydroxyurea blocks at specific stages of the cell cycle (Mariani and Schimke, 1984). These authors postulated that the amplification event is general, and DHFR gene amplification is merely being selected afterwards with all the DNA that is replicated before the HU block being amplified.

The non-specificity of gene amplification has serious clinical implications since it indicates that all drugs that inhibit DNA synthesis may cause gene amplification. Research involving the pretreatment of B16 murine melanoma cells with a variety of cytotoxic agents (methotrexate, cytosine arabinoside, 5-azacytidine and aphidicolin) showed that metastatic capacity increased (McMillan and Hart, 1986). After drug injection, the tumour cells produced significantly raised numbers of lung nodules, with up to a 4-fold enhancement seen at higher drug concentrations.

The effects of hydroxyurea have been studied by many groups in relation to DHFR gene amplification. Hydroxyurea was used to inhibit DNA synthesis to interrupt the natural progression of cells into mitosis (Hoy et al., 1987). Hydroxyurea effectively prevents the progression of the cells from S-phase into mitosis (such that mitosis does not occur until the ensuing S-phase). When the hydroxyurea is removed, the cell cycle may once again commence but is dependent upon the stage in the cycle reached at which inhibition occurred. The result is that cells in G1, G2 and M during G1 inhibition of DNA synthesis accumulate and are accelerated through the cell cycle upon return to normal growth, approximately halving their cycle time. The opposite effect is seen, however, with cells arrested in S phase whose progression through the cell cycle is delayed. Consequently, prior to these cells progressing to mitosis they contain greater than the normal G2/M DNA content per cell. This suggests the possibility of overreplication occurring during the transient delay between S and M phases of the cell cycle. This theory supports earlier evidence of chromosomal aberrations observed (Hill and Schimke, 1985) in the first M phase after DNA inhibition and subsequent restoration.

Rapid gene amplification can be obtained by treating cells under selective conditions but by making the "step-

ups" in drug concentration extremely shallow (Rath et al., 1984).

The frequency of amplification can also be raised by using dual selection and obtaining dual amplifications at higher rates than predicted from the individual frequencies (Giulotto et al., 1987). By selecting baby hamster kidney cells (BHK) simultaneously with methotrexate and N-phosphonacetyl-L-aspartate (PALA), doubly resistant colonies were obtained with frequencies as high as 260 times greater than the product of independent selection frequencies.

1.5 GENE AMPLIFICATION.

1.5.1 Early Events of Gene Amplification.

Early amplification events have been studied using models where the frequency of amplification is higher than that obtained merely by plating cells under selective conditions. Chinese hamster ovary cells with a defect in pyrimidine synthesis which has been corrected by transfection with a CAD gene containing plasmid have been studied (Carroll et al., 1988). The defective cells lack the CAD gene which codes for the first three enzymes of pyrimidine synthesis (carbamyl-phosphate synthetase, aspartate transcarbamylase and dihydroorotase). The defect occurs in 1-2% of CHO cells (Giulotto et al.,

1986). In one mutant the integrated CAD gene was deleted, suggesting that a deletion is the first step in the amplification process. This was followed by replication, possibly by the rolling circle method, allowing the amplified DNA to exist either extrachromosomally or as integrated DNA (Biedler, 1982). This study offers a possible mechanism for all amplification events but does not exclude the possibility of amplification occurring by means of overreplication or sister chromatid exchange and mismatch.

The extrachromosomal elements which are frequently seen may be obligatory intermediates in the production of HSRs (Vogelstein et al., 1980). Under this hypothesis, amplification involves a replication and/or recombination event within a single S-phase without ever leaving the confines of a theoretical replication loop structure.

1.5.2 Mechanisms of Gene Amplification.

Any mechanism suggested for gene amplification must satisfy certain criteria:-

(1) Amplified DNA may occur as DMs or HSRs.

(2) The amplification rate in established cell lines is high.

(3) Agents that interfere with DNA synthesis may increase the amplification frequency.

(4) Stepwise selection is required to produce high levels of resistance with high gene copy numbers.

(5) Amplified DNA which is integrated into the chromosome occurs in large repeated units which are heterogeneous in sequence, content and size.

(6) Sequences at the centre of the amplified unit are amplified more frequently than those at the edges.

(7) Sublines selected exhibit novel joints that are created during amplification.

No model for gene amplification has so far satisfied all of these criteria. The model currently thought most pertinent is unscheduled DNA synthesis followed by recombination, or "saltatory or onionskin replication", (see Figure 1.6). Multiple rounds of DNA synthesis from a single origin within a single cell cycle can generate an unstable set of replication bubbles (hence onion skin). Replication and elongation of this DNA slows down or stops before meeting another replication fork from an adjacent origin (Stark and Wahl, 1984). In this model the only two strands which are aligned correctly are the parental strands. The DNA strands produced by these extra rounds of replication are associated, but not attached to, the chromosome. This allows the formation of DMs by recombination between and within these strands and HSRs by recombination of these strands into the chromosome. Furthermore, the close proximity of these unattached strands may allow the formation of loops and circles

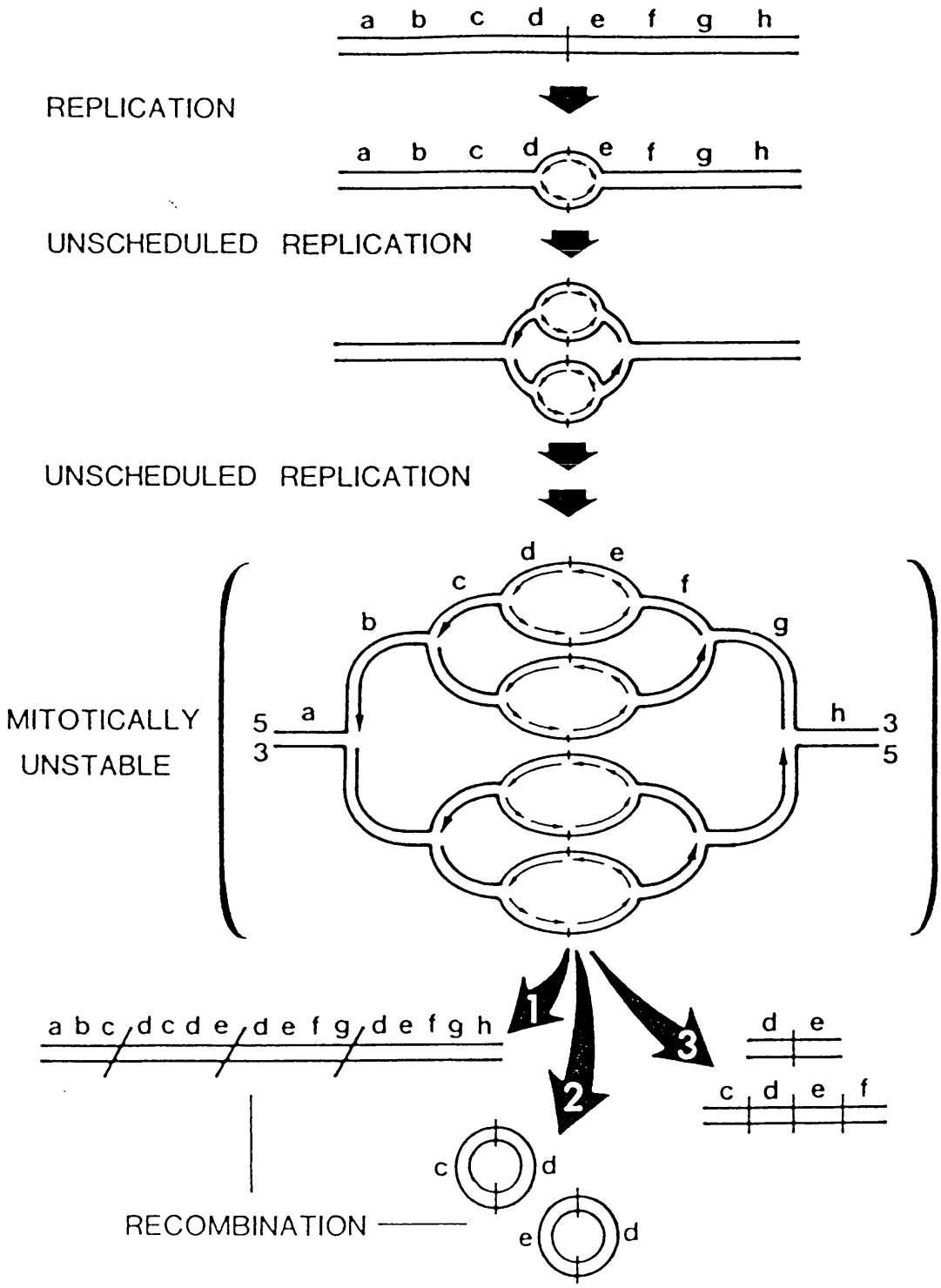


Figure 1.6 **Model of Onionskin Replication.**

(Stark and Wahl, 1984)

facilitating more DNA replication by the rolling circle mechanism (see Figure 1.7) (Gilbert and Dressler, 1968). In rolling circle DNA amplification, the amplification origin only initiates replication in one strand of the duplex DNA (in the DM). First, a single strand nick is introduced, allowing this strand to peel away from the intact strand as it is displaced by chain extension. The displaced strand then acts as a template for synthesis of a complementary DNA strand. This allows for the formation of long direct repeats of DNA extremely rapidly.

This mechanism fits the preset criteria for gene amplification. It clearly tolerates the formation of DMs and HSRs with a preference for their formation in that order. One of the problems with this interpretation is that amplicon sizes far in excess of the predicted upper limit of 100 Kb have been seen (Warr and Atkinson, 1988). Formation of multiple replication origins would explain large replicons. Another problem with the hypothesis is that the rate of chain elongation in mammalian cells does not allow for the formation of large replicons in the average S-phase.

Another mechanism of gene amplification is unequal sister chromatid exchange, due to the misalignment of chromatids during mitosis, which may be caused by pairing in a small region of homology (Szostak et al., 1983). Once misalignment has occurred a heteroduplex structure is formed by either a single- or double-strand nick.

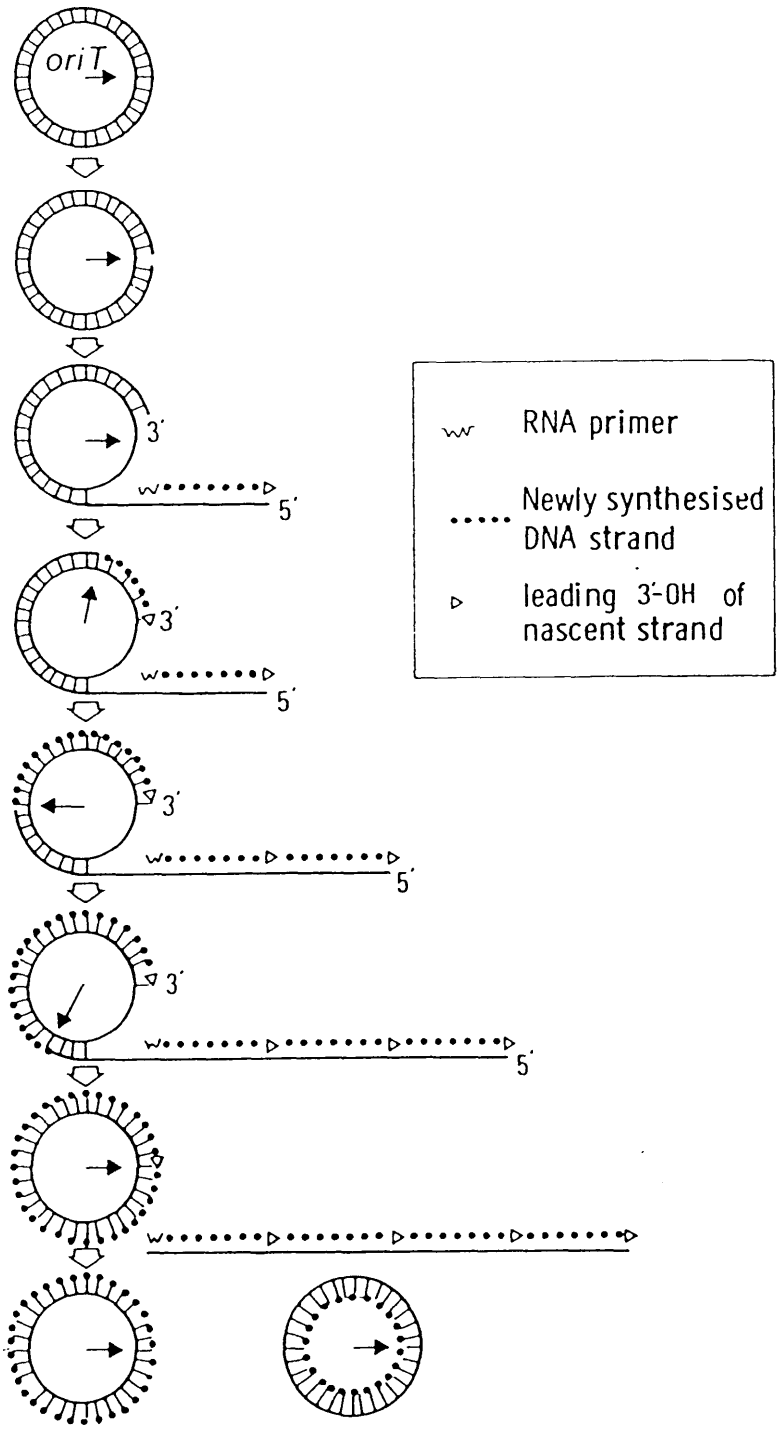


Figure 1.7 **The Rolling Circle Model of Replication.**

(Glass, 1983)

1.5.3 Structure of Amplified Genes.

Amplified DNA is primarily a linear representation of the normal DNA sequence (Van der Bliek et al., 1986). The length of amplified DNA is highly variable and the genes may occur in a head-to-head orientation (Ford and Fried, 1986).

Looney and Hamlin observed the presence of two completely different units of amplification, amplicons, in a methotrexate resistant CHO cell line, CHOC400 (Looney and Hamlin, 1987). This cell line is highly resistant and contains between 1,000 to 1,200 copies of the DHFR genes in the form of an HSR. Using chromosome walking techniques, the two amplicon types have been shown to differ in size, end-to-end arrangement and representation within the genome. Type I amplicons were 260 kb long, arranged in head to tail fashion, encompassing 10 to 15% of the CHOC400 genome. Type II amplicons were 220 kb long, arranged in head to head and tail to tail fashion, and represent the majority of the remaining amplicons. Amplicon II was represented in its entirety within the type I amplicon. Abnormal banding patterns were seen on three different chromosomes (one major and two minor), one of which may have been the original site for the type I amplicon before a fragmentation event occurred, involving loss of part of the amplicon and giving rise to

the type II amplicon. The purpose of these two distinct forms is unclear.

The simplest model for the structure of amplified DNA is that the DHFR gene is amplified in an amplicon which is repeated several times producing multiple copies of the gene in a linear tandem repeat array. By monitoring the size of HSRs and the number of gene copies, estimates were made of the size of the amplicon. The initial estimates were inaccurate and there were large discrepancies between the findings of different groups. The size of the amplicon was estimated variously between 500 - 1,000 kb in MTX-resistant CHO cells (Nunberg et al., 1980) and 3,000 kb in MTX-resistant murine lymphoma cells (Bostock and Clark, 1980).

The amount of DNA found in double minutes in MTX-resistant human neuroblastoma cell lines was quantified by electron microscopy to determine the dry mass of the DNA (Bahr et al., 1983). The double minutes were uniform in size, therefore 32 pairs were quantified and the average calculated. The mean dry weight of the "single minute" was found to be 4.71×10^{-14}g, thereby giving a DM weight of 9.4×10^{-14}g. On the basis of DNA constituting 15% of the dry mass, the corrected dry weight for a DM was 1.41×10^{-14}g. This is approximately 6.38×10^{3} kb of DNA contained within the DM. Estimating that each DM may contain up to five DHFR gene copies, the amplicon size found on these DMs was approximately 1,300

kb. More speculative estimates were also made about the size of the HSRs and this was found to be approximately 2.26×10^5 kb (Bahr et al., 1983).

The amplified unit in resistant cells shows evidence of novel DNA rearrangements. The DHFR gene in murine cells is only 32 kb in length, but amplified regions of 240 kb have been isolated in methotrexate resistant murine cells. Chromosome "walking" was employed to analyse this 240 kb unit of amplified DNA (Federspiel et al., 1984). Many different DNA rearrangements were isolated in the regions surrounding the DHFR gene. It was found that the rearrangements (and corresponding junctions) were unique to each cell line and many different rearrangements were isolated in each cell line. It was also noted that the degree of amplification of sequences adjacent to the DHFR gene varied among and within cell lines. In addition, following prolonged maintenance in selective culture conditions the arrangement of these sequences varied. The conclusion from this study was that a dynamic and complex arrangement of the amplified sequences exists and that no static repetitive unit exists to be amplified.

In general, a gradient of amplification has been seen in MTX resistant cells where the closer the sequence is found to the DHFR gene, the more frequently it is amplified compared to fragments distal to the gene (Caizzi and Bostok, 1982).

CHAPTER 2. MATERIALS AND METHODS.

2.1 MAMMALIAN CELL CULTURE.

MAWI, a human colorectal adenocarcinoma cell line, was established from a "spill" culture from a mucoid adenocarcinoma with signet ring cells (Lewis et al., 1983) at Charing Cross Hospital. The cell line is a low producer of carcinoembryonic antigen (CEA) and expresses HLA A, B, and C. Doubling time is approximately 18 hours.

JAR was established from a trophoblastic tumour of the placenta following pregnancy. Under certain conditions JAR secretes protein hormones, such as progesterone and oestrogen, at high levels (Patillo et al., 1971).

Chinese hamster ovary cells (CHO) were a gift from Dr. Victor Ling, (Ontario Cancer Institute, Toronto, Canada) in both sensitive form, AUXB1, and multidrug resistant mutant, $CH^{R}C5$, and are auxotrophs requiring glycine, adenosine, thymidine and proline for growth (Ling and Thompson, 1974).

L1210 mouse leukemic cells were a gift from Dr. Anne Jackman, (Institute of Cancer Research, Sutton, Surrey) both sensitive and resistant lines. The resistant cells, L1210 R7A, grow in 1.0×10^{-6} M MTX and require continuous drug selection (Jackson et al., 1975).

MAWI cells were grown as monolayers in DMEM containing 25mM HEPES supplemented with 10% foetal calf serum, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Subculturing was performed when cells reached confluence, approximately every 3-4 days, by removing the culture medium, washing the cells with saline and covering the cells with trypsin-EDTA at a concentration of 0.5g trypsin and 0.2g EDTA per litre. After 5 minutes incubation at 37°C, the cells were detached from the flask and could then be treated, split or frozen. Reseeding was done in fresh medium at a concentration of approximately 2 x 10^4 cells/ml and cells were incubated at 37°C.

JAR cells were grown as above except that they were seeded at a concentration of approximately 5 x 10^5 cells/ml.

CHO cells were maintained in α-MEM medium supplemented with 25mM HEPES, 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were passaged once a week and replated at a concentration of approximately 1 x 10^6 cells/ml.

Murine leukemic cells, L1210 and L1210 R7A, were maintained as suspension cultures in RPMI1640 containing 25mM HEPES. The medium was supplemented with 10% dialysed donor horse serum, 2mM L-glutamine, 100 U/ml penicillin

and 100 μg/ml streptomycin. L1210 R7A cells were maintained in 1.0 x 10^{-3} M MTX. These cells were passaged once a week and reseeded at an approximate concentration of 10^6 cells/ml.

All cells were counted using an Improved Neubauer Haemacytometer and a Reichert Nr.259 840 microscope. Once counted, the cells were frozen in growth medium containing 10% DMSO in cryogenic vials (NBL) at a rate of approximately 1°C drop per minute. Once frozen, stocks were transferred to liquid nitrogen. For retrieval, vials were thawed rapidly in a 37°C incubator and resuspended in 20 ml medium plus serum. Cells were centrifuged immediately to remove DMSO (MSE chilspin 2, 1,500 r.p.m. [≈1,500 x g_{av}]) for 5 minutes and resuspended in the appropriate volume of medium.

2.1.1 IC_{50} Determination.

Before treating with methotrexate IC_{50} concentrations were determined for MAWI and JAR cell lines. Cells were plated with a separate 6-well plate for each days counting so that the cells were only removed from the incubator on the days they were to be counted. Before counting, dead cells were removed by saline washing and then the live cells detached by trypsinisation. Trypan blue was used such that only viable cells were counted.

JAR and MAWI were counted and plated at a concentration of 4 x 10^5 cells/ml, allowed to settle overnight in 9 well plates, and then exposed to a range of drug concentrations. Controls were included with no drug exposure. Each well was counted in duplicate with three replicate wells for each count.

After counting, the percentage cell survival was calculated. Percentage survival against time was plotted for each drug concentration and the 50% inhibitory concentration was interpolated from the graph.

2.1.2 Production of resistant cells.

The MAWI cells were synchronised by pulsing for 6 hours with 1.0 mM hydroxyurea, in an attempt to increase the probability of obtaining amplification and concomitant resistance to methotrexate (Brown et al., 1983; Mariani and Schimke, 1984; Johnston et al., 1983). Hydroxyurea is a potent inhibitor of DNA synthesis (Timson, 1975). Tumour cells were seeded and allowed to settle overnight before the hydroxyurea pulse. The hydroxyurea containing medium was removed and the cells then exposed to various drug concentrations. Two treatment regimens were then followed:

(a) Cells were exposed to a range of drug concentrations for 3 days and then the drug removed. Once the cells had recovered, which was estimated by return to

normal morphology and growth pattern, (3 days - 3 weeks) the treatment was repeated either using the same or an increased drug concentration.

(b) Cells were continuously exposed to IC_{50} drug concentrations in medium containing dialysed foetal calf serum. This treatment was followed until the doubling time of the cells in drug was equivalent to the doubling time of the sensitive parental cells when grown in drug free medium. Only then was the drug concentration raised by stepwise increments.

2.2 CHARACTERISATION OF RESISTANT LINES.

2.2.1 MTT Assay.

The MTT assay is based on the reduction of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a formazan, by living cells. Cells were plated in 96-well microtitre plates at 1 x 10^4 cells/ml in DMEM containing dialysed foetal calf serum and 2,200 mg/L sodium bicarbonate (HEPES free). The cells were incubated in a 5% CO_2 atmosphere at 37^{o}C and allowed to settle overnight. The following day, the medium was removed and 50 μl fresh medium containing the relevant drug concentration was added and the cells reincubated as before. The cells were left to grow for 5 days. The drug

containing medium was removed and the cells were incubated for six hours in 0.5 mg/ml MTT. Formazan crystals were solubilised in DMSO and the absorbance was quantitated with a microplate spectrophotometer (Titertek Multiscan MCC 340) at 540 nm (Pieters et al., 1989).

2.2.2 Uptake of Radioactively-Labelled Drugs.

Exponentially growing cells were harvested using trypsin-EDTA and resuspended at 5 x 10^6 cells/ml in DMEM containing dialysed foetal calf serum. Radiolabelled methotrexate ([^{3}H]-MTX) was added at the appropriate concentration. Incubation was at 37°C with shaking and 1 ml aliquots were removed at pre-determined time intervals and the cells pelleted through an oil mixture (0.5 ml). The mixture consisted of 4 parts silicone oil (Dow Corning 704 diffusion pump fluid) and 1 part mineral oil (3 in 1). Centrifugation was for 1 minute in a MSE Micro Centrifuge at 13,000 r.p.m. [≈13,400 x g_{av}]. The cell pellets were dissolved in 0.5 ml Protosol, added to 5 ml scintillation fluid and counted in duplicate on a liquid scintillation counter (Packard 1900CA Tri-carb liquid scintillation counter) (Millar et al., 1989).

2.2.3 Dihydrofolate Reductase Level Determination.

2.2.3.1 Dihydrofolate Reductase Preparation.

A crude preparation of dihydrofolate reductase was prepared following a published method (Taylor et al., 1985). Cells were harvested and washed twice in 10 ml ice-cold phosphate buffered saline. The pellets were resuspended in 5 ml 0.15 M potassium phosphate buffer, pH 7.0, and homogenised on a bed of ice using a Dounce homogeniser. The homogenate was then sonicated for 4 minutes using the Ultrasonics Inc. W375 sonicator. The sonicated solution was centrifuged (Sorvall Technospin R, 3,500 rpm [$\approx$ 2285 x g_{av}]) at 4°C for 15 minutes to remove cell debris. The supernatant was then centrifuged at high speed (Sorvall OTB 55B, TFT 50.38 rotor, 24,000 rpm [$\approx$ 52,100 x g_{av}]) for 1 hour at 4°C. The supernatant contained the crude preparation of dihydrofolate reductase and this was stored at -70°C until required.

2.2.3.2 Dihydrofolate Reductase Assay.

The assay was based on the decrease in absorbance at $O.D._{340nm}$ due to the conversion of NADPH to NADP which occurs stoichiometrically with the conversion of dihydrofolate (DF_2) to tetrahydrofolate (TF_4).

Dihydrofolate + NADPH ----> Tetrahydrofolate + NADP

Dihydrofolate reductase was quantified using the following enzyme unit definition:

> One unit will convert 1.0×10^{-6} M of 7,8 Dihydrofolate and NADPH to 5,6,7,8 Tetrahydrofolate and NADP per minute at pH 7.0 at 30°C. The assay was performed using a Pye Unicam SP8-400 UV/VIS spectrophotometer and 3 ml silica cuvettes.

2.7 ml 0.15 M potassium phosphate buffer, pH 7.0, 0.1 ml 2 mg/ml NADPH and 0.1 ml enzyme preparation were mixed and added to a cuvette. This was incubated in a 30°C water bath and the temperature within the spectrophotometer was also controlled to 30°C. The reaction was initiated by the addition of 0.1 ml 1 mg/ml dihydrofolic acid (dissolved in 0.25M 2-mercaptoethanol).

Once the dihydrofolate was added, the decrease in absorbance at $O.D._{340nm}$ was monitored and the rate calculated. Estimates of enzyme concentration in DHFR preparations were made by comparing rates calculated to those calculated from known enzyme concentrations on a standard curve.

2.3 PREPARATION OF PLASMIDS.

Plasmids were prepared and analysed for use in the production of cDNA probes and riboprobes. These were required to investigate gene copy numbers and mRNA

expression levels in DNA and RNA extracted from drug resistant cells and tumour material.

2.3.1 Bacterial Stocks: Strains and storage.

All plasmids were propagated in Esherichia coli (E.coli) K12 strains HB101 or JM101.
The genotypes of these strains are as follows:

HB101 F^-, hsdS20, rB^-, mB^-, $recA^-$, supE44, ara-14, gal K-2, lacY1, proA2, rps L20, str^r, xyl-5, mt1-1.

JM101 (lacpro), thi, F'traD36, proAB, supE, lacIqZ M15.

All bacterial stocks were stored as exponential cultures, both in 50% v/v glycerol at -20°C and 15% v/v glycerol at -70°C. The stocks at -70°C were not used routinely but were set aside to form a consistent seed lot. Stocks were reselected and grown every six months.

2.3.2 Plasmids.

The plasmid pSVDHFR was a gift from Adrian Brasnett, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, England. The plasmid contains a mouse DHFR mini-gene on an SV40 based vector containing the pBR322 genome (Chernajovsky et al., 1984).

The plasmid pCHP1 was obtained from the American Type Culture Collection, ATCC Number: 39839. The plasmid was constructed from a portion of the coding sequence of the P-glycoprotein inserted into the EcoRI site of pUC9 (Riordan et al., 1985).

2.3.3 Cell Transformation with Recombinant Plasmids.

Two methods for transformation were used: a simple and relatively quick calcium chloride method and a more complex, but also more efficient, method using the Hanahan protocol. Transformation was required in order to introduce plasmid DNA into bacterial hosts such that large quantities of the required plasmids may be produced.

2.3.3.1 Transformation by Calcium Chloride.

Competent cells were prepared from E.Coli K-12 strains JM101, using a published method (Mandel and Higa, 1970). A bacterial culture was prepared by inoculating 10 ml of antibiotic free L-broth with a single colony of JM101 and grown overnight, with shaking, at 37°C. 1 ml of the resultant resting culture was removed and used to inoculate 100 ml of L-broth. This was grown, with shaking, at 37°C, until an $O.D._{550nm}$ of 0.4 was reached ($\approx 5 \times 10^7$ cells/ml). The bacterial culture was chilled on ice for 10 minutes and harvested by centrifugation

(Sorvall RC-5B, HB-4 rotor, 5,000 rpm [≈ 2,705 x g_{av}]) at 4°C for 6 minutes. The supernatant was removed and the cells resuspended in 50 ml of an ice cold sterile solution of 50 mM calcium chloride ($CaCl_2$), 10 mM Tris-HCl, pH 8.0. The cells were then placed on ice for 15 minutes followed by a repeat of the centrifugation step (Sorvall RC-5B, HB-4 rotor, 5,000 rpm) at 4°C for 6 minutes. Once more the supernatant was discarded and the cells resuspended in 6.67 ml ice cold sterile 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0 before being aliquoted into pre-chilled, sterile Eppendorf tubes. The tubes were incubated at 4°C for 4 hours prior to being used for transformation.

Up to 25 ng of plasmid DNA was added to each aliquot of cells in 0.1 volume x10 ligation buffer (0.67 M Tris-HCl, pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP, 1 mg/ml BSA). The cells and DNA mixture were gently mixed by inverting the tube and stored on ice for 30 minutes. The cells were then heat shocked at 42°C for 2 minutes and 1 ml of L-broth containing ampicillin, 50 μg/ml, was added to each tube and incubated at 37°C for one hour. This allowed the bacteria to recover and express antibiotic drug resistance. After incubation 100 μl aliquots were plated by spreading onto L-agar/ampicillin media, containing 0.004% w/v X-gal, and 0.008% w/v IPTG. Once the liquid had completely soaked into the agar, the plates were inverted and incubated overnight in the dark at 37°C.

2.3.3.2 Transformation by standard high efficiency method.

This is a modification of a published method (Hanahan, 1985). Frozen bacterial stocks were streaked onto SOB (2% bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM Mg SO_4) agar plates (1.3% agarose) and grown overnight at 37°C. Single colonies were used to inoculate 10 ml of SOB culture medium and these were incubated, with shaking, at 37°C until an $O.D._{550nm}$ of 0.4 was reached. The culture was transferred to a 50 ml polypropylene centrifuge tube (Falcon-2098) and stored on ice for 15 minutes. The cells were then pelleted by centrifugation (Sorvall Technospin R, 2,300 r.p.m. [$\approx$ 990 x g_{av}]) at 10°C for 3 minutes. The pellet was drained thoroughly and the cells resuspended in 1/3 original culture volume of standard transformation buffer (TFB - 100 mM KCl, 45 mM $MnCl_2.4H_2O$, 10 mM $CaCl_2.2H_2O$, 3 mM $HACoCl_3$, 10 mM K-MES). The mixture was vortexed and re-incubated on ice for 15 minutes before centrifugation and draining of the pellet. The pellet was then resuspended in TFB to 8% original culture volume, effectively concentrating the cells from 2.5 ml culture medium to 200 μl TFB. DnD (1 M DTT, 90% v/v DMSO, 10 mM KOAc) was added to 3.5% (v/v) into the centre of the cell suspension and the contents mixed gently, followed by incubation on ice for 10 minutes. Another aliquot (the same volume) of DnD was then added and the tubes

incubated on ice for a further 20 minutes before pipetting 210 µl aliquots into prechilled sterile Eppendorf tubes. At this stage, the bacteria could be stored at 4°C for up to 12 hours without affecting the efficiency of transformation, or snap frozen and stored at -70°C.

Up to 0.2 µg vector DNA was added to the competent cells. After incubation on ice for 30-40 minutes, the cells were heat shocked at 42°C for 90 seconds and then returned to ice for 2 minutes. After heat shock and cooling, 800 µl SOC (SOB + 20 mM glucose) was added and the cells incubated at 37°C for 60 minutes with gentle shaking. The cells were then harvested by centrifugation (MSE Microcentaur, 6,500 rpm) at room temperature for 2 minutes. The pellets were resuspended in 100 µl SOC and plated onto SOB plates containing ampicillin (50 µg/ml), X-gal (0.004% w/v) and IPTG (0.008% w/v) and incubated overnight at 37°C.

2.3.3.3 Characterisation of Recombinant Plasmids.

Bacteria containing recombinant plasmids were white in colour, as opposed to blue non-recombinants. Confirmation that the plasmid from the white colonies contained a DNA insert was provided using restriction enzyme digestion on DNA isolated by the 'miniprep' method (section 2.3.5), followed by restriction enzyme digestion and agarose gel

electrophoresis. This allowed the characterisation of the insert based on size and presence of restriction sites.

2.3.4. Large Scale Preparation of Recombinant Plasmid DNA.

This procedure was used to grow up large quantities of plasmid for use in restriction analysis and the production of radiolabelled probes.

Plasmid DNA was prepared by density gradient centrifugation using a modification of a published method (Ish-Horowicz and Burke, 1981). All techniques and manipulations involving live bacterial cultures were performed using safe microbiological procedures as described by the GMAG regulations.

Fresh colonies of plasmid carrying E.Coli strains were grown overnight on agar plates prior to each plasmid preparation. A single colony was picked and used to inoculate 10 ml of L-broth (5 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract, pH 7.2) containing 50 μg/ml ampicillin. The 10 ml 'starter' culture was incubated at 37°C, with shaking, until an $O.D._{550nm}$ of 0.4 was reached. 1 ml of this culture was used to inoculate each 500 ml of L-broth containing ampicillin.

A 1 litre culture was grown overnight, with shaking, at 37°C, and the bacterial cells were harvested in 250 ml

GSA bottles by centrifugation in the Sorvall RC5B (GSA rotor, 7,000 rpm [≈ 5019 x g_{av}]) at 4°C for 6 minutes, and the pellets resuspended in 4 ml of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). These were pooled into a GSA bottle and lysozyme was added at 5 mg/ml and the bacteria incubated at room temperature for 10 minutes. A fresh solution of 0.2 M NaOH, 1% w/v SDS was prepared and 40 ml added to the cells. After gentle mixing the GSA bottles were cooled on ice for 10 minutes. The addition of 20 ml of 3M potassium acetate [KOAc], pH 4.8, precipitated the high molecular weight nucleic acid and cellular protein, but not the plasmid DNA. The resultant solution was vigorously vortexed and incubated on ice for 30 minutes. The cell debris was removed by centrifugation (GSA rotor, 10,000 rpm [≈ 10,240 x g_{av}]) at 4°C for 10 minutes. The supernatant was decanted into a clean GSA bottle, and 0.6 volume of propan-2-ol was added to precipitate the remaining nucleic acid. After a 10 minute incubation at room temperature, centrifugation at 4°C for 10 minutes was used to pellet the precipitate (GSA rotor, 10,000 rpm [≈ 10,240 x g_{av}]). The supernatant was once more gently removed and the pellet resuspended in 20 ml of TE buffer. The DNA solution was transferred to a 30 ml polypropylene tube and approximately 21 g (7g solid / 6.5g of DNA solution) of caesium chloride added. Ethidium bromide was added at 0.5 mg/ml and remaining debris removed by centrifugation (HB4 rotor, 12,000 rpm [≈ 15,570 x g_{av}]) at RT for 30 minutes. After

centrifugation and transfer of supernatant to a fresh tube the specific gravity was adjusted to 1.56 by the addition of solid caesium chloride. The solution was transferred to a 35 ml Sorvall Ultracrimp centrifuge tube and centrifuged in a Sorvall OTD 65B ultracentrifuge (TFT 50.38 rotor, 42,000 rpm[≈ 159,600 x gav]) for 36 hours at 18°C. The tubes were carefully removed from the rotor, taking care not to disturb any gradient banding, and the plasmid band was inspected under a uv lamp (UVSL-58 Mineralight Lamp, Ultra-Violet Products Incorporated, California, U.S.A.). The plasmid band was harvested and the ethidium bromide removed by extraction with TE/caesium chloride saturated propan-2-ol, involving at least 2 further extractions once no colour is visible. DNA was dialysed against 2 litres of TE buffer, pH 8.0, overnight with 3 changes of TE buffer at 4°C. The DNA solution was extracted once with phenol (saturated against TE, pH 8.0), once with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1), before being ethanol precipitated (see section 2.7.1) and resuspended in an appropriate volume of TE buffer, pH 8.0, and stored at -20°C.

2.3.5 Small-scale Preparation of Recombinant Plasmid DNA.

This procedure enabled the analysis of several recombinant products at the same time where a high yield was not essential. An alkaline lysis 'miniprep' technique

was used, producing up to 5 μg DNA (Birnboim and Doly, 1979).

A single plasmid-containing bacterial colony was selected to inoculate a 5 ml L-broth culture containing ampicillin (50 μg/ml) and incubated at 37°C, with vigorous shaking, overnight. 1.4 ml was transferred to a sterile 1.5 ml Eppendorf tube and the remainder stored at 4°C until required. The bacterial cells were harvested by centrifugation (MSE Microcentaur, 13,000 rpm [≈ 13,400 x g_{av}]) for 1 minute at RT. The supernatant was removed by aspiration, and the pellet dried by inverting the tube for a few minutes. DNA was extracted using a scaled down version of the maxi-prep procedure, allowing all procedures to be performed in an Eppendorf tube. No caesium chloride gradient was required as the first ethanol precipitation was sufficient to produce DNA clean enough for further analysis.

2.3.6 Purification of Restriction Fragments.

Restriction fragments were purified from plasmid DNA by two different methods, depending on the yield required and the amount of available material.

2.3.6.1 Extraction from Low Melting Point (LMP) Agarose.

This technique was used to purify small amounts of DNA , between 1 and 10 μg. DNA restriction fragment separation was performed as for normal agarose gels (see sections 2.7.3 and 2.7.4) except that the gels were poured and electrophoresed at 4°C. After electrophoresis, the desired band was identified by inspection under short wave uv light and cut out using a scalpel blade. The volume of the gel slice was estimated and an equal volume of extraction buffer (0.1 M Tris-acetate, pH 7.5, 5 mM EDTA, 0.5 M NaCl) added. The gel slice was then macerated in the extraction buffer using an autoclaved glass rod and the resultant semi-solid mass was heated at 65°C, with shaking, for at least 15 minutes, until the agarose had fully melted. Once melted, an equal volume of phenol (saturated in TE, pH 8.0 at room temperature) was added and the tube contents mixed gently, by inversion. Centrifugation was used to separate the organic and aqueous phases (MSE Microcentaur, 13,000 rpm) at room temperature for 10 minutes. The aqueous phase was transferred to a clean sterile Eppendorf and the lower layer was back extracted with an equal volume of TE buffer to remove any remaining DNA. After centrifugation, as before, the second aqueous phase was pooled with the initial one. The combined aqueous phases were extracted with an equal volume of phenol/chloroform (1:1) and once with chloroform/isoamyl-alcohol (24:1). After ethanol

precipitation (section 2.7.1) the extracted DNA was resuspended in an appropriate volume of TE buffer, pH 8.0.

2.3.6.2 Extraction by Electroelution.

This technique was used to extract quantities of restriction fragment greater than 10 μg. Recombinant plasmids were restriction digested and electrophoresed to size fractionate the DNA fragments (see sections 2.7.3 and 2.7.4). The required band was identified and cut from the gel as before (section 2.3.6.1). DNA was electroeluted from the gel using an electroelution manifold, Biotrap BT 1000 (Schleicher and Schuell, supplied by Anderman and Co. Ltd., Surrey, England). The gel slice was placed in a chamber and just covered in TBE running buffer. The elution chamber was then placed in a horizontal gel tank (BRL H5) and the tank filled with TBE to the same level as the manifold. The gel slice was then gently moved so that it rested against the permeable filter membrane and a 200V current was passed through the tank for 30 minutes. The DNA migrated out of the agarose into a small cavity between the permeable membrane and a non permeable membrane. The gel slice was removed and uv irradiated again to check that all the DNA had emigrated. Once the agarose was free of DNA, the current was reversed for 20 seconds to remove any DNA stuck to the back filter. The DNA solution was then carefully removed, using a Gilson pipette tip, and its purity analysed.

2.3.7 Production of Riboprobes.

Both plasmids, pCHP1 and pSVDHFR, were restriction digested and the insert cDNA isolated. The insert was sub-cloned into riboprobe vectors. The vectors used, pGEM3Z and pGEM4Z, were obtained from Promega Biological Research Products, Wisconsin, U.S.A. Both vectors contain multiple cloning sites, colour recombinant product indicators (lacZ) and SP6 and T7 RNA polymerase promoters.

The riboprobe vector and insert DNA were restriction endonuclease digested and the vector DNA treated with calf intestinal alkaline phosphatase (CIAP). The insert and vector were ligated in a molar ratio of 1:3, vector:insert using approximately 40 ng insert DNA. The ligation mixture contained 1 unit of T4 DNA ligase and 1 μl 10x ligation buffer (400 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 500 μg/ml BSA) and DNA in a total volume of 10 μl. The ligation reactions were incubated either at 25°C for 1 hour or 15°C for 5 hours, depending on the temperature found to be most suitable for individual reactions. After incubation, 2μl of the ligation mix was used to transform bacterial cells using the high efficiency transformation method (section 2.3.3.2).

2.3.8 Radiolabelling of DNA.

DNA was radiolabelled by random oligonucleotide priming (Feinberg and Vogelstein, 1984). DNA fragments were purified either as described (in section 2.3.6) or as follows. The recombinant plasmid containing the 'probe' insert was restriction endonuclease digested and separated by electrophoresis through a 1% w/v LMP agarose gel. The DNA fragment required was cut from the gel, inspected under uv and, removing as little as possible of the agarose but all of the DNA, placed in a sterile preweighed Eppendorf tube. 3 ml distilled water was added for every gram wet weight of agarose, and the sample heated in a boiling water bath for 10 minutes to melt the agarose and denature the DNA. An aliquot from this sample was removed for use and the rest stored at -20°C for future use.

The aliquot removed, containing 50 μg DNA in 30μl, was transferred to a sterile Eppendorf. The following solutions were added in this order: 10 μl oligolabelling buffer (OLB - 200 mM HEPES, pH 6.6, 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 mM each of dATP, dGTP, dTTP, 150 μg/ml hexadeoxyribonucleotide primer, $p(dN)_6$, 400 μg/ml BSA); 25 μCi α^{32}P-dCTP (>3,000 Ci/mmol); sterile distilled water to 49 μl; 5 units of Klenow polymerase. The labelling mixture was incubated at RT for approximately 6 hours and then spin column chromatography

was used to separate the labelled DNA from the unincorporated nucleotides (see section 2.7.6).

2.3.9 Radiolabelling of Riboprobes.

RNA was radiolabelled by random oligonucleotide labelling using RNA transcripts prepared from cDNA inserts into pGEM vectors as discussed in section 2.3.7.

Radiolabelling is performed using the following mix added in this order: 4.0 μl 5x transcription buffer (prepared by Promega and distributed with RNA polymerases), 2.0 μl 100 mM DTT, 1 unit/μl RNasin, 4 μl 2.5 mM each of ATP, GTP and UTP, 1.2 x 10^{-5} M CTP, 1 μg linearised template (vector containing insert), 50 μCi α^{32}P-CTP, 1 μl (1 unit/μl) SP6 of T7 RNA polymerase. The final volume was adjusted to 20 μl and the mixture incubated at 37°C, for 60 minutes. Following incubation, the DNA template was removed by DNase digestion and the digested DNA removed by phenol/chloroform extraction. The labelled riboprobe was ethanol precipitated (section 2.7.1) and resuspended in 100 μl DEPC-water.

2.4 NUCLEIC ACID ANALYSIS.

2.4.1 Extraction of DNA from Tumour Material and Tissue.

Tumour samples were snap frozen and stored at -70°C and, before extraction, were thawed and rinsed quickly in sterile phosphate buffered saline (PBS). Samples, between 0.01 and 0.3g wet weight, were suspended in 0.5 ml TE buffer, pH 8.0, and an equal volume of 2x lysis buffer (200 mM Tris-HCl, pH 7.5, 2% sarcosyl, 0.2% SDS, 20 mM EDTA, 140 mM 2-mercaptoethanol), and incubated on a revolving wheel at 55°C overnight. The following day, the cell debris was removed by centrifugation (MSE Microcentaur, 13,000 rpm [≈ 13,400 x g_{av}]) at room temperature for 10 minutes. The supernatant was phenol extracted (phenol saturated with TE, pH 8.0) twice. The sample was then phenol/chloroform (1:1) extracted once and chloroform/iso-amyl alcohol (24:1) extracted, followed by ethanol precipitation (section 2.7.1) and resuspended in an appropriate volume of TE, pH 8.0, and stored at -20°C.

2.4.2 Extraction of DNA from Cultured Cells.

Approximately 10^7 cells were washed 3 times in 10 ml PBS and the cells harvested by centrifugation (Sorvall Technospin R, 1,500 rpm [≈ 1,500 x g_{av}]) at 4°C for 5 minutes. The cell pellet was then incubated in 2 ml lysis

buffer (0.33 M sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100) on a revolving wheel at 4°C for at least 2 hours. The lysed cells were pelleted by centrifugation (Sorvall Technospin R, 4,000 rpm [≈ 3000 x g_{av}]) for 10 minutes at 4°C, then resuspended in 2 ml incubation buffer (75 mM NaCl, 25 mM EDTA, 0.5% SDS, 200 μg/ml proteinase K) and incubated overnight at 37°C with gentle shaking. The following day, the cell debris was removed by centrifugation (Sorvall Technospin R, 4,000 rpm [≈ 3000 x g_{av}]) for 10 minutes at 4°C and DNA purified as in 2.4.1.

2.4.3 Extraction of RNA from Tumour Material and Tissue.

The tumour material was minced on a tile placed on dry ice, then suspended in 1 ml denaturing solution (4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and homogenised (Polytron PT 10-35) on ice for 1 minute. To the homogenate the following were added, in order, with mixing at each stage: 0.1 ml 2M sodium acetate, pH 4.0, 1 ml phenol saturated in DEPC H_2O, 0.2 ml chloroform/iso-amyl alcohol (49:1). The mixture was vortexed and incubated on ice for 15 minutes, followed by centrifugation (Sorvall RC-5B, GSA rotor, 10,000 rpm) at 4°C for 20 minutes. This produced two phases, with the RNA in the upper aqueous phase which was removed and transferred to a fresh tube. RNA was iso-propanol

precipitated at -20°C for 1 hour, then pelleted by centrifugation as before and resuspended in 0.3 ml denaturing solution and transferred to a sterile 1.5 ml Eppendorf. An equal volume of isopropanol was added and the RNA was re-precipitated at -20°C for another hour. The RNA was pelleted (MSE Microcentaur, 13,000 rpm) at 4°C for 10 minutes, dried in a Speed Vac concentrator and resuspended in an appropriate volume of DEPC H_2O.

2.4.4 Extraction of RNA from Cultured Cells.

Approximately 2 x 10^7 cells were harvested and washed in ice cold PBS at least twice. Each time, the cells were pelleted by centrifugation (Sorvall Technospin R, 1,500 rpm) at 4°C for 5 minutes. The cell pellet was resuspended in 0.4 ml lysis buffer (0.15M NaCl, 10 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 1 mM $MgCl_2$) and transferred to a sterile 1.5 ml Eppendorf tube. The cells were incubated on ice for 5 minutes and then centrifuged (MSE Microcentaur, 13,000 rpm) at 4°C for 5 minutes. The resultant supernatant was transferred to a sterile Eppendorf tube containing 50 μl 10% SDS and 200 μl phenol (saturated with TE, pH 8.0), vortexed and recentrifuged. The aqueous layer was transferred to another Eppendorf tube containing 200 μl buffered phenol, vortexed and centrifuged again. The RNA was precipitated from the aqueous layer by the addition of 40 μl 3M potassium

acetate, pH 5.4, and 1 ml absolute ethanol at -20°C overnight. The RNA pellet was washed in 75% ethanol, recentrifuged, dried and resuspended in an appropriate volume of DEPC H_2O.

2.4.5 Quantitation of RNA and DNA.

Purity and quantity of DNA and RNA were determined using a Pye Unicam SP8-400 UV/VIS spectrophotometer. The solution was scanned and the readings at 260nm used to calculate the concentration of nucleic acid. An $O.D._{260nm}$ of 1 corresponds to approximately 50 μg/ml double-stranded DNA and 40 μg/ml single-stranded RNA. The $O.D._{260nm}$:$O.D._{280nm}$ ratio provided an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have a ratio of 1.8 and 2.0 respectively. All RNA and DNA samples, where possible, were made up to working concentrations of 1 mg/ml before storage.

2.5 TRANSFER OF NUCLEIC ACIDS TO SOLID SUPPORTS.

2.5.1 Slot Blotting and Hybridisation.

Nucleic acid samples were denatured before slot blotting. DNA samples were treated with 0.1 volume 3M sodium hydroxide and incubated at 65°C for 1 hour. The samples were allowed to cool to room temperature before the

addition of 1 volume of 2M ammonium acetate, pH 7.0, and then diluted to the correct volume in 1M ammonium acetate. RNA samples were denatured by the addition of 0.3 volume 20 x SSC (saline sodium citrate), 0.2 volume 37% formaldehyde, and incubated at 60°C for 15 minutes, then diluted in 15 x SSC to the appropriate volume.

Slot blotting was performed using a modification of a published technique (Wickenden et al., 1985), using a 72-well suction manifold (Schleicher and Schuell GmbH, Dassel, West Germany). A piece of nitrocellulose was pre-wet in distilled water and soaked in either 1M ammonium acetate (DNA) or 6 x SSC (RNA). The membrane was then placed on the manifold, vacuum suction applied and the wells washed through prior to application of samples in 1M ammonium acetate (DNA) or 6 x SSC (RNA). The samples were then loaded in a volume of 250 μl/well. The liquid is drawn through the membrane by the vacuum and the nucleic acid deposited on the surface of the membrane in a limited area defined by the well. Once all the liquid has passed through the membrane the slots are once again washed through as before. The manifold was then dismantled and the nitrocellulose membrane removed, air dried and baked at 80°C for 2 hours.

Membranes were prehybridised for at least 4 hours at 65°C in 30 ml pre-hybridisation buffer consisting of: 5 x SSC; 10 mM phosphate buffer, pH 7.0; 1.0 mM EDTA, pH 8.0; 5%

dextran sulphate; 0.1% SDS; 100 μg/ml denatured sonicated salmon sperm DNA; 0.1 volume 10 x Denharts (2% BSA fraction V, 2% polyvinyl-pyrrolidone [PVP], 2% Ficoll, 1% SDS. Moist filters were then transferred to plastic bags, heat sealed and 15 ml pre-hybridisation buffer was added with single-stranded ^{32}P-labelled DNA probe. The bags were sealed, taking care to remove any air bubbles, and incubated overnight at 65°C, with shaking. After hybridisation, the excess probe was removed by repeated washes at 65°C with 0.1% SDS and stepwise decreases in SSC, 2 x SSC - 0.1 x SSC, which increased stringency. The effectiveness of the washes was monitored using a Mini-Instruments series 900 mini-monitor. Once excess probe had been removed, the filters were air dried at room temperature, covered in Saran Wrap and autoradiographed.

Hybridisation signals were quantitated by densitometric scanning of autoradiographs using a Chromoscan III integrating densitometer (Joyce-Loebl).

2.5.2 Southern Blotting and Hybridisation.

Southern blotting was performed using the published technique (Southern, 1975). DNA fragments were size fractionated by agarose gel electrophoresis. The gel was photographed, marker fragment (Lambda-HindIII; PhiX-174-HaeIII) migration measured and then the DNA denatured in situ by soaking for 1 hour, with gentle shaking, in 0.5 M

NaOH, 1.5 M NaCl at room temperature. The gel was rinsed in 2 x SSC and then neutralised by soaking for 1 hour, with gentle shaking, in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 0.001 M EDTA. DNA fragments were transferred to Hybond-N (Nylon blotting membrane supplied by Amersham International) by capillary action using the apparatus described by Southern (1975). The efficiency of transfer was monitored by restaining the gel in TAE buffer containing 0.5 μg/ml EtBr for 30 minutes and looking for untransferred DNA. The membrane was rinsed in 2 x SSC, to remove any agarose, and partially air dried for 5 minutes. The DNA was then covalently immobilised by exposure of the membrane, face down and wrapped in Saran Wrap, for 5 minutes, to short wave uv light, generated from a Fotodyne 3-3002 transilluminator. The nylon membrane was then baked for 2 hours at 80°C and either stored at 4°C or prehybridised and hybridised immediately as described above for slot blots.

2.5.3 Northern Blotting and Hybridisation.

This procedure was much the same as Southern blotting, only RNA is transferred. The RNA samples are denatured by glyoxylation and a glyoxal gel run. RNA ladders (0.16-1.8 kb and 0.24-9.4 kb)are run alongside the gel as well as glyoxylated lambda-HindIII markers. The DNA gel markers were removed, stained in EtBr, measured and photographed, and the RNA gel tracks blotted immediately

by capillary action onto Hybond-N as described (Southern, 1975). After blotting overnight, the filter was uv irradiated and baked as above. The marker tracks were cut off and stained by soaking for 10 minutes in 1 M acetic acid, followed by soaking for a further 10 minutes in staining solution (0.4 M acetic acid, 0.4 M sodium acetate, 0.2% w/v methylene blue), followed by washing to remove excess dye in distilled water. The filter, after baking, was prehybridised and hybridised as described for slot blots.

2.6 IN SITU HYBRIDISATION.

2.6.1 Fixing of Cell Cultures.

Cells were grown on glass slides and fixed in 10% (phosphate) buffered formalin for 10 minutes. After removal from the formalin, they were washed in tap water for 10 minutes and rinsed twice in DEPC treated water.

2.6.2 Cutting and Fixing of Tissue Sections.

5 micron thick sections were cut from formalin fixed paraffin embedded blocks of tissue, mounted onto slides pretreated with 3-aminoalkylsilane and dried overnight at 37°C. Sections were dewaxed in inhibisol (Penetone Chemicals) and rehydrated through graded alcohols to DEPC treated water.

2.6.3 In Situ Hybridisation to Riboprobes.

The slides were soaked in 2 x SSC at room temperature for 20 minutes, and then at 70°C for 10 minutes before being rewashed twice in DEPC treated water. This was followed by incubation in 50 mM Tris-HCl, pH 7.6, at room temperature for 10 minutes. The slides were then wiped dry and placed in an immunostaining tray and covered with a proteinase K solution, 10 μg/ml for tissue and 1 μg/ml for cells. These were incubated at 37°C for 1 hour and then washed in 0.2% glycine, 1 x PBS (DEPC treated) for 1 minute. This was followed by a PBS wash and 2 further DEPC treated water washes. The slides were covered with 0.5 ml 50 mM Tris-HCl, pH 7.6, and incubated for 1 hour at 37°C. After incubation the samples were prehybridised in 0.5 ml prehybridisation buffer (50% formamide, 0.72M NaCl, 1 x PE [5 x PE: 0.25M Tris-HCl, pH 7.5, 0.5% w/v sodium pyrophosphate, 1% w/v polyvinyl-pyrrolidone, 1% w/v Ficoll, 25 mM EDTA], 150 μg/ml denatured salmon sperm and DEPC H_2O up to 6 ml) and incubated at 37°C for 2 hours. After 2 hours, the buffer was removed and the slides hybridised overnight at 37°C. Hybridisation was performed by pipetting 80 μl hybridisation solution, (50% formamide, 0.6M NaCl, 1 x PE, 10% dextran sulphate, 150 μg/ml denatured salmon sperm) containing a radiolabelled riboprobe, prepared as described in section 2.3.9, onto a coverslip and gently lowering the slide onto the inverted

coverslip. The following day the coverslips were discarded and the slides washed twice at room temperature in 2 x SSC and twice at 37°C in 2 x SSC and 50% formamide. The slides were dehydrated by consecutive immersion in 70% and 90% industrial methylated spirits followed by absolute alcohol and then allowed to air dry before autoradiography.

2.6.4 In Situ Autoradiography.

Slides were dipped in K5 nuclear emulsion (Ilford) and air dried overnight. They were then placed in a light proof box at 4°C for 14 days. After 14 days, the autoradiographs were developed in a darkroom as follows: the slides were soaked in distilled water for 5 minutes, developer for 2.5 minutes, 1M acetic acid for 4 minutes, and fixed in 1:10 fixer for 12 minutes. They were then washed for 5 minutes with several changes of distilled water and then in running tap water for at least 30 minutes. The slides were then left to air dry overnight.

2.6.5 Staining of Slides.

The slides were rehydrated in distilled water for 5 minutes and then immersed for 30 minutes in Cole's haematoxylin (diluted 1:4 in distilled water). Stain differentiation was produced by placing the slides in acidic water (2 ml HCl, 350 ml H_2O). Slides were then

washed under running water for 5 minutes and then immersed in 0.25% aqueous Eosin for 5 minutes. This was followed by a 10 seconds rinse in running water, and the slides were then left to air dry overnight. Once dry the slides were placed in Inhibisol and finally mounted in Ralmount.

2.7 GENERAL MOLECULAR BIOLOGY METHODS.

2.7.1 Ethanol Precipitation of Nucleic Acids.

DNA and RNA were precipitated from aqueous solution by the addition of 0.1 volume 3M sodium acetate (pH 4.8 with glacial acetic acid) and >2.5 volumes ice-cold absolute ethanol. Precipitation was completed by leaving to stand at -20°C for at least 4 hours. DNA was recovered by centrifugation for 10 minutes at 4°C, either in a micro-centrifuge (MSE Microcentaur, 13,000 rpm [≈ 13,400 x g_{av}]) or in the Sorvall RC5B (HB-4 rotor, 10,000 rpm [≈ 10,815 x g_{av}]). The supernatant was removed and the nucleic acid pellet washed in ice-cold 75% ethanol. The DNA or RNA was centrifuged, as before, and the supernatant again removed. The nucleic acid pellet was dried in a Speed Vac concentrator (Savant Instruments Inc., Hicksville, New York, U.S.A.). The dry DNA pellets were resuspended in an appropriate volume of TE (10 mM Tris.Cl pH 8.0; 1 mM EDTA pH 8.0) buffer (pH 8.0) and

stored at -20°C. Dry RNA pellets were resuspended in DEPC treated sterile water and stored at -70°C. Samples that required long term storage were stored under 75% ethanol and the RNA pelleted and resuspended as required.

2.7.2 Random Shearing of High Molecular Weight DNA.

Salmon sperm DNA was dissolved in sterile deionised water to a concentration of 10 mg/ml by gentle stirring for 16 hours at 4°C. Random shearing of DNA was obtained by sonication in an MSE Soniprep 150. Shearing was performed on ice, using 30 second pulses with at least 60 seconds between pulses. An aliquot of the sonicated DNA was electrophoresed on a 1% agarose gel alongside molecular weight markers. The size was an indication of the extent of breakdown achieved by sonication. The procedure was repeated until the DNA fragments were below 500 base pairs (bp) in size. The sheared DNA was aliquoted in 1 ml fractions and stored at -20°C. Before use, the sonicated salmon sperm DNA was thawed and denatured by heating in a boiling water bath for 10 minutes and then snap cooled in a dry ice/ethanol bath.

2.7.3 Restriction Endonuclease Digestion of DNA.

Restriction digests were performed in sterile 1.5 ml polypropylene Eppendorf tubes. The components were added in the following order: DNA (dissolved in TE, pH 8.0);

0.1 volume appropriate x10 restriction buffer (supplied with enzyme); DNase and RNase free bovine serum albumin (BSA) to a working concentration of 100 μg/ml; sterile water to appropriate volume; restriction enzyme (2-10 units/μg DNA). The final DNA concentration was kept, whenever possible, between 0.1 and 0.2 mg/ml. The total volume of restriction enzyme in the digest was kept below one tenth total volume to limit glycerol levels. Digests were performed at 37°C, except for BclI, which was incubated at 50°C as per manufacturers instructions.

2.7.4 Agarose Gel Electrophoresis.

All gel electrophoresis was carried out using horizontal submerged gel apparatus supplied by Bethesda Research Laboratories, Maryland, U.S.A. (H4, H5, and H6) or Cambridge Electrophoresis Limited, Cambridge, England (10 x 10 cm, 20 x 17 cm). 0.8%-1.2% agarose gels were prepared by dissolving the appropriate mass of solid agarose in Tris-acetate electrophoresis (TAE) buffer (40mM Tris-HCl, pH 8.0, 20 mM NaOAc, 2mM EDTA). Higher percentage gels, (1.5%) were made up in Tris-borate electrophoresis (TBE) buffer (89 mM Tris-HCl, pH 8.0, 89 mM sodium borate, 2 mM EDTA). The solid agarose was fully dissolved by boiling the solution in a microwave oven (Tricity 2003 T). The solution was then cooled and the gel poured and set at room temperature (4°C for low melting temperature gels). Once set, the tank was filled (with the same buffer as the agarose was dissolved in) to

a level at least 0.5 cm above the surface of the gel. Ethidium bromide (EtBr, 5 mg/ml) solution was added to the running buffer to a final concentration of 5 μg/ml prior to the loading of the samples. Each sample was mixed with 0.1 volume stop/loading buffer (20% w/v Ficoll, 0.1M EDTA pH 8.0, 1.0% sodium dodecyl sulphate [SDS], 0.25% w/v bromophenol blue, 0.25% w/v Xylene Cyanol), then loaded into the wells and electrophoresed in a field of 2-10 V/cm (40-107 mA), until the nucleic acid fragments had separated as required. Separation was monitored by exposing the gel to short wave ultra violet (uv) light from a transilluminator (Fotodyne 3-3002, Fotodyne, Wisconsin, U.S.A.) and compared to the distribution of standard molecular weight markers. Gels were photographed either on Ilford FP4 negative film using a Plan Polaroid MP-4 Land camera or on Polaroid-665 film using an IBI Quick Shooter Model QSP photosystem.

2.7.5 Glyoxal Gel Electrophoresis.

Glyoxal gels were used for the electrophoresis of RNA samples under denaturing conditions. RNA samples were denatured in 5 μl/μg glyoxal buffer (DMSO:Deionised glyoxal:0.125M sodium phosphate, pH 6.8; [25:6:4]) at 50°C for 1 hour. The samples were allowed to cool, and 0.1 volume loading buffer (50% glycerol, 10 mM phosphate buffer, pH 6.8, 0.05% bromophenol blue) was added. The samples were loaded onto a 1% agarose gel in 10 mM sodium

phosphate, pH 6.8, and run in a buffer containing 10 mM phosphate, pH 6.8. Once the samples had run into the gel the buffer was circulated. The gel was run for approximately 3 hours at 100 V.

If the RNA was to be visualised, the gel was first washed for 30 minutes in 50 mM NaOH, then 30 minutes in 0.1 M Tris-HCl, pH 7.5 and 0.1M NaCl and finally stained in a fresh Tris/NaCl mix containing 5 μg/ml EtBr. The RNA was then visible using uv transillumination.

2.7.6 Spin Column Chromatography.

Spin column chromatography was used to separate unincorporated nucleotides from radio-labelled DNA. The tip of a sterile 1 ml syringe was plugged with siliconised glass wool and the syringe filled with Sephadex G-50, preswollen in 5mM Tris.HCl pH 7.2, 5 mM NaCl and 0.1% SDS. The syringe was placed in a polypropylene 15 ml tube and centrifuged for 4 minutes (Sorvall Technospin R, 3000 rpm) to pack the gel matrix. A 1.5 ml Eppendorf was then placed in the bottom of the centrifuge tube, beneath the tip of the syringe. The samples were applied to the top of the column and recentrifuged for 4 minutes (Sorvall Technospin R, 3000 rpm). The Eppendorf tube was removed and an aliquot, usually 5 or 10 μl, was removed and made up to 100 μl with TE. This aliquot was counted, using the Cerenkov

method, in an LKB model 1214 Rackbeta liquid scintillation counter (Berger and Krug, 1985), and the specific activity of the labelled probe was calculated.

2.7.7 Autoradiography.

All autoradiography was performed at -70°C, using a pair of intensifying screens and pre-flashed X-ray film. Pre-flashing was obtained using a filtered electronic flash gun.

2.8 List of chemicals and equipment used and suppliers.

Apart from all those listed below all chemicals were Analar grade, supplied by British Drug Houses Limited (BDH), Poole, Dorset, England.

Agar	Difco Laboratories, Detroit, Michigan USA.
Agarose	Gibco-BRL., Trident House, Paisley, Scotland.
Ampicillin	Sigma Chemical Company Ltd., St.Louis, Missouri, USA.
Autophen developer	Ilford, Basildon, Essex, England.
Bijoux vials (7 ml)	Sterilin Ltd., Teddington, Gloucester, England.
Bio-freeze vials	Costar, supplied by NBL.
Bovine serum albumin	Sigma Chemical Company Ltd.
Caesium chloride	Pharmacia LKB Biotechnology, Uppsala, Sweeden.
Citric acid	Sigma Chemical Company Ltd.
D FCS	Gibco-BRL.
dATP	Pharmacia LKB Biotechnology.
dCTP	Pharmacia LKB Biotechnology.
Deoxyribonuclease 1	Boehringer Corp., Manheim, W.Germany.
Dextran sulphate	Pharmacia LKB Biotechnology.
dGTP	Pharmacia LKB Biotechnology.
DHS	Gibco-BRL.
Diethyl pyrocarbonate	Sigma Chemical Company Ltd.
Dimethyl sulphoxide	BDH Limited, Pole, Dorset, England.
Dithiothreitol	Sigma Chemical Company Ltd.
DMEM	Flow Laboratories, Rickmansworth, Herts., England.
DNA polymerase 1	Gibco-BRL.
dTTP	Pharmacia LKB Biotechnology.
Ethidium bromide	Sigma Chemical Company Ltd.
FCS	Gibco-BRL.
FP4 film	Ilford.
Guanidine thiocyanate	Sigma Chemical Company Ltd.
HEPES	Gibco-BRL.
Hybond-N	Amersham International plc.
Hydroxyurea	Sigma Chemical Company Ltd.
Hypam fixer	Ilford.
Hyperfilm-MP	Amersham International plc.
IPTG	Northumbria Biologicals Ltd., Northumberland, England.
Iso-amyl alcohol	Sigma Chemical Company Ltd.

L-glutamine	Northumbria Biologicals Limited.
Lamda DNA	Gibco-BRL.
LMP-agarose	Gibco-BRL.
MEM Alpha	Gibco-BRL.
Mercaptoethanol(2-)	Sigma Chemical Company Ltd.
Methotrexate	Lederle Laboratories Division, Gosport, Hampshire, England.
Methotrexate-3H	Amersham International plc., Amersham, England.
Nitrocellulose	Schleicher and Schuell GmbH. Dassel, W.Germany.
p[dN]6	Pharmacia LKB Biotechnology.
Penicillin	Glaxo, Greenford, England.
Petri dishes	Sterilin Ltd.
Phenisol	Ilford.
phiX174 DNA	Gibco-BRL.
Phosphorus-32	Amersham International plc.
Polaroid 665 film	International Biotechnologies Inc
Potassium acetate	Sigma Chemical Company Ltd.
Proteinase K	Boehringer Corp.
Restriction enzymes	Gibco-BRL.
RNase-inhibitor	Boehringer Corp.
RPMI	Flow Laboratories.
RQ1 DNase	Promega Corp.
Saline	Ivex Pharmaceuticals.
Salmon sperm DNA	Sigma Chemical Company Ltd
Sephadex G-50	Pharmacia LKB Biotechnology.
Sodium chloride	Sigma Chemical Company Ltd.
SP6 RNA polymerase	Promega Corp.
Streptomycin	Evans Medical Limited.
T7 RNA polymerase	Promega Corp.
Tetracycline	Sigma Chemical Company Ltd.
Tissue culture flasks	Costar.
Transfer RNA	Sigma Chemical Company Ltd
Tris(hydroxymethyl) aminoethane	Sigma Chemical Company Ltd.
Tris(hydroxymethyl) aminoethane hydrochloride	Sigma Chemical Company Ltd.
Trypan blue	Sigma Chemical Company Ltd.
Trypsin-EDTA	Gibco-BRL.
Tryptone	Difco Laboratories.
Universals (30ml)	Sterilin Ltd.
Whatman 3MM	Whatman Limited.
X-Gal	Northumbria Biologicals Ltd.
Yeast extract	Difco Laboratories.

CHAPTER 3. DERIVATION OF RESISTANT CELL LINES.

3.1 INTRODUCTION.

Methotrexate has a broad range of antitumour activity but its value is limited by the presence or development of resistant disease (Dorr and Fritz, 1980). Resistance to methotrexate is usually a result of transport alterations (Underhill and Flintoff, 1989) or amplification of dihydrofolate reductase (Schimke et al., 1985). The aim of this project was to produce methotrexate resistant cell lines and study their mechanisms of resistance.

Methotrexate is used as a single agent in the treatment of choriocarcinoma. Remission is obtained in nearly 99% of "low-risk" cases treated by methotrexate alone (Bagshawe et al., 1976, 1989). Resistance to methotrexate, however, is the main cause of treatment failure. No methotrexate resistant choriocarcinoma cell lines were available.

Colonic tumours are generally not treated with chemotherapy due to their inherent resistance to chemotherapeutic drugs. It was considered that colonic tumour cells would be a suitable model with which to induce resistance easily to individual drugs and that they would be a good model with which to study gene amplification (Bagshawe and Malcolm, personal communication).

Two regimes for the treatment of JAR (choriocarcinoma) and MAWI (colon carcinoma) cells were tested. Cells were treated in vitro either by intermittent or continuous exposure to methotrexate. Intermittent exposure was used as this more closely resembles clinical treatment than does continuous exposure. Continuous exposure to methotrexate with stepwise increments in methotrexate concentration was used to maximise the probability of obtaining methotrexate resistant sublines in vitro (Bostock et al., 1979; Schimke et al., 1985).

3.2 RESULTS.

3.2.1 IC50 determination.

Each cell count is the mean of six wells, each well counted in duplicate. From total cell counts, Tables 3.1 and 3.2, percentage viable cell number was calculated relative to that of controls. For each individual drug concentration used, cell viability (100 - % arrested growth or death) was plotted against time (Figures 3.1 and 3.2).

Cells numbers relative to controls at the various drug concentrations was calculated at 72 hours exposure time, Figure 3.3. From these data the respective IC_{50} values were estimated by interpolation as 7.5×10^{-9}M MTX for JAR and 50×10^{-9}M MTX for MAWI.

3.2.2 Intermittent exposure - JAR.

After cell cycle synchronisation by pulsing with hydroxyurea, JAR cells were exposed to a range of methotrexate concentrations for three days in an attempt to induce methotrexate resistance. The IC_{50} drug concentration killed 50% of cells after three days exposure, as calculated by viable cell counts. After being allowed to recover and grow, the cells were retreated at the same drug level with identical cell kill, based on cell counts. On further repetitions of this procedure the same percentage cell viability was observed, but the time for recovery increased from a mean of 5.6 days with 2 exposures to 12.6 days with 8 exposures. No methotrexate resistant JAR cells were obtained using intermittent exposure to IC_{50} concentrations of methotrexate (Table 3.3).

In order to increase the likelihood of producing resistant JAR cells the methotrexate concentration was increased. When cells were treated with 2 - 6.7 x IC_{50} methotrexate there were proportional increases in cell inhibition and the recovery period increased, ranging from 7.0 - 21.8 days (Table 3.3). With exposure to higher methotrexate concentrations, 13.3 and 66 x IC_{50}, remaining cell numbers were negligible and the cells eventually died either as a result of the methotrexate or of failure to survive at low cell density.

3.2.3 Continuous exposure - JAR.

JAR cells were exposed continuously to IC_{50} levels of methotrexate (7.5×10^{-9} M) with fresh drug and medium added at least twice a week. After 45 passages over approximately 4 months a sub-line, B^0, was derived. This line was considered to be resistant to 7.5×10^{-9} M MTX as it had the same growth rate in drug as the parental cell line had in the absence of methotrexate. Upon initial exposure to methotrexate the cells took on a fibroblastoid appearance, in contrast to the normal epithelioid morphology. The growth rate also slowed initially, but during continued exposure reverted as resistance was achieved. With continued exposure, the resistant cells resumed an epithelial-like appearance and growth rates approached those of the parental cell line.

The B^0 methotrexate resistant sub-line was then exposed continuously to a higher drug concentration, 5×10^{-8} M methotrexate. This procedure was repeated to give higher levels of resistance (see table 3.4).

Sublines B^0, B^1, and B^2 exhibited no morphological alterations upon exposure to their respective methotrexate concentrations. Sub-line B^3, which had not been exposed to the stepwise increment in MTX concentration for as many passages, still exhibited slight morphological changes, from epithelial to fibroblast-like, upon drug selection.

3.2.4 Intermittent exposure - MAWI.

MAWI cells were exposed to a range of methotrexate concentrations for three days. After three days the methotrexate was removed and the cells allowed to recover. This recovery period varied greatly in length, ranging from 4 days to 5 weeks, with no correlation between recovery time and total dose of methotrexate (Table 3.5).

Attempts to induce resistance to methotrexate in the MAWI cell line were unsuccessful. The cells remained adherent to the flask following exposure, but did not divide. This phenomenon occurred at all stages of treatment and was initially interpreted as resistance to the drug concentration to which the cells were being exposed. On further exposure to the same methotrexate levels or stepwise increments in methotrexate concentrations, however, after a drug free interval, the non-dividing cells eventually died. No correlation was noted between the onset of the block in cell division and either methotrexate concentration or the number of exposures to any given drug level.

Another problem with the MAWI cell line was density dependence, and a minimum cell number was required for viability. This was confirmed by plating cells in 75 cm^2 flasks at low concentrations, 1-5 x 10^2 cells/ml ($\approx$ 2-10 x 10^3 cells per flask), with no growth achieved from these levels.

3.2.5 Continuous exposure - MAWI.

When MAWI cells were exposed continuously to methotrexate the results were very similar to those obtained with intermittent exposure. When $\geq 10^4$ cells/ml ($\approx 2 \times 10^5$ cells/flask) were plated in IC_{50} methotrexate concentrations, the cells exhibited a morphological change and then stopped growing 4-14 days after the commencement of drug exposure. As a result, no methotrexate resistant MAWI cells were obtained using this procedure.

3.3 DISCUSSION.

Intermittent exposure to methotrexate did not induce drug resistance in either JAR or MAWI cells. It is possible that the intermittent exposure may have produced unstable temporary resistance, for instance with amplified DHFR genes present in the form of double minutes (Schimke, 1988). The recovery period between successive treatment schedules could have allowed these unstably resistant cells to revert due to loss of non-segregating DMs (Snapka and Varshavsky, 1983).

Continuous exposure of MAWI cells to methotrexate did not produce resistant cells. MAWI cells are derived from a colon cancer and, generally, colonic tumours are not treated with drugs due to their intrinsic resistance. MAWI is intrinsically 7-fold methotrexate resistant relative to JAR

cells. Methotrexate resistant cells have been produced from human colon carcinoma cells (Srimatkandada et al., 1989). The HTC-8 cells were grown in stepwise increases in methotrexate concentrations producing a 10,000-fold resistant cell line capable of growth in 1 x 10^{-4}M MTX. Although the resistant cell line HCT-8R4 was highly resistant, 10,000 fold, the DHFR protein, DNA and mRNA were only found to be increased 25-fold. No transport defect was detected.

Continuous exposure of JAR cells to an IC_{50} concentration of methotrexate (7.5 x 10^{-9} M) produced methotrexate resistant cells. Elevation of the drug concentration in a stepwise fashion resulted in further drug resistant sublines. This is similar to the findings of others who have produced methotrexate resistant cell lines by continuous exposure to stepwise increases in methotrexate concentrations (see Tables 3.6 and 3.7). Methotrexate resistant human leukemic cell lines were produced by stepwise selection with 100,000-fold resistance to methotrexate (Srimatkandada et al., 1983). The cells, K562, were grown in progressively increasing concentrations of methotrexate, starting at nanomolar concentrations and, with 2-fold increases in methotrexate, were eventually capable of continued growth in 1 x 10^{-4} M MTX. The resistant cells, K-562/R4, contained a 240-fold increase in DHFR activity with no increase in uptake or alteration in DHFR observed. Although not quantified, both DHFR gene copy number and mRNA levels were amplified. Most studies of methotrexate resistance have used

cell lines originating from mouse, hamster or rats. There are 8 published studies using human cell lines (Tables 3.6 and 3.7). These studies indicate that there are four main mechanisms controlling methotrexate resistance:

(1) Increase in DHFR levels.
(2) Alteration in DHFR leading to decreased MTX binding.
(3) Impaired transport of methotrexate.
(4) Reduced polyglutamylation of methotrexate.

Resistance in these published cell lines was produced by stepwise increments as were the methotrexate resistant JAR cells. The JAR cells took longer to induce resistance than other resistant cell lines reported. Although no other methotrexate resistant choriocarcinoma cell lines were available for comparison, another group produced a series of squamous carcinoma cells resistant to different methotrexate concentrations (Frei et al., 1984). The increments used for JAR cells were 6.7-, 2.0- and 5-fold methotrexate concentrations. The resistant squamous cell line, SSC-15, was produced using 1.2- to 2.0-fold increases. Although these figures seem comparable, the method of resistance induction varied. The JAR sublines were only exposed to an increase in drug concentration once resistance had been induced at that concentration, whereas the SSC-15 cells were exposed to an increment every 1 to 4 weeks regardless of the resistant state of the cells. Thus, the JAR sublines were produced by exposure to relatively large increases in drug concentration over a longer time period as opposed to

smaller steps in drug concentration over a shorter time period. The SSC-15 parental cell line was 4 times less sensitive than the parental JAR cell line and the cells were made resistant to higher levels of methotrexate. The only pair of resistant sublines which are comparable are the SSC-15 subline resistant to 7.5×10^{-7}M MTX and the B^2 subline, resistant to 5.0×10^{-7}M MTX, which were found to be 17- and 13.3- fold resistant to methotrexate respectively.

A similar method for induction of methotrexate resistance was also used with human lymphoblastoid cells, W1-L2, (Niethammer and Jackson, 1975). As with the resistant JAR sublines the cells were continually exposed to methotrexate. The drug concentration was not increased until the cells' growth rate had increased to that of the parental cells and no further dead cells were seen. The drug concentration was then doubled. This mirrors the definition of resistance used with the JAR cells before the methotrexate concentration was increased. Although there was only a 1.7-fold difference in sensitivity in the two parental cell lines (JAR and W1-L2), the resistant W1-L2 cells were resistant to much higher levels of methotrexate over a much shorter time period.

Both acute and T-lymphoblastoid leukemia cells lines possess similar inherent sensitivity to methotrexate as do the choriocarcinoma cell lines (Ohnoshi et al., 1982; Mini et al., 1985). The sensitive acute lymphoblastoid leukemic cells, MOLT-3, have a methotrexate IC_{50} of 1.0×10^{-8}M (Ohnoshi et al., 1982), and the sensitive T-lymphoblast

leukemia cells, CCRF-CEM, have an IC_{50} of 1.2 x 10^{-8}M (Mini et al., 1985). Both of these compare well with JAR cells which have an IC_{50} of 7.5 x 10^{-9}M MTX. In both studies cells were continuously maintained in methotrexate, with 1.5- to 2-fold increases in methotrexate once growth rates had increased to the normal level. This took 2-4 months for the MOLT-3 cells and 2-3 weeks for the CCRF-CEM cells. Consequently, the MOLT-3 cells took 10 months before they were resistant to 1 x 10^{-7}M MTX whereas the CCRF-CEM cells only took 4 months before they were resistant to 2.0 x 10^{-7}M MTX. These figures both represent a more rapid induction of resistance to methotrexate than JAR cells where subline B^2 took 12 months to be resistant to 1 x 10^{-7}M MTX.

An important factor which must be considered in the comparison of resistant cell lines and their production is how resistance is measured. Most studies quantify degrees of resistance by the increase in IC_{50} value of the resistant cell line compared to the sensitive parental cells but it is the method used to measure these values which differs. Clonogenic assays are frequently used (Koizumi et al., 1988; Frei et al., 1984). The most common method is growth inhibition analysis involving counting cell viability by a variety of methods including Coulter counters (Ohnoshi et al., 1982), haemocytometer counting (Jackson and Neithammer, 1977) and the MTT assay (Table 3.7).

Comparison of published methotrexate resistant human tumour cell lines indicates that the methods used to induce

resistance in choricarcinoma and the levels of resistance produced are similar, with the only obvious difference being the time taken to induce resistance. This may, however, merely reflect the inherent sensitivity of choriocarcinoma cells to methotrexate and help to explain why methotrexate is so successful as a single agent in the treatment of choriocarcinoma.

Tumour cell lines have been identified with more than one mechanism of methotrexate resistance (Srimatkandada et al., 1989).

In the next chapter I have characterised the resistant JAR cells and attempted to determine the mechanism of resistance.

Time (hours)	Cell Counts at Different Drug Concentrations (x 10^6)				
	Methotrexate concentration (M)				
	Control	$1x10^{-9}$	$5x10^{-9}$	$1x10^{-8}$	$5x10^{-8}$
24	120[a]±4[b]	116±4	112±7	106±6	102±7
48	241±8	230±15	204±13	173±10	151±12
72	531±37	415±26	259±21	120±23	75±26
96	814±43	524±49	234±30	70±7	27±10
120	1549±110	875±100	339±35	131±13	29±46

TABLE 3.1 **Cell Counts - JAR.**

Time (hours)	Cell Counts at Different Drug Concentrations (x 10^6)					
	Methotrexate concentration (M)					
	Control	$1x10^{-8}$	$5x10^{-8}$	$1x10^{-7}$	$5x10^{-7}$	$1x10^{-6}$
24	120[a]±3[b]	116±4	120±4	111±3	94±4	87±4
48	212±4	187±13	149±8	90±9	86±9	78±7
72	381±6	309±21	234±23	102±23	75±14	49±16
96	544±8	377±19	288±32	150±18	50±10	17±8
120	898±37	598±53	520±35	210±52	83±13	12±11

TABLE 3.2 **Cell Counts - MAWI.**

(a) Mean cell count [n=6].
(b) ± Standard deviation.

Cell counts after daily exposure to methotrexate with exposure time increasing from 1 - 5 days. Cells counted using a haemocytometer with trypan blue exclusion and each count performed in duplicate.

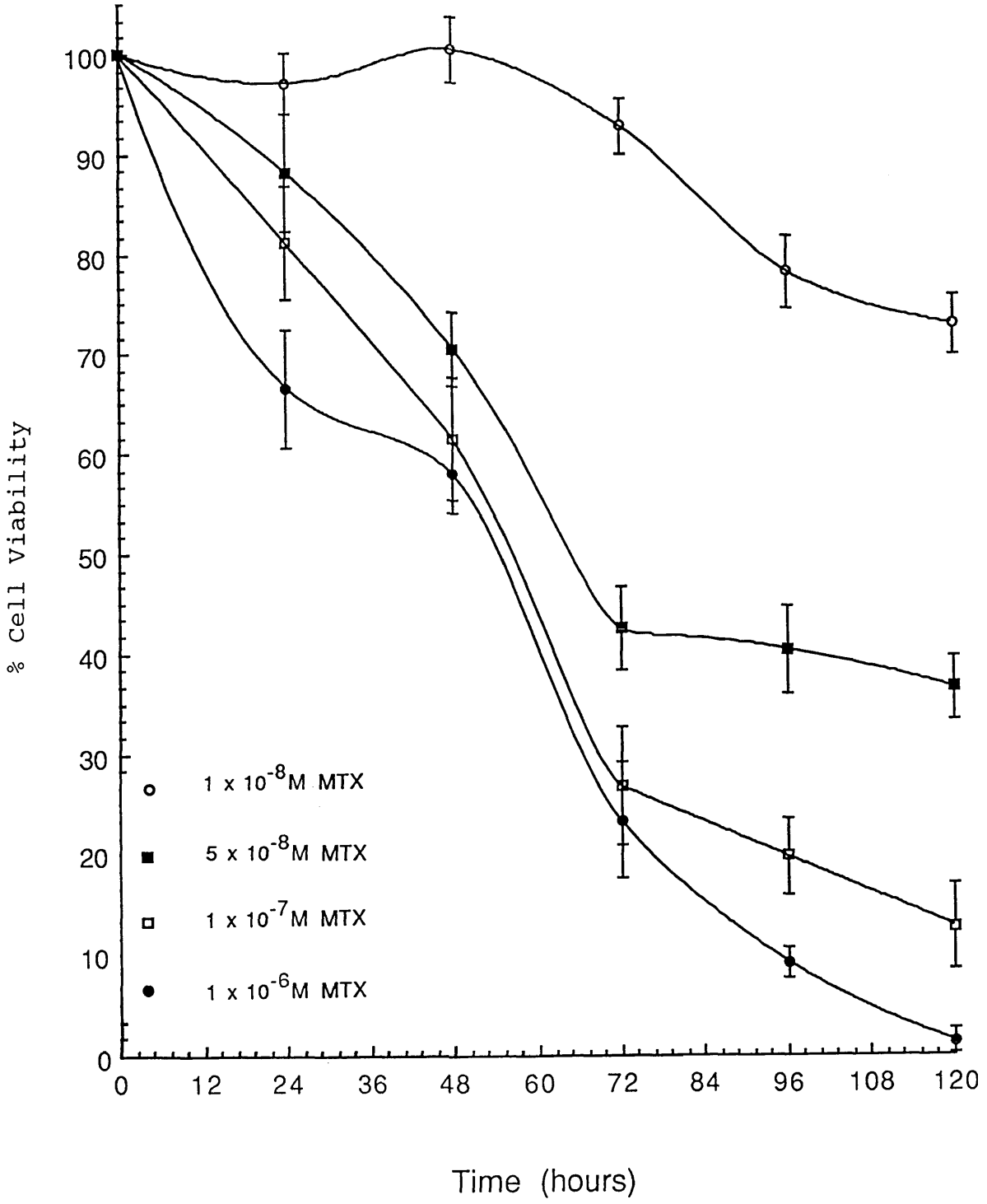


Figure 3.1 **Viability of MAWI Cells at a Range of Methotrexate Concentrations.**

Cells plated at 4 x 10^5 cells/ml in DMEM and treated with methotrexate the following day. Counts, using trypan blue exclusion, represent the average of triplicate wells, each counted in duplicate.

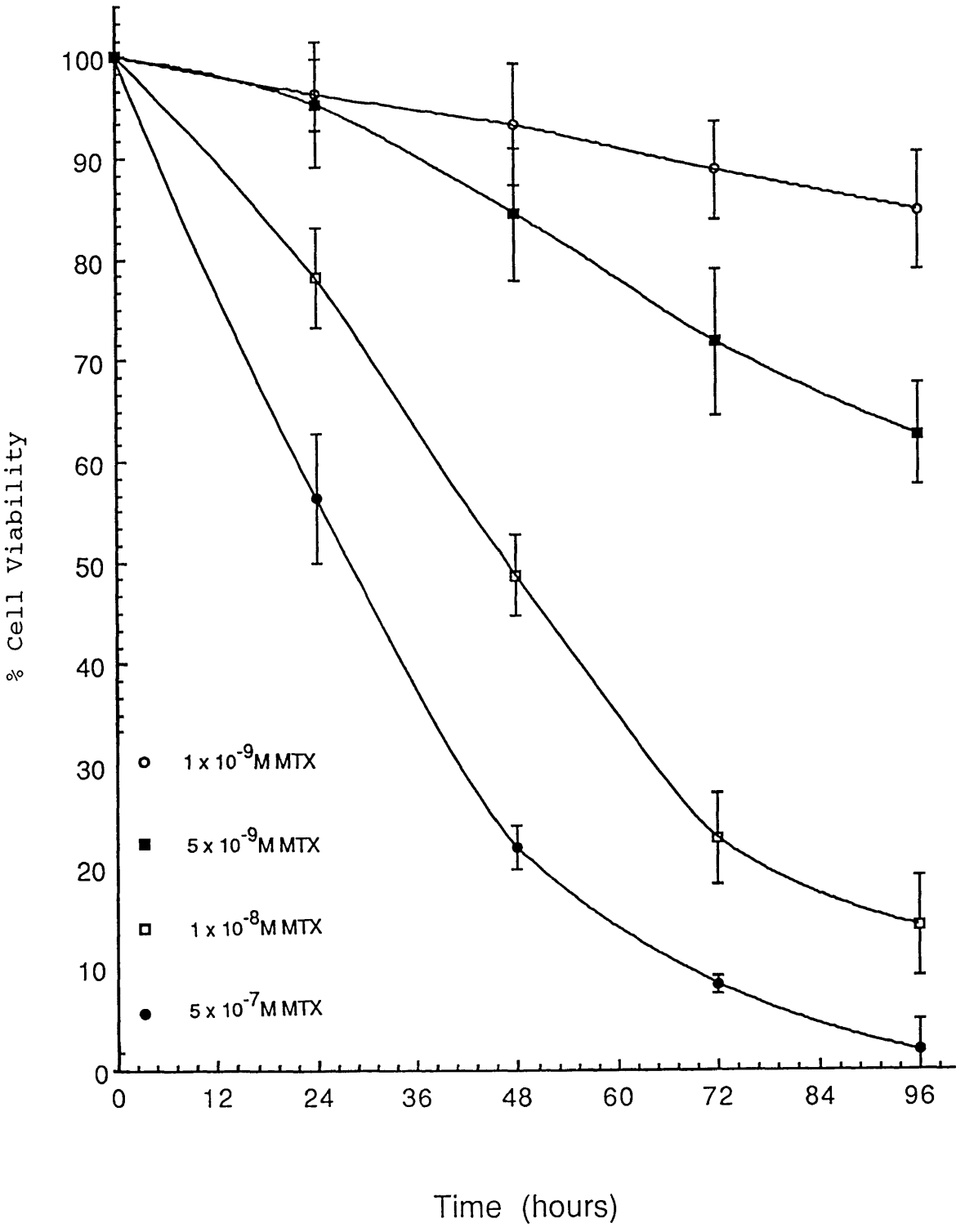


Figure 3.2 **Viability of JAR Cells at a Range of Methotrexate Concentrations.**

Cells plated at 4 x 10^5 cells/ml in DMEM and treated with methotrexate the following day. Counts, using trypan blue exclusion, represent the average of triplicate wells, each counted in duplicate.

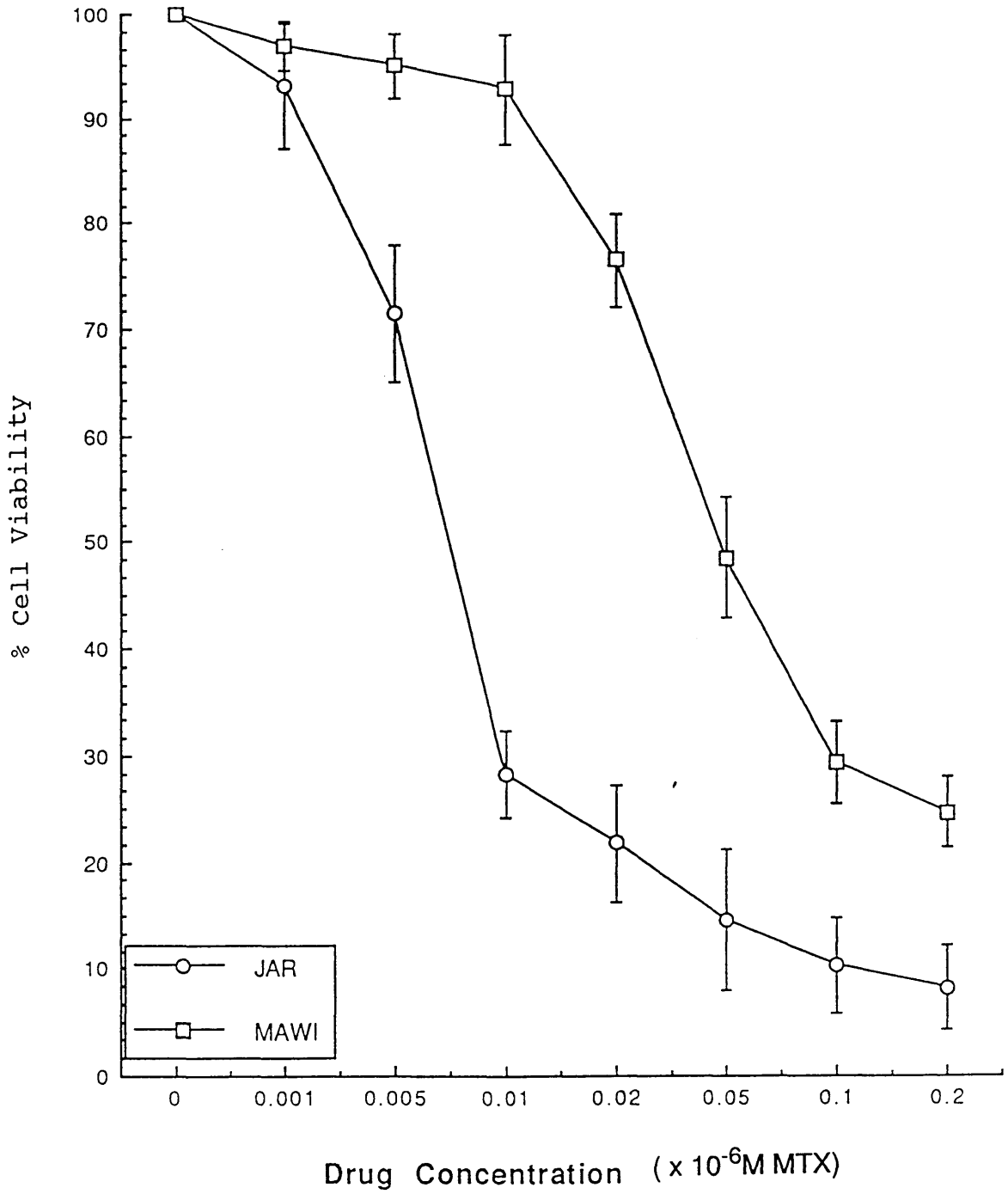
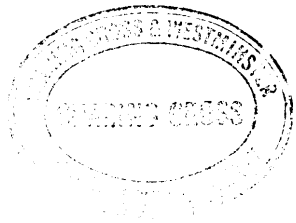


Figure 3.3 **Viabilityof MAWI and JAR Cells After 72 Hours Incubation in a Range of Methotrexate Concentrations.**

Cells plated at 4 x 10^5 cells/ml in DMEM and treated with methotrexate the following day. Surviving cells counted, using trypan blue exclusion, after 72 hours using triplicate wells, each counted in duplicate. Graphs used to estimate IC_{50} values for the cell lines. Error bars represent one standard deviation from the mean.

MTX (M)	xIC$_{50}$	Number of treatments	Number of expts	Recovery Time (days)	Max/Min (days)
$7.5x10^{-9}$	1.0	2	10	5.60 ± 1.2	4 / 8
	1.0	3	12	6.60 ± 2.0	4 / 11
	1.0	4	5	7.20 ± 1.2	6 / 8
	1.0	5	7	9.28 ± 1.3	8 / 12
	1.0	6	4	9.25 ± 1.5	7 / 11
	1.0	7	6	10.60 ± 3.3	7 / 15
	1.0	8	8	12.63 ± 3.9	8 / 18
$1.5x10^{-8}$	2.0	2	7	7.57 ± 1.8	6 / 11
	2.0	3	8	7.00 ± 2.3	7 / 14
	2.0	4	6	11.67 ± 4.1	7 / 18
	2.0	5	6	13.17 ± 3.3	9 / 15
	2.0	6	8	14.38 ± 2.5	11 / 18
	2.0	7	4	16.50 ± 3.4	13 / 22
	2.0	8	4	16.75 ± 1.1	15 / 18
$2.5x10^{-8}$	3.3	2	8	10.38 ± 1.8	8 / 14
	3.3	3	6	13.17 ± 2.4	10 / 17
	3.3	4	4	14.25 ± 1.3	13 / 16
	3.3	5	5	17.00 ± 2.8	12 / 20
	3.3	6	6	14.67 ± 1.0	13 / 16
	3.3	7	3	18.67 ± 1.3	17 / 20
$5.0x10^{-8}$	6.7	2	7	15.14 ± 2.7	11 / 19
	6.7	3	5	16.50 ± 2.0	13 / 19
	6.7	4	6	18.50 ± 2.3	16 / 22
	6.7	6	4	21.75 ± 2.4	19 / 25
$1.0x10^{-7}$	13.3	2	8	40.38 ± 10.1	27 / 56
	13.3	3	5	52.20 ± 8.4	39 / 64
	13.3	4	5	73.80 ± 8.4	65 / 89
$5.0x10^{-7}$	66.7	2	7	63.57 ± 16.4	41 / 87
	66.7	3	6	70.50 ± 14.2	53 / 91
	66.7	4	3	86.00 ± 11.6	71 / 99

TABLE 3.3 JAR cells - Intermittent Exposure to Methotrexate.



Subline	MTX conc'n (M)	Passages	Time (days)	x IC_{50}
B^0	7.5×10^{-9}	45	120	--
B^1	5.0×10^{-8}	+37	110	6.70
B^2	1.0×10^{-7}	+42	140	13.3
B^3	5.0×10^{-7}	+26	140	67.0

TABLE 3.4 **Methotrexate Resistant JAR - sublines.**

Methotrexate resistant JAR sublines produced by continuous exposure to varying drug concentrations. The increases in methotrexate concentration were achieved in a stepwise fashion commencing with an IC_{50} drug concentration and the resistant lines were produced sequentially.

MTX/M	xIC_{50}	Number of treatments	Number of expts	Recovery Time / days	Max/Min /days
$4.5x10^{-8}$	1.0	3	4	26.25 ± 6.9	1 / 36
	1.0	4	6	33.75 ± 14.2	18 / 56
	1.0	5	5	48.00 ± 14.7	32 / 75
	1.0	6	9	78.11 ± 37.0	42 / 167
	1.0	7	7	92.57 ± 35.3	49 / 154
	1.0	8	6	102.00 ± 16.9	57 / 118
	1.0	9	8	136.00 ± 43.7	73 / 202
	1.0	10	3	190.00 ± 30.7	157 / 231
	1.0	12	5	182.80 ± 87.6	86 / 302
$5.0x10^{-8}$	1.1	4	6	47.50 ± 13.7	26 / 64
	1.1	5	4	65.50 ± 31.1	27 / 107
	1.1	6	9	79.22 ± 31.5	40 / 138
	1.1	7	7	79.43 ± 26.6	36 / 127
	1.1	8	3	98.00 ± 43.6	51 / 156
	1.1	9	5	95.80 ± 14.1	78 / 118
	1.1	11	4	157.25 ± 69.7	75 / 267
	1.1	12	3	136.67 ± 42.2	83 / 186
$1.0x10^{-7}$	2.2	3	6	50.83 ± 17.5	28 / 79
	2.2	4	3	44.00 ± 16.9	22 / 63
	2.2	5	4	65.50 ± 24.2	27 / 92
	2.2	7	2	73.00 ± 31.0	42 / 104
$5.0x10^{-7}$	11.1	2	5	31.60 ± 12.7	12 / 51
	11.1	3	6	44.17 ± 17.1	21 / 71
	11.1	4	3	58.33 ± 19.9	40 / 86
$1.0x10^{-6}$	22.2	1	6	39.67 ± 11.3	27 / 53
	22.2	2	2	51.30 ± 22.7	26 / 81

TABLE 3.5 MAWI cells -Intermittent Exposure to Methotrexate.

Cell line	Cell type	Initial MTX Conc'n	Final MTX Conc'n	Time /months	Reference
K562	Chronic myelogenous leukemia	3.0 x 10-9M	5.0 x 10-6M	6	Koizumi, 1988
K562	Chronic myelogenous leukemia	N/A	1.0 x 10-4M	N/A	Srimatkandada et al., 1983
W1-L2	Lymphoblastoid	2.0 x 10-8M	1.6 x 10-6M 3.2 x 10-6M	4 4	Jackson and Niethammer, 1977
MOLT-3	Acute lymphoblastoid leukemia	5.0 x 10-9M	1.0 x 10-7M	10	Ohnoshi et al., 1982
CCRF-CEM	T-lymphoblast leukemia	5.0 x 10-9M	2.0 x 10-7M	4	Mini et al., 1985
CCRF-CEM	T-lymphoblast leukemia	N/A	1.0 x 10-6M	7	Jansen et al., 1989
SCC15	Squamous cell carcinoma of head and neck	5.0 x 10-8M	7.5 x 10-7M 4.5 x 10-6M 2.2 x 10-5M 2.7 x 10-4M	4 6 10 13	Frei et al., 1984
HCT-8	Colon carcinoma	N/A	1.0 x 10-4M	N/A	Srimatkandada et al., 1989
JAR	Choriocarcinoma	7.5 x 10-9M	7.5 x 10-9M 5.0 x 10-8M 1.0 x 10-7M 5.0 x 10-7M	4 8 12 17	Present work

TABLE 3.6 **Methotrexate resistant human tumour cell lines** - levels of selection and time taken to induce drug resistance.

Cell line	Initial IC_{50}	Final IC_{50}	Stability /months	Degree of resistance	Method of measurement	Reference
K562	4.3×10^{-9}M	2.2×10^{-5}M 1.4×10^{-5}M 2.0×10^{-5}M 1.3×10^{-5}M 1.2×10^{-5}M	>1 >1 >1 >1 >1	5,100 3,300 5,000 3,000 2,800	Clonogenic assay	Koizumi, 1988
K562	1.0×10^{-8}M	1.0×10^{-3}M	N/A	100,000	Survival in methotrexate	Srimatkandada et al., 1983
W1-L2	1.3×10^{-8}M	2.2×10^{-6}M 6.8×10^{-6}M	N/A N/A	170 523	Cell counts, haemocytometer	Jackson and Niethammer, 1977
MOLT-3	1.0×10^{-8}M	3.0×10^{-7}M	>6	30	Cell counts, coulter counter	Ohnoshi et al., 1982
CCRF-CEM	1.2×10^{-8}M	9.0×10^{-7}M	>4	75	Cell counts	Mini et al.,1985
CCRF-CEM	2.9×10^{-8}M	3.4×10^{-6}M	>7	120	Growth inhibition	Jansen et al., 1989
SSC-15	3.0×10^{-8}M	5.0×10^{-7}M 3.6×10^{-6}M 2.2×10^{-5}M 2.7×10^{-4}M	>1 N/A N/A >4	17 120 730 9000	Clonogenic assay	Frei et al.,1984
HCT-8	N/A	N/A	N/A	10,000	Survival in methotrexate	Srimatkandada et al., 1989
JAR	7.5×10^{-9}M	1.2×10^{-8}M 3.0×10^{-8}M 4.3×10^{-8}M $>1. \times 10^{-7}$M	N/A N/A N/A N/A	1.6 4.0 5.7 >13.3	Growth inhibition, MTT assay	Present work

TABLE 3.7 **Methotrexate resistant human tumour cell lines** - Stability of resistant lines, degree of resistance and how resistance was measured.

CHAPTER 4. CHARACTERISATION OF METHOTREXATE RESISTANT JAR SUBLINES.

4.1 INTRODUCTION.

Having produced methotrexate resistant JAR sublines (see chapter 3) it was then necessary to characterise the cells.

Growth rates can influence the drug sensitivity of cell lines (Flintoff et al., 1976). Resistant cells may possess a slower growth rate than the parental cell lines (Mini et al., 1985). A cell population with a higher growth rate will contain a higher proportion of cells entering S-phase over a given time period. Conversely, a population with a slower growth rate will contain a lower proportion of cells going through S-phase during the same time period. Methotrexate kills cells during S-phase, but the inhibition of RNA and protein synthesisis caused by the drug slows the entry of cells into S-phase, making methotrexate a self-limiting S-phase specific drug (Pratt and Ruddon, 1979). Consequently, the action of methotrexate is growth rate specific. The growth rates of the parent and resistant sublines were similar.

Cross-contamination of cell lines in culture is a common problem (Nelson-Rees et al., 1981; Dickson, 1981). DNA fingerprinting (Jeffreys et al., 1985), or the use of locus specific hypervariable probes, can provide evidence that cells share a common parentage (van Helden et al., 1988,

Masters et al., 1988). The use of a locus-specific hypervariable probe was required to indicate that the methotrexate resistant sublines were derived from the JAR cell line.

Reduced drug uptake is the most common transport defect found to cause methotrexate resistance (Bertino et al., 1985; Ohnoshi et al., 1982). Transport deficiencies, however, may also involve defects in intracellular release of the drug (Neithammer and Jackson, 1975), cell membrane binding (Flintoff and Nagainis, 1983), translocation across the membrane (McCormick et al., 1981) and efflux from the cell (Dembo and Sirotnak, 1984).

4.2 RESULTS.

4.2.1 Methotrexate Sensitivity.

The results are shown in Table 4.1. From the data, IC_{50} values were interpolated for each resistant subline (Table 4.2). The resistant JAR sublines, B^0, B^1, B^2 and B^3 had elevated IC_{50} values of 3.0, 4.3, 8.2 and $\geq 10.0 \times 10^{-8}$ M methotrexate respectively, compared with 1.2×10^{-8} M for the parental cell line.

4.2.2 Growth Rates.

Growth rates for the parental cell line, JAR, and the four methotrexate resistant sublines, B^0 - B^3, were calculated using the MTT assay. As shown in Table 4.3 and Figure 4.1 all cell lines had similar growth rates.

4.2.3 Locus Specific Hybridisation in Methotrexate Resistant JAR Sublines.

Analysis of DNA extracted from the parental JAR line and the methotrexate resistant sublines indicated no difference when hybridised to a locus specific probe. This confirms that the resistant JAR sublines were derived from the parental JAR cell line (Figure 4.2).

4.2.4 ^{3}H-Methotrexate Uptake in Resistant JAR Cells.

Methotrexate uptake was measured in the resistant series of JAR cells, B^0 - B^3.

Methotrexate entered the cells at similar rates and there was little difference in total uptake (Table 4.4).

4.3 DISCUSSION.

The resistant sublines had growth rates similar to that of the parental cell line. This similarity in growth rates was only seen once resistance was achieved. During the development of resistance the cells grew at a slower rate, see Chapter 3. Upon each exposure to a higher drug concentration the population doubling time slowed as a higher percentage cell death occured. Slower growth rates may be a result of a longer cell cycle. It is quite common for resistant cell lines to possess growth rates slightly slower than that of the parental cells (Mini et al., 1985). Analysis of growth rates and population doubling times must be considered in conjunction with cell cycles. The methotrexate resistant sublines studied here were sufficiently similar to warrant no further analysis. Had significant differences been seen, then the proportion of cells from the population in G_0 and S-phase would have been analysed. Methotrexate is most effective against populations with a high proportion of cells entering S-phase and least effective against populations with a high percentage of cells in G_0 phase (Johns and Bertino, 1982). When studying population doubling times, cells were exposed to methotrexate for 7 days to minimise any cell cycle effects. That is, exposure to the drug was long enough to allow all cells originally to pass through S-phase at least once during the experiment. If this is not achieved, some cells will not be killed, but will not be "resistant".

Measurement of radiolabelled methotrexate uptake indicated no difference between the sensitive and resistant cells. Long time periods, 120 hours, were used to avoid differences in short term uptake. For example, initial uptake, under 15 minutes, in resistant cells is occasionally higher in drug resistant cells but consistent with parental cells over a longer time period (Mini et al., 1985; Frei et al., 1984).

Although no differences in tritiated methotrexate uptake in resistant JAR cells was noted, the destination of the drug is unknown (Flintoff, 1986; Dembo and Sirotnak, 1984). How the drug is being released intracellularly and the rate of drug efflux from the cell were not measured. Consequently cells with no detectable transport defect, as detected by drug uptake studies, may be resistant by other less easily detectable transport deficiencies.

Exclusion of defective uptake as the mechanism of methotrexate resistance in the JAR sublines does not remove the possibility of resistance by defective polyglutamation of the drug (Cowan and Jolivet, 1983). Normally, once inside a cell, methotrexate is rapidly polyglutamated (Whitehead, 1977). Polyglutamate levels are measured using sephadex chromatography to analyse radiolabelled polyglutamate formation from methotrexate. Customarily, the methotrexate derivatives accumulate intracellularly and, in the absence of extracellular drug they are preferentially retained causing delayed cytotoxicity (Jolivet et al., 1982).

Analysis of methotrexate polyglutamation was not performed on the resistant JAR cells.

Locus specific probes are used to identify cross-contamination of cell lines (Masters et al., 1988). The technique identifies regions of tandemly repeated DNA sequences which are unique to each individual (Jeffreys et al., 1985). Cross contamination can lead to genetically identical cell lines being considered as separate populations or vice versa (van Helden et al., 1988). Locus specific hybridisation analysis of the methotrexate resistant JAR sublines confirmed that these lines are derived from the JAR parental cell line, validating the conclusions drawn from studies on the resistant cells.

	Cell Line - % Cell Viability				
MTX (M)	JAR	B^0	B^1	B^2	B^3
0	100±3.9	100±7.3	100±5.8	100±5.4	100±3.2
$1x10^{-9}$	75.3±4.2	76.5±2.9	93.3±6.9	92.3±3.3	85.7±7.3
$5x10^{-9}$	65.7±5.0	65.9±6.3	86.7±7.1	88.5±9.2	78.6±5.2
$1x10^{-8}$	55.2±4.5	52.9±4.8	66.7±5.8	84.6±3.8	75.0±6.1
$5x10^{-8}$	5.14±3.8	42.1±5.1	46.7±5.5	82.6±4.6	71.4±3.7
$1x10^{-7}$	4.30±2.5	29.4±3.7	33.3±2.0	30.8±2.5	70.0±5.2
$5x10^{-7}$	3.80±4.1	19.9±3.9	29.1±5.7	27.3±3.6	68.7±6.1
$1x10^{-6}$	3.10±4.0	11.8±3.6	26.7±6.5	21.5±2.3	67.8±4.4

Table 4.1 **Cell Viability of Methotrexate Resistant JAR Sublines Exposed to a Range of Drug Concentrations.**

Cell viability determined using the MTT assay. Each value represents the mean of 7 wells counted for each sample with the standard deviation of that mean. Results are representative of repeated experiments. Initial cell concentration was 1 x 10^4 cells/ml and absorbance was read after 7 days incubation at 540 nanometers.

Cell Line	MTX (M)	IC_{50} (M MTX)	Relative resistance
JAR	0	$\approx 1.2 \times 10^{-8}$	1.0
B^0	7.5×10^{-9}	$\approx 3.0 \times 10^{-8}$	2.5
B^1	5.0×10^{-8}	$\approx 4.3 \times 10^{-8}$	3.6
B^2	1.0×10^{-7}	$\approx 8.2 \times 10^{-8}$	6.8
B^3	5.0×10^{-7}	$\geq 1.0 \times 10^{-7}$	$\geq$ 8.3

Table 4.2 **IC_{50} Values for Methotrexate Resistant JAR Sublines.**

Results determined using the MTT assay. Cells were plated at 1×10^4 cells/ml and incubated under standard conditions at various drug concentrations for seven days. The IC_{50} values were obtained by extrapolation from the graph (Figure 4.1).

Cell Line	MTX (M)	Absorbance (540nm)			
		24 hours	48 hours	72 hours	96 hours
JAR	0	0.27±0.03	0.58±0.08	0.81±0.12	1.0±0.20
B^0	7.5×10^{-9}	0.21±0.06	0.39±0.09	0.67±0.06	0.89±0.16
B^1	5.0×10^{-8}	0.19±0.10	0.34±0.07	0.67±0.14	0.93±0.15
B^2	1.0×10^{-7}	0.30±0.09	0.37±0.13	0.68±0.06	0.96±0.10
B^3	5.0×10^{-7}	0.18±0.06	0.32±0.16	0.60±0.12	0.78±0.11

TABLE 4.3 **Growth rates of methotrexate resistant JAR sublines.**

Growth rates of methotrexate resistant cell lines measured using the MTT assay. Each value represents the average of 10 wells, with absorbance values normalised by comparison to growth of parental JAR cells after 96 hours.

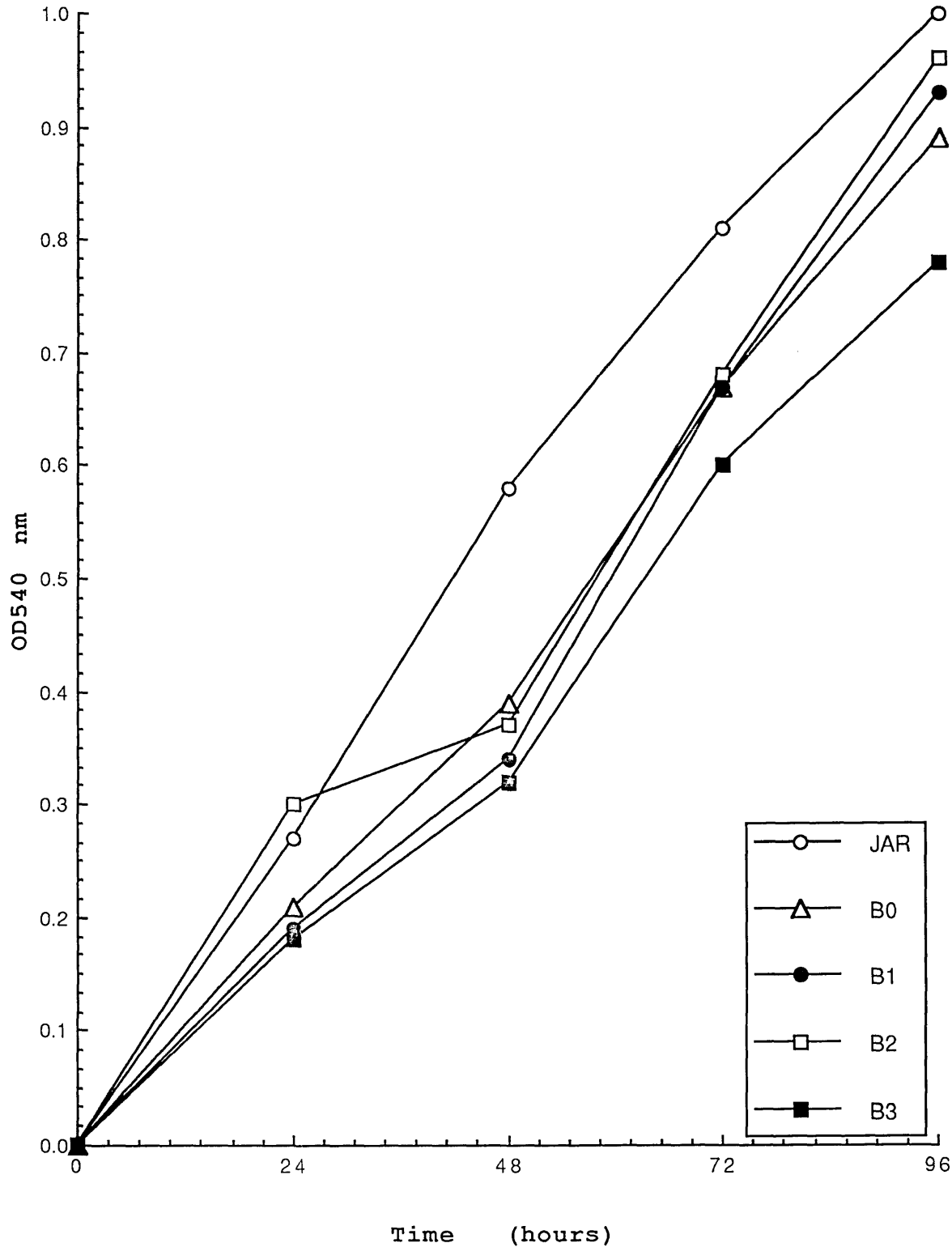
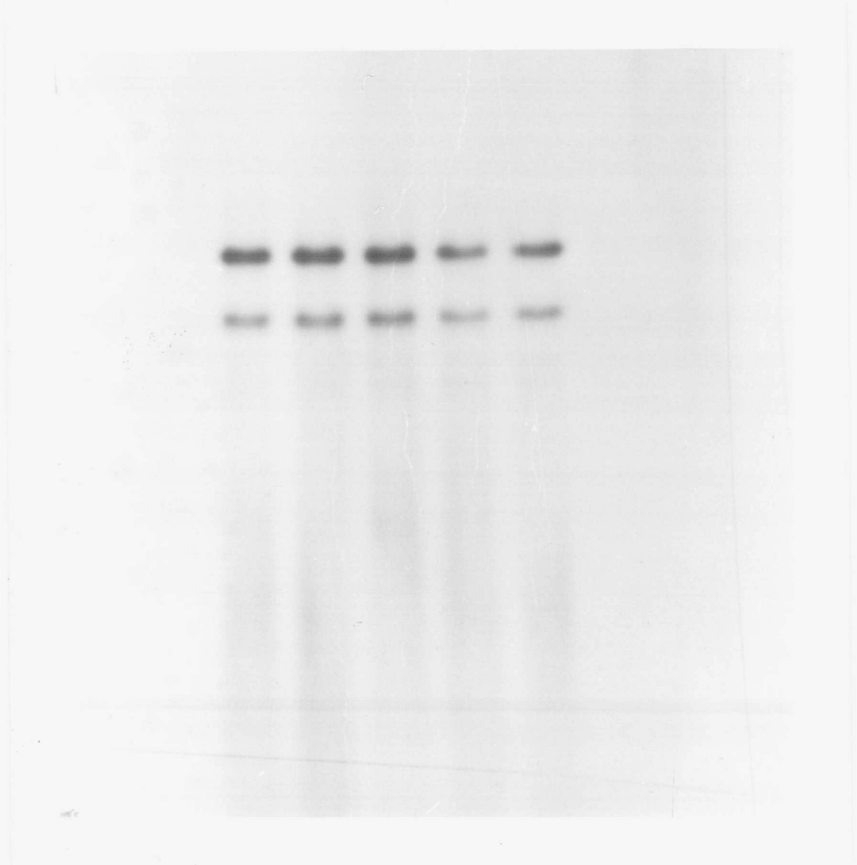


Figure 4.1 **Growth Rates of Parental and Methotrexate Resistant JAR Cell Lines.**

Growth rates calculated using the MTT assay. Cells plated at 1 x 10^4 cells/ml in 96-well plates and incubated for 96 hours. Absorbance quantified using a microplate spectrophotometer at 540 nm. Values represent average of 10 wells. Error bars removed for clarity (see Table 4.3).

1 2 3 4 5



Lane 1: JAR
Lane 2: B^0
Lane 3: B^1
Lane 4: B^2
Lane 5: B^3

Figure 4.2 **Locus Specific Hybridisation Analysis of Parental JAR and Resistant JAR sublines.**

DNA extracted from sensitive and resistant JAR cells. 5μg digested with HinfI, electrophoresed on 0.8% agarose gel and blotted onto Genescreen as per manufacturers instructions and hybridised to a random oligonucleotide labelled locus specific probe, p(lambda)g3, and hybridised overnight at 65°C. Filter washed in 0.1xSSC/0.1%SDS at 65°C and autoradiographed overnight.

	Cell Line - ^{3}H-MTX Uptake (dpm)				
Time (mins)	JAR	B^0	B^1	B^2	B^3
0	0	0	0	0	0
15	598 ± 35	375 ± 31	438 ± 37	579 ± 26	426 ± 56
20	519 ± 38	404 ± 50	318 ± 35	375 ± 31	554 ± 61
30	429 ± 34	318 ± 19	454 ± 27	758 ± 82	478 ± 48
60	506 ± 66	375 ± 38	716 ± 87	793 ± 93	532 ± 35
120	559 ± 57	554 ± 34	699 ± 93	617 ± 84	432 ± 49

Table 4.4 **Uptake of Methotrexate in Drug Resistant JAR Sublines.**

Uptake determined using tritiated methotrexate and exponentially growing cells shaking at 37°C. Aliquots removed at time intervals and centrifuged through an oil mixture (4 parts silicon oil to 1 part mineral oil). Resultant pellets were dissolved in protosol and counted in duplicate on a liquid scintillation counter. Cells pulsed before uptake analysis with 5-[^{125}I]Iodo-2'-deoxyuridine (^{125}I-dU) for 4 hours to standardise for cell number. Tritiated methotrexate used at 16.7 Ci/mM at a concentration of 5.0×10^{-6} M. ^{125}I-dU used at 0.1 mCi/ml.

CHAPTER 5. AMPLIFICATION IN RESISTANT CELLS.

5.1 INTRODUCTION.

Gene amplification is often associated with experimentally generated resistance in mammalian cells (Schimke, 1980, 1986, 1988). For example, squamous cell carcinoma lines resistant to methotrexate have been isolated with increased DHFR content and amplified gene copy numbers (Frei et al., 1984). Similarly, the numbers of amplified DHFR genes in methotrexate resistant murine sarcoma 180 cells were proportional to the increased levels of dihydrofolate reductase protein and mRNA levels (Alt et al., 1978). Amplification of the dihydrofolate reductase gene has been seen in methotrexate resistant small-cell lung carcinoma cells in the form of double minutes (Curt et al., 1983), and as HSRs in methotrexate resistant CHO cells (Nunberg et al., 1978).

Having treated MAWI and JAR cell lines with a range of doses of methotrexate, as shown in chapter 3, and characterised the resistant cell lines produced in chapter 4, the cells were then analysed for dihydrofolate reductase DNA, mRNA and enzyme activity levels.

5.2 RESULTS.

5.2.1 DHFR Gene Amplification.

The leukemic cell line, L1210, and its methotrexate-resistant sub-line, L1210 R7A, were used as baseline and positive controls for amplification of the DHFR genes. L1210 R7A is resistant to 1.0×10^{-6} M methotrexate and slot blot analysis indicated that this was associated with a 44-fold amplification of the DHFR genes compared to the sensitive parental cell line.

5.2.1.1 MAWI - Intermittent Exposure.

Although MAWI cells treated by intermittent exposure were not resistant (section 3.2.4), slot blot analysis was performed to measure any variation in DHFR gene copy number.

Slot blot analysis indicated a range of relative DHFR gene copy number (relative to the sensitive parental cells) from approximately 0.5 - 2.0, Figure 5.1. Copy number was calculated individually for each DNA extraction, and then gene copy numbers were grouped according to the highest level of methotrexate to which the cells had been exposed. There was no significant difference in gene copy number between exposure levels, Table 5.1.

5.2.1.2 MAWI - Continuous Exposure.

No resistant cells were produced using this procedure and at no stage were cell numbers high enough to allow the extraction of DNA or RNA from these cells for slot blot quantitative analysis.

5.2.1.3 JAR - Intermittent Exposure.

Slot blot analysis of treated JAR cell DNA indicated a broader range of gene copy numbers than MAWI (see Figure 5.2). The gene copy number is increased following exposure to threshold concentrations greater than 1.0×10^{-7} M methotrexate. Above this value the gene copy number is more variable and the mean increased. Little variation is seen in untreated cells. The mean copy number of DHFR genes is proportional to the level of drug to which the cells have been exposed, as shown in Table 5.2, but these do not differ significantly due to the high variance, as tested using regression analysis.

5.2.1.4 JAR - Continuous Exposure.

DNA was extracted from the methotrexate resistant JAR cells which were produced as described in section 3.2.3. Slot blot analysis, as shown in Figure 5.3, indicated 1.12-, 1.68-, 1.82- and 2.68-fold amplification of the DHFR genes in lines

B^0, B^1, B^2 and B^3 respectively when hybridised with the DHFR probe, Table 5.3.

Hybridisation to the dihydrofolate reductase probe, as shown in Figure 5.4, produced the following relative mRNA copy numbers for lines B^0, B^1, B^2 and B^3; 1.10-, 1.41-, 1.77- and 2.18-fold elevation respectively, Table 5.3.

Analysis of enzyme activity in the methotrexate resistant cell lines mirrored the amplification levels and elevation levels indicated in the DNA and RNA from these cells. The enzyme activity levels were elevated 1.16-, 1.50-, and 2.46-fold for sublines B^0, B^1 and B^2 respectively, Table 5.3.

Statistical analysis of the correlation between these three variables, DNA, RNA and enzyme activity, in the resistant sublines was performed by calculating correlation coefficients. Significance was tested using the exact t-test for the existence of correlation in small samples (Bailey, 1959). The correlation between DNA and RNA is 0.845, which is significant at the 5% level. The correlation between DNA and enzyme activity is 0.877, which is significant at the 10% level. The correlation between RNA and enzyme activity is 0.979 which carries a significance of 2.5%, Table 5.3a.

5.2.2 P-glycoprotein Gene Amplification.

A multidrug resistant Chinese hamster ovary cell line, CH^RC5, and its sensitive parental cell line, AUXB1, were used as positive controls. CH^RC5 is a highly resistant cell line selected by growth in colchicine and is cross resistant to a variety of drugs including vinblastine, erythromycin and adriamycin. Slot blot hybridisation of DNA extracted from AUXB1 and CH^RC5 cells to the P-glycoprotein probe indicated 58-fold gene amplification relative to the sensitive parental cells.

5.2.2.1 MAWI - Intermittent Exposure.

The range of P-glycoprotein cDNA copy number was similar to that for DHFR (see Figure 5.5). Mean copy numbers did not change with increasing exposure to methotrexate (Table 5.4).

A set of anomalous results was obtained with a MAWI subline, treated with 15 series of exposures to 1.0×10^{-6} M methotrexate (Figure 5.5). This is a 20 x IC_{50} dose of methotrexate. Hybridisation studies indicate an 18.3 fold amplification of the P-glycoprotein gene. This subline was not resistant to methotrexate and the cells died after further treatment at the same concentration of methotrexate. The three values shown in Figure 5.5 represent extractions performed at different stages of treatment;

After 9 exposures relative copy number = 18.7.

After 12 exposures relative copy number = 20.4.

After 15 exposures relative copy number = 16.2.

This result is anomalous since the cell line was not resistant to methotrexate, nor did it contain amplified DHFR genes. Attempts to re-treat several more sublines in exactly the same manner resulted in the low level variation reported with all the other MAWI sublines.

5.2.2.2 JAR - Intermittent Exposure.

When DNA extracted from JAR cells was hybridised to the P-glycoprotein probe little variation in gene copy number was observed (Figure 5.6), and the level of drug to which the cells had been exposed did not affect gene copy number, as shown in Table 5.5.

5.2.2.3 JAR - Continuous Exposure.

No amplification of P-glycoprotein was seen in the methotrexate resistant JAR sublines.

5.2.3 In Situ Hybridisation.

Hybridisation of sense and antisense riboprobes for dihydrofolate reductase to methotrexate resistant L1210 cells indicated the specificity of the in situ technique (Figures 5.8 and 5.9). No hybridisation was seen with the antisense riboprobe to the sensitive L1210 cells (Figure 5.10). The signal on these suspension culture cells is possibly weakened by the cells being prepared using a cytospin preparation which may damage the cells. Hybridisation of the antisense DHFR riboprobe to the methotrexate resistant JAR subline B^3 indicated good localisation, (Figure 5.11), with negative hybridisation using the sense riboprobe (Figure 5.12). No significant difference in hybridisation was seen with the other less resistant sublines or the parental cell line, all of which were much lower than the B^3 cells.

5.3 DISCUSSION.

The results described in section 5.2 indicate that low levels of gene amplification in choriocarcinoma cells may be sufficient to confer methotrexate resistance. No further genetic control is seen in these resistant cells with mRNA and enzyme activity levels directly reflecting gene amplification.

Intermittent exposure to methotrexate produced a range of DHFR gene copy levels in cells which were not considered to be resistant. The initial event in acquiring resistance is an unstable one (Schimke et al., 1981). Early stages of methotrexate resistance may be temporary and coupled with temporary amplification of dihydrofolate reductase genes in the form of double minutes. These are readily lost through mitosis during the recovery period when there is no selection (Kaufman et al., 1979).

The range of DHFR copy numbers may be a result of unstable gene amplification in a proportion of the JAR cells. Initial gene amplification producing unstable resistance is associated with the appearance of amplified genes in the form of double minutes (Schimke, 1988). By definition, double minutes, lacking centromeres, are lost without continued selection (Kaufman et al., 1981). Studies with methotrexate resistant S-180 cells indicated that growth out of methotrexate resulted in reversion to sensitivity, with concomitant loss of amplified DHFR genes (Alt et al., 1978). One possibility is that the JAR cells exposed intermittently to methotrexate may have contained some cells with DMs, but these were not further selected due to the amplified genes causing higher metabolic demands on the cells (Kaufman et al., 1981). In the absence of selecting agents, resistant S-180 cells had a lower growth rate than revertant cells which had a lower DHFR gene copy number. This implies that once methotrexate is removed, as in intermittent exposure, there

is positive selection for cells not containing double minutes with amplified genes.

One possible explanation for temporary resistance is that intermittent exposure may allow cells to maintain a lower than normal pool of drug intracellularly due to impaired polyglutamate formation (Pizzorno et al., 1988). Human leukemia T-lymphoblast cells, CCRF-CEM, have been given 6 or 7 exposures to a 24-hour exposure to methotrexate at constant concentrations (3 and 30 x 10^{-6} M respectively). The resistant cells possessed a polyglutamate level approximately 50% lower than the parent cells. No other difference was noted between these resistant cells and their sensitive parent cells. Defective polyglutamate formation was not tested in the JAR cells exposed to intermittent methotrexate.

High level resistance to methotrexate in human colon carcinoma cell lines may not be correlated with high level amplification of the DHFR gene (Srimatkandada et al., 1989). Colon carcinoma cells, HCT-8R4, were produced with 10,000 fold resistance to methotrexate as defined by survival in increasing concentrations of methotrexate. The resistant cells only contained a 25-fold amplification of the DHFR gene with corresponding elevation in enzyme activity and mRNA levels. Further analysis indicated a single base change in the gene, resulting in a protein with decreased methotrexate binding. It was this mutated gene that was further amplified (Srimatkandada et al., 1989).

Amplification seen in methotrexate resistant JAR cells, sublines B^0, B^1, B^2 and B^3, increased proportionally with the degree of resistance. The low levels of amplification were not coupled with impaired transport (see chapter 4), although polyglutamate formation was not studied. The increase in dihydrofolate reductase gene copy number with progressively increasing methotrexate concentration is consistent with the gene amplification model (Schimke et al., 1977). In murine cells, methotrexate resistant cells have been isolated with amplified DHFR genes in which DHFR contributes up to 20% of the cells' total soluble protein (Bostock et al., 1979). These cells are 30,000 fold resistant to methotrexate, with 1,000 fold increase in DHFR activity. CHO cells have been produced with up to 1,200 copies of the DHFR gene (Looney and Hamlin, 1987). Although the resistant JAR sublines do not approach these levels of amplification, the levels monitored may still be relevant when considering clinical resistance. Small-cell lung carcinoma cells were grown from a bone-marrow aspirate of a patient with recurrent disease following initial response to high dose methotrexate (Curt et al., 1983). The cells were methotrexate resistant with no uptake alterations but only contained a 2.4-fold amplification of the DHFR genes in the form of double minutes. Consequently, although the resistant sublines, B^0, B^1, B^2 and B^3, only contained 1.12, 1.68, 1.82 and 2.68-fold DHFR gene amplification respectively, these may be sufficient to cause drug resistance. Also they may be more relevant to the clinical situation than levels of

resistance and amplification exhibited in murine and CHO cell models. Cells have also been identified where the amount of DHFR was not proportional to DHFR gene copy number (Frei et al., 1984). This implies that changes in regulation may occur as well as gene amplification (Warr and Atkinson, 1988).

It appears that there is a 1:1 relationship between gene copy number and mRNA copy number (see Figure 5.7 and Table 5.3). The correlation between the two sets of data, shown in Figure 5.7, illustrates that the elevation in mRNA levels are a direct result of transcription of amplified gene copy number. The high correlation between DNA copy number, mRNA levels and enzyme activity in the resistant JAR sublines (Table 5.3a) indicates that gene copy number is the point of control of cellular enzyme levels rather than transcription or translation.

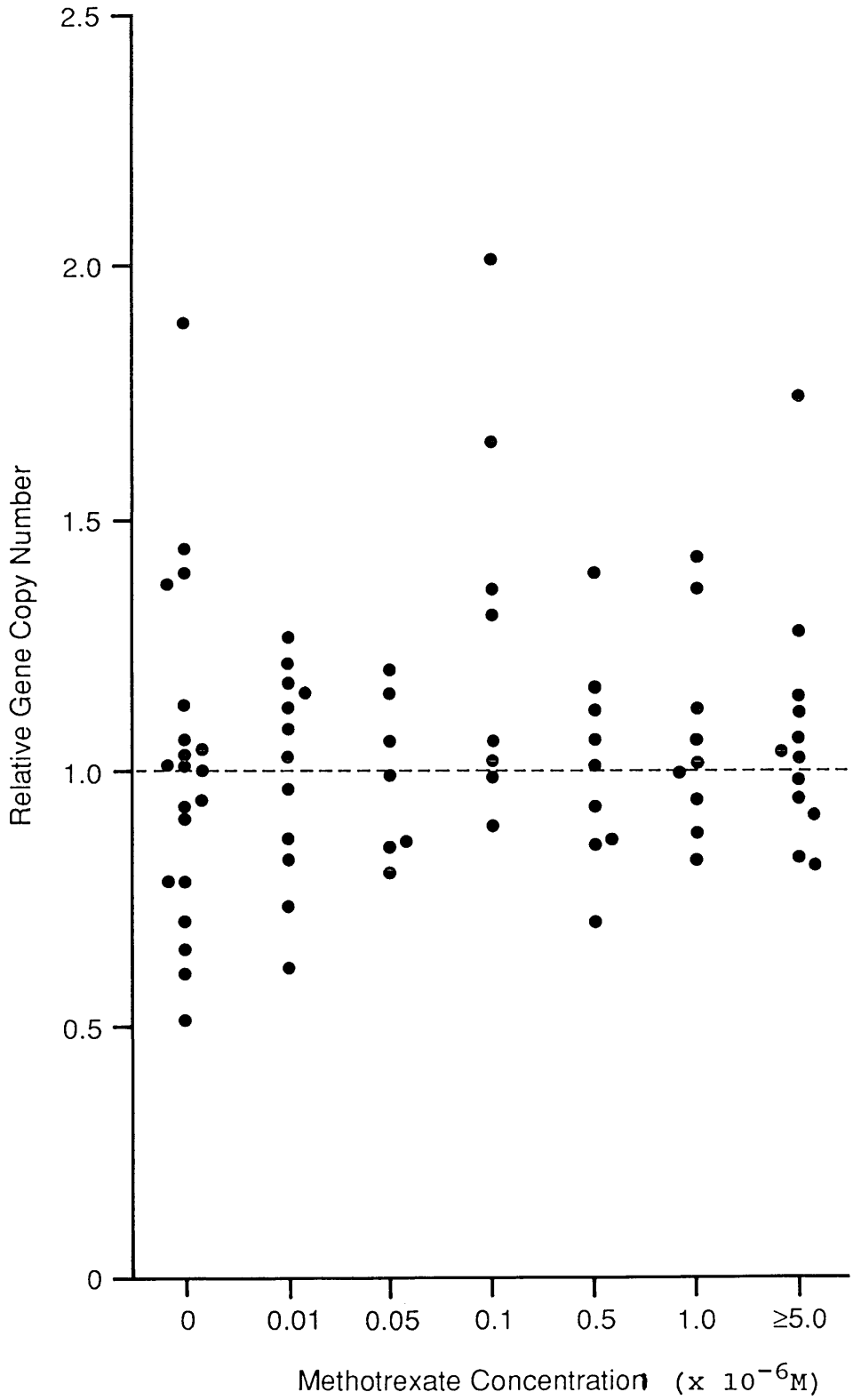


Figure 5.1 **Relative DHFR Gene Copy Number of MAWI Cells Treated with Intermittent Exposure to a Range of Methotrexate Concentrations.**

• = Single extraction of DNA from cultured cells. Data represents several extractions, with all samples represented above slot blotted and hybridised in one experiment.

MAWI cells - Dihydrofolate reductase probe.		
Methotrexate concentration (M)	Mean	S.D.
0 (n=20)	1.00	0.31
1.0×10^{-8} (n=12)	1.00	0.20
5.0×10^{-8} (n=7)	0.99	0.15
1.0×10^{-7} (n=8)	1.19	0.36
5.0×10^{-7} (n=10)	0.99	0.19
1.0×10^{-6} (n=9)	1.07	0.19
$\geq 5.0 \times 10^{-6}$ (n=12)	1.07	0.24

Table 5.1 **Dihydrofolate reductase gene copy number of MAWI cells exposed to intermittent methotrexate.**

The numbers are mean gene copy numbers, relative to the parental cell line, as detected by slot blot analysis. Samples were extracted from MAWI cells and hybridised to a probe fragment from the plasmid pSVDHFR. Each sample represents extraction of DNA from separately treated cells which have been exposed to the same treatment regimen. S.D. = Standard Deviation.

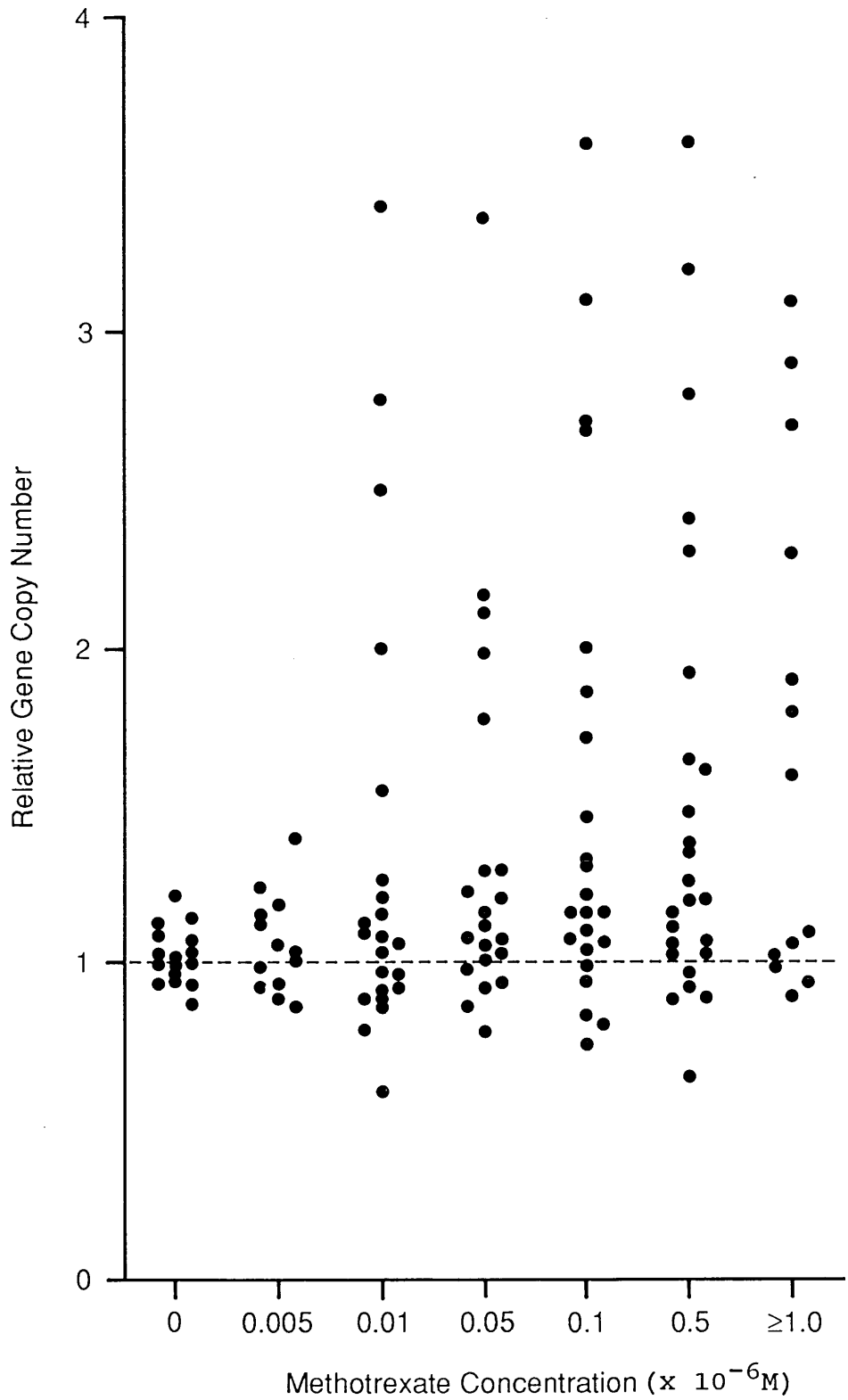


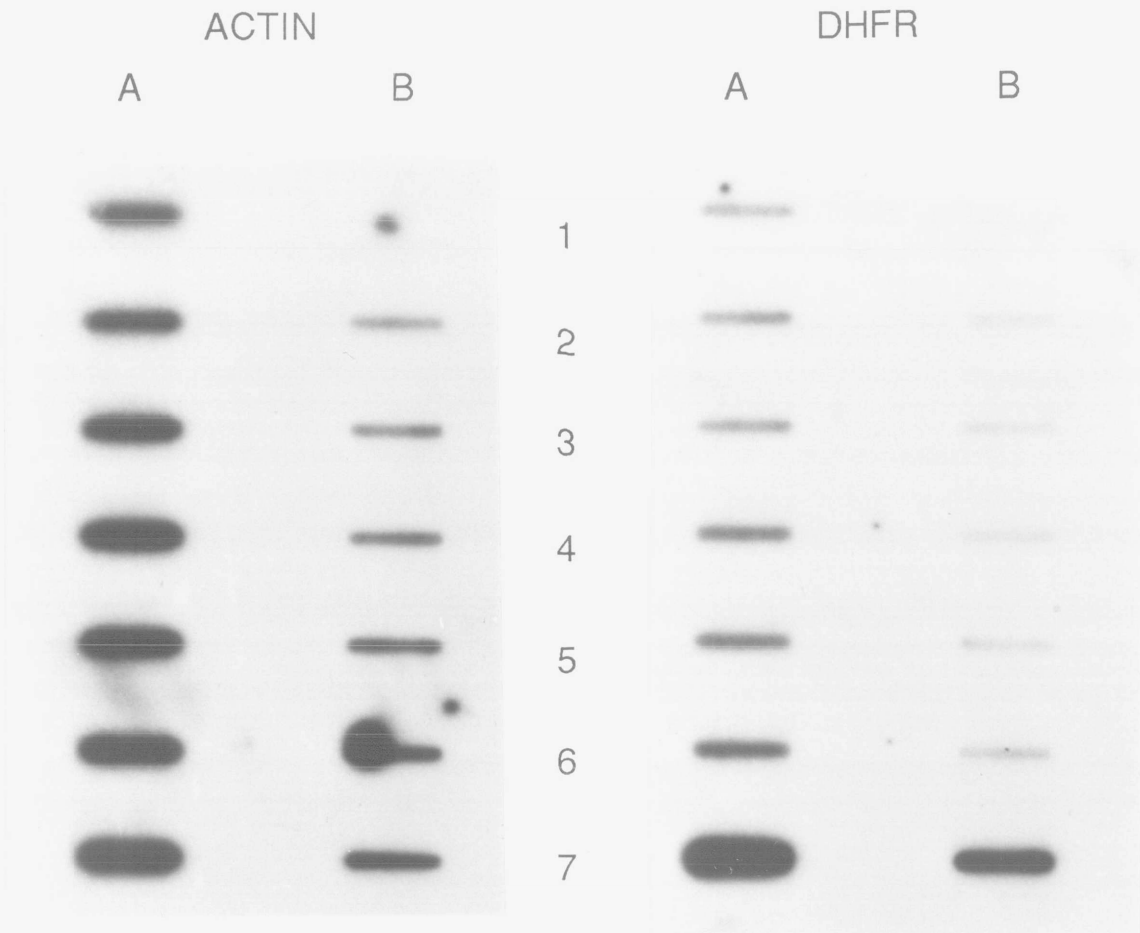
Figure 5.2 **Relative DHFR Gene Copy Number of JAR Cells Treated with Intermittent Exposure to a Range of Methotrexate Concentrations.**

• = Single extraction of DNA from cultured cells. Data represents several extractions, with all samples represented above slot blotted and hybridised in one experiment.

JAR cells - Dihydrofolate reductase probe.		
Methotrexate concentration (M)	Mean	S.D.
0 (n=16)	1.00	0.08
5.0×10^{-9} (n=13)	1.06	0.15
1.0×10^{-8} (n=22)	1.29	0.69
5.0×10^{-8} (n=21)	1.38	0.71
1.0×10^{-7} (n=23)	1.52	0.77
5.0×10^{-7} (n=25)	1.52	0.76
$\geq 1.0 \times 10^{-6}$ (n=13)	1.71	0.77

Table 5.2 **Dihydrofolate reductase gene copy number of JAR cells exposed to intermittent methotrexate.**

Mean gene copy numbers, relative to the parental cell line are shown, as detected by slot blot analysis. Samples were extracted from JAR (choriocarcinoma) cells and hybridised to a probe fragment from the plasmid pSVDHFR. Each sample represents extraction of DNA from separately treated cells which have been exposed to the same treatment regimen. S.D. = Standard Deviation.



A1. 5 μg DNA extracted from JAR cells.
A2. 5 μg DNA extracted from B^0 cells.
A3. 5 μg DNA extracted from B^1 cells.
A4. 5 μg DNA extracted from B^2 cells.
A5. 5 μg DNA extracted from B^3 cells.
A6. 5 μg DNA extracted from L1210 cells.
A7. 5 μg DNA extracted from L1210 R7A cells.

B1. 1 μg DNA extracted from JAR cells.
B2. 1 μg DNA extracted from B^0 cells.
B3. 1 μg DNA extracted from B^1 cells.
B4. 1 μg DNA extracted from B^2 cells.
B5. 1 μg DNA extracted from B^3 cells.
B6. 1 μg DNA extracted from L1210 cells.
B7. 1 μg DNA extracted from L1210 R7A cells.

Figure 5.3 **Slot Blot Analysis of DNA Extracted from Methotrexate Resistant JAR Sublines.**

DNA applied to membrane as per manufacturers instructions (Schleicher and Schuell). Prehybridisation, hybridisation and washing performed at 65°C. Autoradiography at -70°C for 24 hours with intensifying screens, and signal quantified by scanning densitometry.

	Relative Amplification/Elevation		
Cell Subline	DNA (n=5)	RNA (n=4)	Enzyme (n=2)
JAR	1.00 ± 0.03	1.00 ± 0.07	1.00 ± 0.09
B^0	1.12 ± 0.14	1.10 ± 0.10	1.16 ± 0.05
B^1	1.68 ± 0.07	1.41 ± 0.13	1.50 ± 0.05
B^2	1.82 ± 0.24	1.77 ± 0.21	2.46 ± 0.03
B^3	2.68 ± 0.22	2.18 ± 0.31	N/C

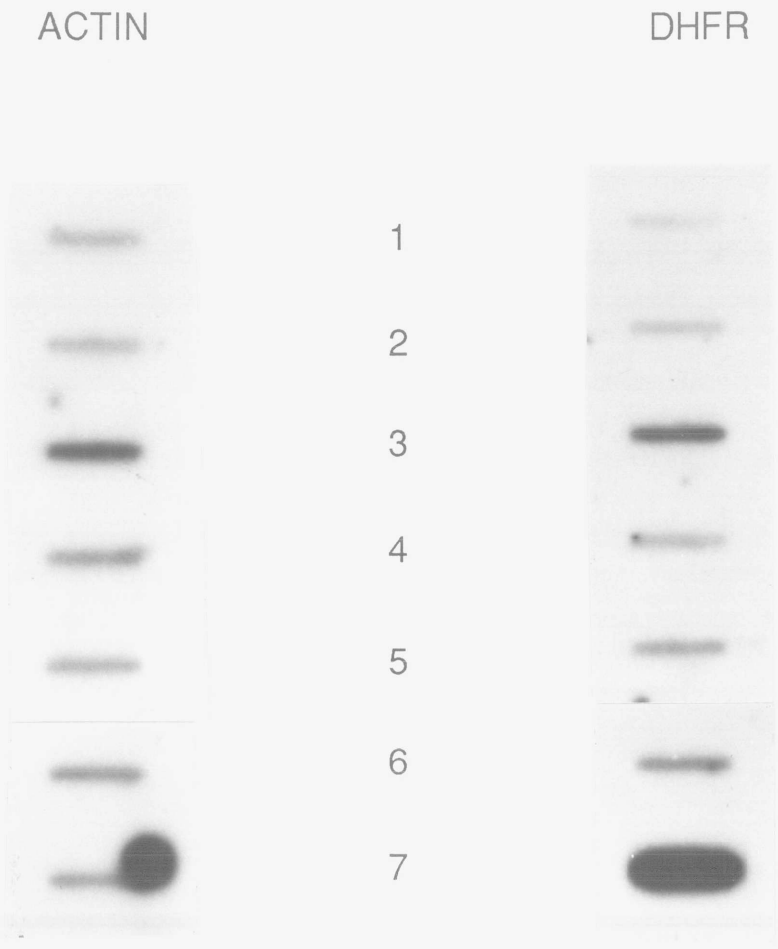
Table 5.3 **Comparison of DNA gene copy number, RNA expression, and dihydrofolate reductase activity levels in methotrexate resistant JAR sublines.**

N/C = Not calculated.

Mean DHFR DNA copy numbers, RNA expression levels and DHFR activity of methotrexate resistant sublines relative to the parental cell line. DNA and RNA levels detected by slot blot analysis, hybridised to a pSVDHFR cDNA oligolabelled probe and quantified by scanning densitometry. Enzyme activity detected by spectrophotometric assay at 340_{nm}.

Relationship	Correlation coefficient	T-value	Significance level
DNA/RNA	0.845	2.737	5%
DNA/Enzyme	0.877	2.581	10%
RNA/Enzyme	0.979	6.79	2.5%

Table 5.3a **Statistical analysis of comparable DNA, RNA and enzyme activity levels in methotrexate resistant JAR sublines.**



1. 10 μg RNA extracted from JAR cells.
2. 10 μg RNA extracted from B^0 cells.
3. 10 μg RNA extracted from B^1 cells.
4. 10 μg RNA extracted from B^2 cells.
5. 10 μg RNA extracted from B^3 cells.
6. 10 μg RNA extracted from L1210 cells.
7. 10 μg RNA extracted from L1210 R7A cells.

Figure 5.4 **Slot Blot Analysis of RNA Extracted from Methotrexate Resistant JAR Sublines.**

RNA applied to membrane as per manufacturers instructions (Schleicher and Schuell). Prehybridisation, hybridisation and washing performed at 65°C. Autoradiography at -70°C for 36 hours with intensifying screens, and signal quantified by scanning densitometry.

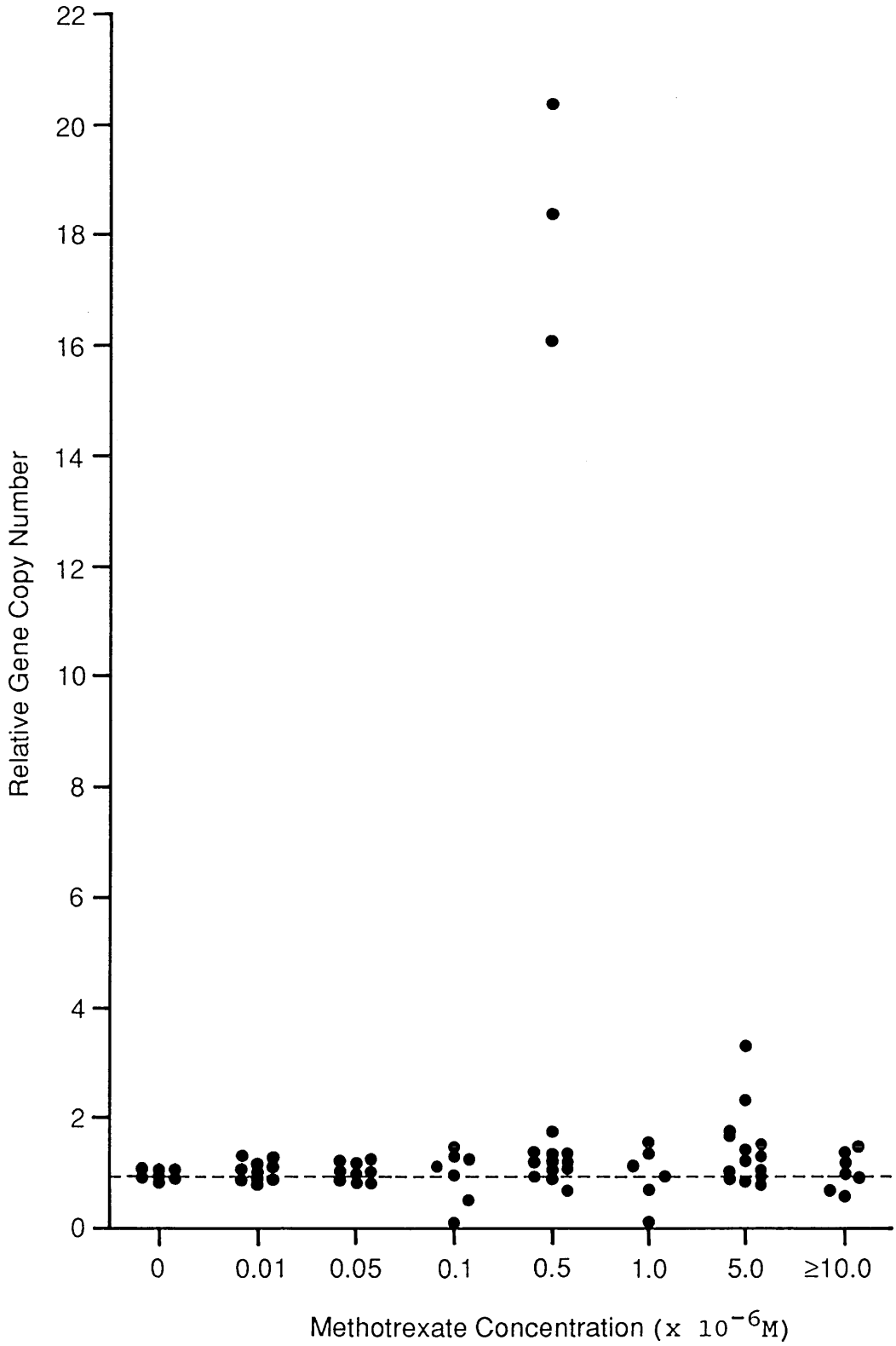


Figure 5.5 **Relative P-glycoprotein Gene Copy Number of MAWI Cells Treated with Intermittent Exposure to a Range of Methotrexate Concentrations.**

• = Single extraction of DNA from cultured cells. Data represents several extractions, with all samples represented above slot blotted and hybridised in one experiment.

MAWI cells - P-glycoprotein probe.		
Methotrexate concentration (M)	Mean	S.D.
0 (n=16)	1.00	0.06
1.0×10^{-8} (n=12)	1.05	0.14
5.0×10^{-8} (n=13)	1.02	0.13
1.0×10^{-7} (n=7)	0.94	0.44
5.0×10^{-7} (n=12)	1.15	0.26
1.0×10^{-6} (n=7)	0.96	0.44
$\geq 5.0 \times 10^{-6}$ (n=21)	1.19	0.38

Table 5.4 **P-glycoprotein gene copy number of MAWI cells exposed to intermittent methotrexate.**

Mean gene copy numbers, relative to the parental cell line, as detected by slot blot analysis. Samples were extracted from MAWI cells and hybridised to a probe fragment from the plasmid pCHP1. Each sample represents extraction of DNA from separately treated cells which have been exposed to the same treatment regimen.
S.D. = Standard Deviation.

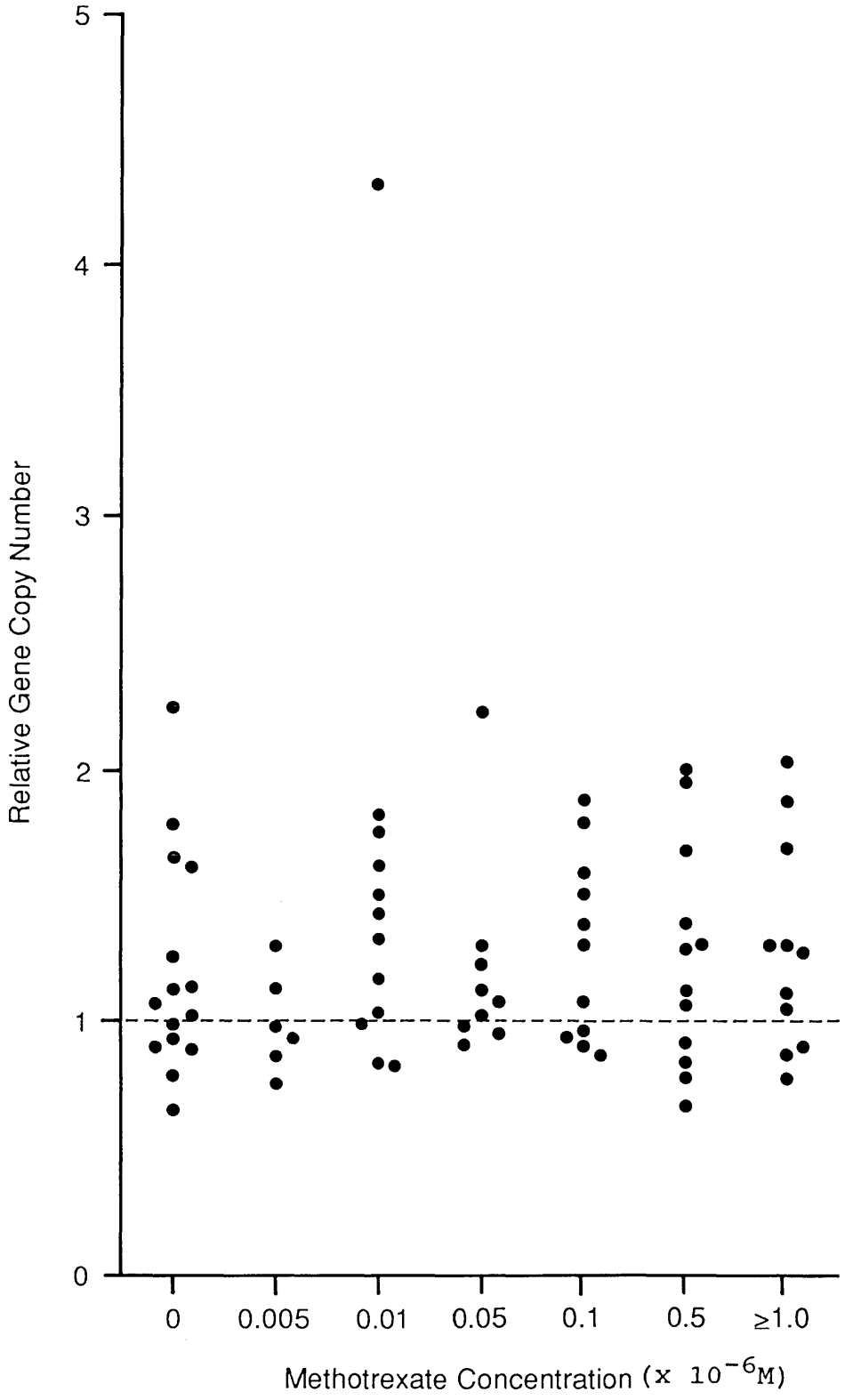


Figure 5.6 **Relative P-glycoprotein Gene Copy Number of JAR Cells Treated with Intermittent Exposure to a Range of Methotrexate Concentrations.**

• = Single extraction of DNA from cultured cells. Data represents several extractions, with all samples represented above slot blotted and hybridised in one experiment.

JAR - cells - P-glycoprotein probe.		
Methotrexate concentration (M)	Mean	S.D.
0 (n=15)	1.00	0.08
5.0×10^{-9} (n=6)	1.06	0.15
1.0×10^{-8} (n=12)	1.30	0.69
5.0×10^{-8} (n=9)	1.36	0.60
1.0×10^{-7} (n=11)	1.52	0.77
5.0×10^{-7} (n=12)	1.53	0.75
$\geq 1.0 \times 10^{-6}$ (n=11)	1.72	0.78

Table 5.5 **P-glycoprotein gene copy number of JAR cells exposed to intermittent methotrexate.**

Mean gene copy numbers are shown relative to the parental cell line, as detected by slot blot analysis. Samples were extracted from JAR (choriocarcinoma) cells and hybridised to a probe fragment from the plasmid pCHP1. Each sample represents extraction of DNA from separately treated cells which have been exposed to the same treatment regimen. S.D. = Standard Deviation.

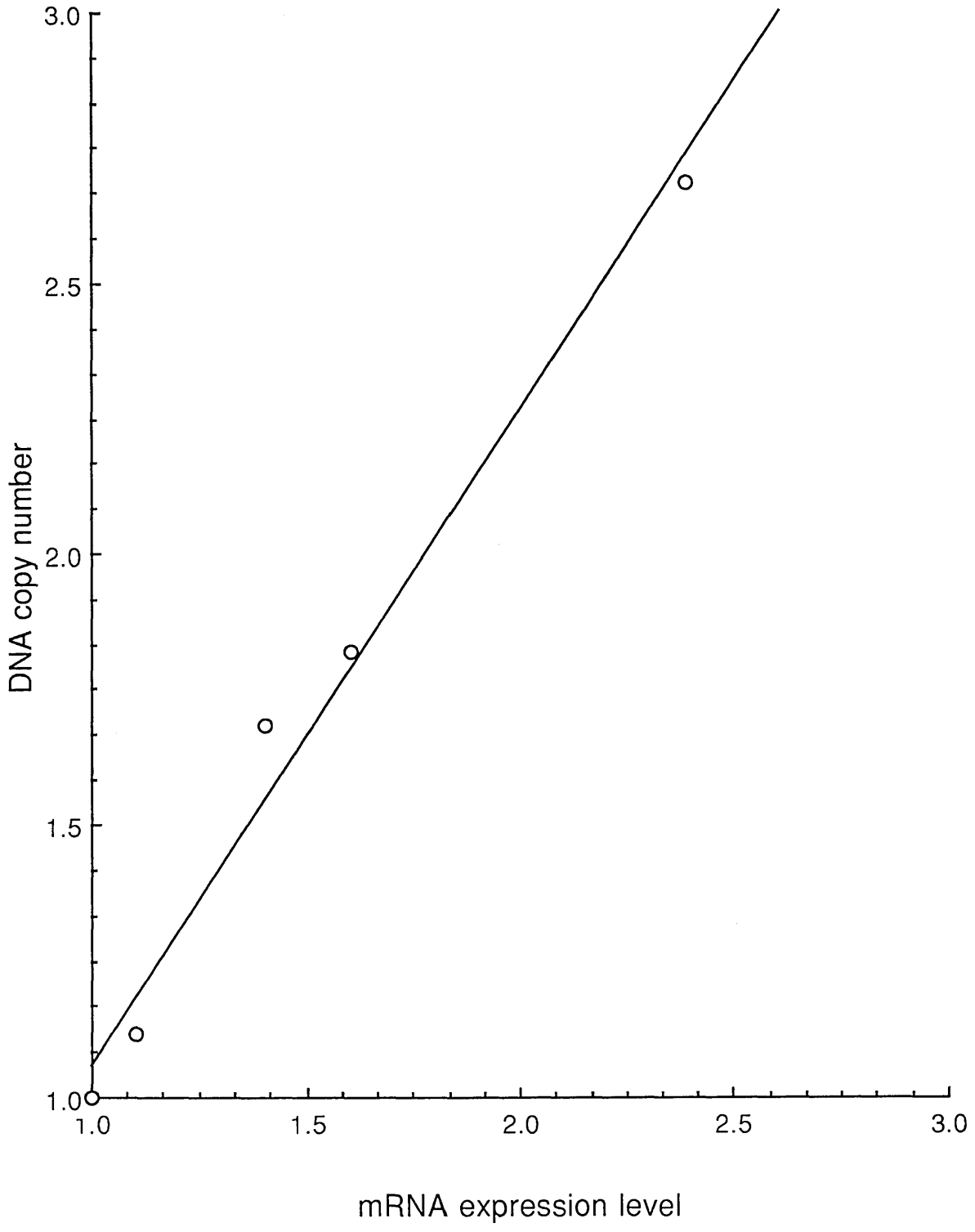


Figure 5.7 **Comparison of Relative DNA Gene Copy Number and mRNA Expression Levels in Methotrexate Resistant JAR Sublines.**

Values represent means as calculated in Table 5.3

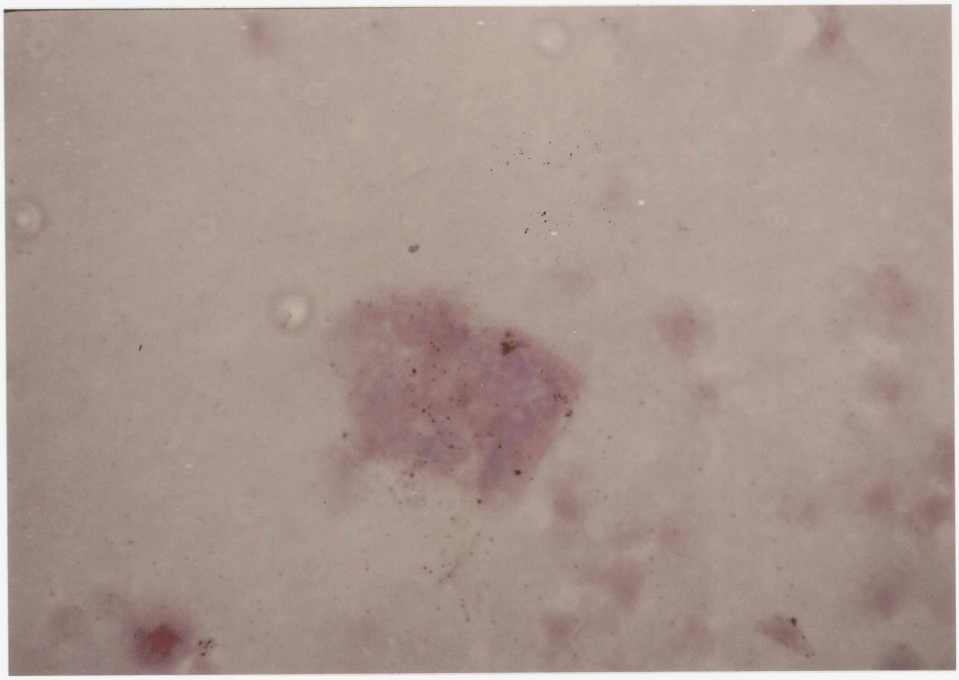


Figure 5.8 **In Situ Hybridisation of Dihydrofolate Reductase Antisense Riboprobe to L1210 R7A Cells.**

Positive hybridisation of radiolabelled (^{32}P) DHFR antisense riboprobe (SP6 polymerase) to methotrexate resistant mouse leukemic cells. Cells centrifuged onto a glass slide using a cytospin and fixed in 10% buffered formalin. Autoradiograph (14 days) counterstained with Haematoxylin. Magnification x 64.

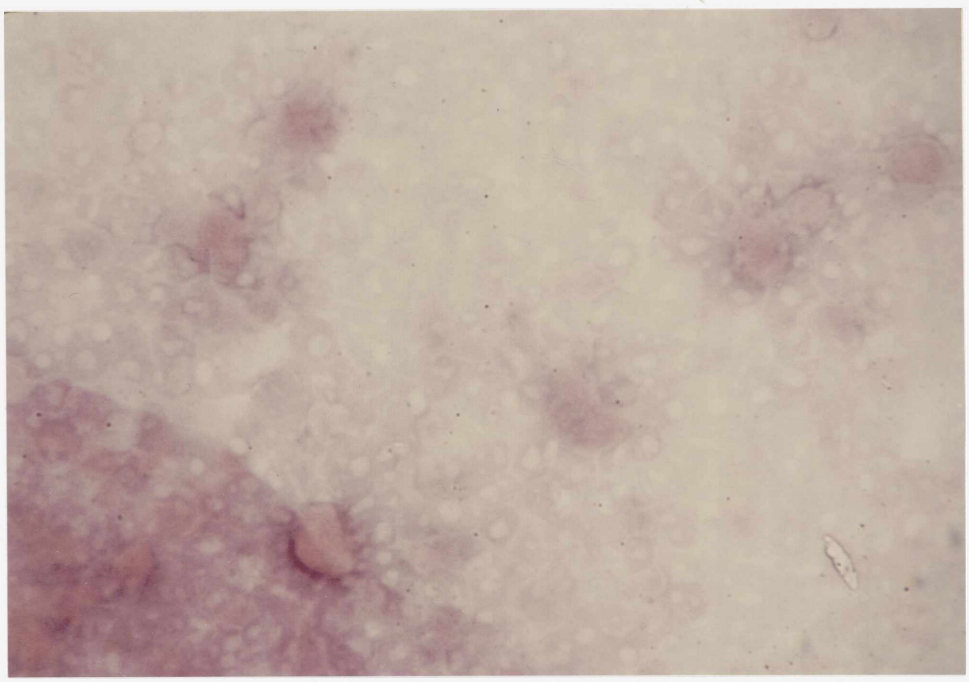


Figure 5.9 **In Situ Hybridisation of Dihydrofolate Reductase Sense Riboprobe to L1210 R7A Cells.**

Negative hybridisation of radiolabelled (^{32}P) DHFR sense riboprobe (T7 polymerase) to methotrexate resistant mouse leukemic cells. Cells centrifuged onto a glass slide using a cytospin and fixed in 10% buffered formalin. Autoradiograph (14 days) counterstained with Haematoxylin. Magnification x 64.

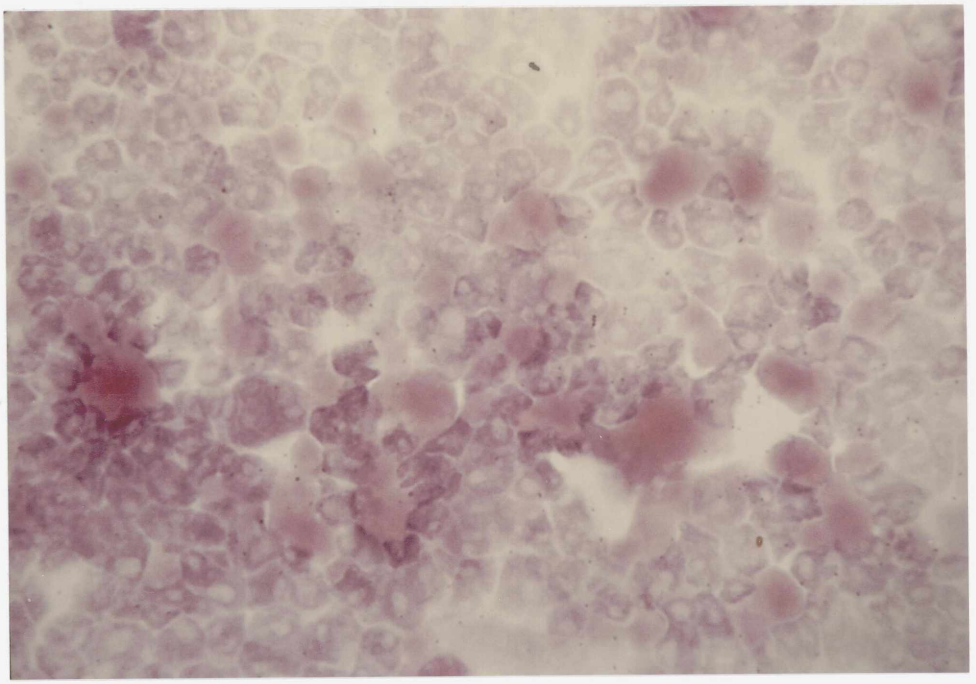


Figure 5.10 **In Situ Hybridisation of Dihydrofolate Reductase Antisense Riboprobe to L1210 Cells.**

Negative hybridisation of radiolabelled (^{32}P) DHFR antisense riboprobe (SP6 polymerase) to methotrexate sensitivee mouse leukemic cells. Cells centrifuged onto a glass slide using a cytospin and fixed in 10% buffered formalin. Autoradiograph (14 days) counterstained with Haematoxylin. Magnification x 64.

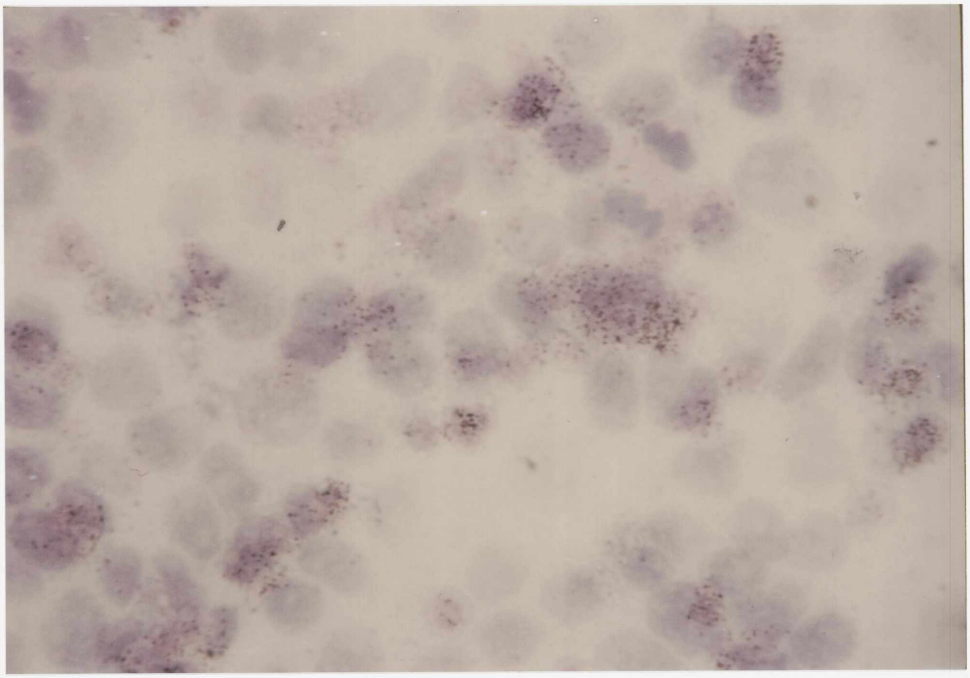


Figure 5.11 **In Situ Hybridisation of Dihydrofolate Reductase Antisense Riboprobe to Methotrexate Resistant JAR B^3 Cells.**

Positive hybridisation of radiolabelled (^{32}P) DHFR antisense riboprobe (SP6 polymerase) to methotrexate resistant human choriocarcinoma cells. Cells grown on a glass slide and fixed in 10% buffered formalin. Autoradiograph (14 days) counterstained with Haematoxylin. Magnification x 100.

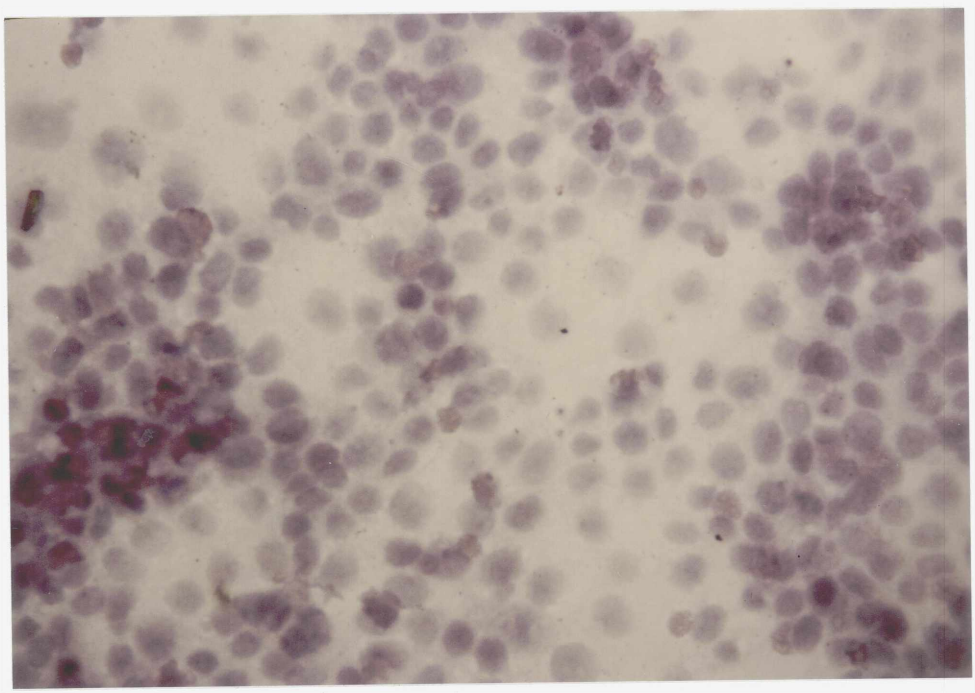


Figure 5.12 **In Situ Hybridisation of Dihydrofolate Reductase Antisense Riboprobe to Methotrexate Resistant JAR B^3 Cells.**

Negative hybridisation of radiolabelled (^{32}P) DHFR antisense riboprobe (SP6 polymerase) to methotrexate resistant human choriocarcinoma cells. Cells grown on a glass slide and fixed in 10% buffered formalin. Autoradiograph (14 days) counterstained with Haematoxylin. Magnification x 64.

CHAPTER 6. GENE AMPLIFICATION IN COLONIC TUMOURS.

6.1 INTRODUCTION.

In this chapter amplification of dihydrofolate reductase and P-glycoprotein DNA and mRNA levels were studied in colonic tumour and normal colon samples from the same patient. Unlike choriocarcinomas, these tumours were readily available from untreated patients.

Normal and neoplastic colon possess high levels of P-glycoprotein DNA, mRNA and protein levels (Fojo et al., 1987; Ueda et al., 1989; Thiebaut et al., 1987; Hitchens et al., 1988). In this study, I compared tumour to normal ratios to monitor whether amplification occurred at the DNA or RNA level in the tumour relative to normal adjacent tissue. Clinical information was recorded so that the prognostic significance of the data could be assessed.

6.2 RESULTS.

The results presented are from 45 tumour and normal samples. All tumours had like histopathological diagnosis of adenocarcinoma, 24 from male patients and 21 from female patients. The mean age for males was 69.4 years with a range of 51 -83; the mean age for females was 69.6 with a range of 41 - 84. The stage and grade of tumours were distributed as follows:-

Differentiation: Poorly differentiated = 3 (7%); Moderately differentiated = 33 (73%); Well differentiated = 9 (20%). Dukes' grading: Dukes' grade A = 5 (11%); Dukes' grade B = 28 (62%); Dukes' grade C = 12 (27%).

6.2.1 Dihydrofolate Reductase.

Tumour and normal DNA were extracted from 45 colonic tumours. In each case, slot blots were performed in duplicate to standardise the amount of DNA loaded, using a probe for β-actin. The ratios of tumour to normal tissue for dihydrofolate reductase gene expression were within the range 0.5 - 1.5 with the exception of one tumour which had a ratio of 2.8. The mean value was 1.00, with a standard deviation of ± 0.35.

RNA was extracted from 31 of the 45 colonic tumour samples and analysed using slot blotting. Ratios were between 0.4 - 1.5 with a mean of 1.03 and a standard deviation of ± 0.32.

6.2.2 P-glycoprotein.

P-glycoprotein tumour to normal ratios in 45 tumour and normal biopsies ranged from 0.57 to 1.59, with a mean of 1.11, and a standard deviation of ± 0.22. The variance was similar to that exhibited in the same tumours for DHFR gene copy number.

The distribution of P-glycoprotein mRNA levels in normal and tumour material was broader than that for DHFR mRNA levels. In 31 samples studied the tumour to normal ratio ranged from 0.4 to 4.4, with a mean copy number of 1.75 and standard deviation of ± 1.11. Using one standard deviation from the mean as the cut off point, 5 samples had elevated P-glycoprotein RNA levels (Table 6.1). Four out of five (80%) of the elevated samples were Dukes' grade C tumours. In the group of 26 samples whose copy numbers were within one standard deviation of the mean, on 19% (5/26) were Dukes' grade C tumours (Table 6.1). This difference, using X^2 and Yates' correction factor, is significant at the 5% level ($p = 0.05$). No difference was seen between degree of differentiation and RNA levels.

6.2.3 In-situ hybridisation.

Hybridisation of sense and antisense riboprobes for P-glycoprotein in sensitive and multidrug resistant CHO cells indicated the specificity of the in-situ technique (Figures 6.1 and 6.2). Hybridisation using the antisense (positive) riboprobe was visible in the tumour section (Figure 6.3). A high level of background hybridisation, however, was seen in the normal tissue section (Figure 6.4).

6.3 DISCUSSION.

Analysis of DHFR DNA and mRNA levels in tumour to normal ratios indicated that there was no amplification of dihydrofolate reductase at either nucleic acid level. This was expected as the colonic tumours had not previously been exposed to methotrexate before surgical excision.

P-glycoprotein was, however, found to be elevated in RNA expression levels in some of the tumour samples, compared to the normal adjacent tissue. Elevation of relative P-glycoprotein mRNA expression levels was seen in a population with mean = 1.75 and with a range of 0.4 - 4.4. This compared to relative gene copy numbers, for the 31 samples from which RNA was extracted, mean = 1.08 with a range of 0.57 to 1.56, which did not indicate any elevation.

The data support the findings of other research groups analysing P-glycoprotein levels in normal colon and colonic tumour material. Relatively high levels of P-glycoprotein mRNA have been observed in normal colon and tumours (Fojo et al., 1987). When other normal tissues were analysed for P-glycoprotein mRNA expression, high levels were found in the adrenal gland and kidney, with intermediate levels present in the lung, liver, rectum and colon. These levels were compared to a sensitive human KB carcinoma cell line, KB-3-1. From 10 individual colon samples the average elevation in mRNA level compared to the

KB-3-1 cell line was 31-fold. Although these levels of mRNA elevation are far greater than those seen in the colon tumour samples in this study they are compared to the KB-3-1 cell line as opposed to adjacent normal tissue. The implication from the data is that colon tissue contains a relatively high "base" level of P-glycoprotein mRNA. Comparison of 8 colon tumours with adjacent normal tissue showed mRNA levels in the range of 0.5 to 3.2 (Fojo et al., 1987). This indicates that some tumours had higher P-glycoprotein mRNA expression in the normal tissue (highest difference was a factor of 2.1), whereas some tumours had higher levels in the tumour compared to the normal tissue (highest factor of 3.2). This ties in well with the data from the 31 colon samples studied here, with P-glycoprotein mRNA expression ranging from 0.4 to 4.4. Several factors may affect P-glycoprotein mRNA levels, including the proportion of cell types within a tissue. The heterogeneity of cell types in a tumour biopsy may lead to underestimation of P-glycoprotein DNA gene copy number and mRNA expression levels (Goldstein et al., 1990). This problem may be overcome using in-situ hybridisation to analyse RNA levels in individual cells (Fojo et al., 1987). High P-glycoprotein levels in the normal colon and in colonic tumours suggests that the expression of the P-glycoprotein encoding gene, mdr1, may contribute to the tumours' intrinsic resistance to antineoplastic agents. In mutant cell lines with low levels of multidrug resistance, elevated P-glycoprotein mRNA expression was detected, without DNA gene amplification (Fojo et al., 1987). This

evidence of increased transcription suggests that in some tumours gene activation may be a more common means of developing resistance than gene amplification.

Elevation of P-glycoprotein mRNA levels compared to gene copy levels may also be explained by elevated expression preceding gene amplification, as seen in multidrug resistant human KB carcinoma cells (Shen et al., 1986). Cells with low level resistance (2- to 6-fold) contained low level elevation of P-glycoprotein mRNA levels but no corresponding amplification in DNA. This implies that clinically, where a tumour may only need low level expression to become drug resistant, mRNA expression should be monitored before gene copy number. Elevated mRNA expression levels, coupled to the fact that multidrug-resistant cells could not be selected without the use of a mutagen, suggests that the initial steps in selection may require activation of the mdr1 gene by a regulatory mutation (Shen et al., 1986). This also supports the findings reported here where the mRNA levels are higher than the DNA copy numbers. Another possibility is increased mRNA stability with differing rates of degradation (Mukhopadhyay et al., 1988).

Studies measuring levels of P-glycoprotein using monoclonal antibodies and western blotting have found elevated levels in tissues such as liver and bowel, with 50% of small bowel mucosa samples staining P-glycoprotein positive (Hitchins et al., 1988). Monoclonal antibodies have also been used to

localise P-glycoprotein within the colon to the apical surfaces of superficial columnar epithelial cells (Thiebaut et al., 1987). This is consistent with the possibility of P-glycoprotein functioning as a drug-transport protein. The nucleic acid sequence of P-glycoprotein was determined from cDNA isolated from both drug-sensitive Chinese hamster ovary (CHO) cells (Endicott et al., 1987) and multidrug-resistant resistant CHO cells (Van der Bliek et al., 1986). Analysis using a nucleic acid sequence database indicated that the P-glycoprotein showed extensive sequence homology with HylB, a bacterial transport protein required for the transport of α-hemolysin (Gerlach et al., 1986).

More sensitive measurement of mRNA expression is the RNA protection assay (Baas and Borst, 1988; Ueda et al., 1989). Isolating a genomic sequence from mdr1 gene in normal human tissue for use as a probe will allow more sensitive analysis of P-glycoproteins in normal tissues (Ueda et al., 1989). This would avoid problems arising from using a probe from resistant cells which may contain a mutation which prevents levels of non-mutated P-glycoprotein being detected in normal human tissues. Another inaccuracy using standard cDNA probes derived from multidrug-resistant cells is that cross hybridisation may occur with the closely related mdr2 gene which has not been shown to be associated with multidrug resistance (Van der Bliek et al., 1986).

The results from this study indicate that monitoring mRNA levels may detect multidrug resistant tumours. Similarly

RNA expression levels in a patient's normal colonic tissue may give an early indication to chemotherapy treatment response. P-glycoprotein gene copy numbers and mRNA expression levels in normal colon tissue may explain why colonic tumours are inherently resistant to most chemotherapy, and the biopsies showed a significant correlation between Dukes' grade C, the worst prognosis, and elevated P-glycoprotein mRNA (80%). This may also be of diagnostic value. Although no correlation was seen between elevated P-glycoprotein mRNA expressionand tumour grade, the sample size studied does not entirely preclude this possibility.

Identification of low level elevation in P-glycoprotein mRNA expression may be of significance in the design of treatment regimens. Although colon tumours are not generally treated chemotherapeutically, the elevated mRNA expression in certain tumours may label them as likely "non-responders." If this could be applied to more sensitive tumours, and a rapid diagnostic assay provided, treatment may be improved. At the onset of elevated P-glycoprotein mRNA expression, verapamil, a known reversing agent in P-glycoprotein-mediated multidrug resistance, could be included in the treatment scedule (Rothenberg and Ling, 1989). Verapamil is not the definitive answer, however, as the levels in humans are limited to approximately 2.0×10^{-6} M, due to effects on heart rhythm and blood pressure (Moscow and Cowan, 1988). This level of verapamil is not sufficient to reverse the affects of in-

vitro tumour models of multidrug resistance (Chabner and Fojo, 1989). P-glycoprotein mRNA expression may provide an early warning of imminent multidrug resistance. This may be of increasing importance if more effective methods for reversing multidrug resistance than verapamil can be found.

	Number	Grade C % (Nº)	Poor Differentiation % (Nº)
Normal	26	19% (5)	16% (4)
Elevated	5	80% (4)	20% (1)

Table 6.1 Comparison and distribution of grade and stage of colon tumours with normal (≤2.85) and elevated (≥2.85) P-glycoprotein mRNA ratios for tumour to normal tissue comparisons.

2.85 = one standard deviation above the mean population value. The correlation between P-glycoprotein mRNA expression and tumour grade was significant with $p \leq 0.05$. Data analyses using X^2 analysis incorporating Yates' correction factor for small sample size.

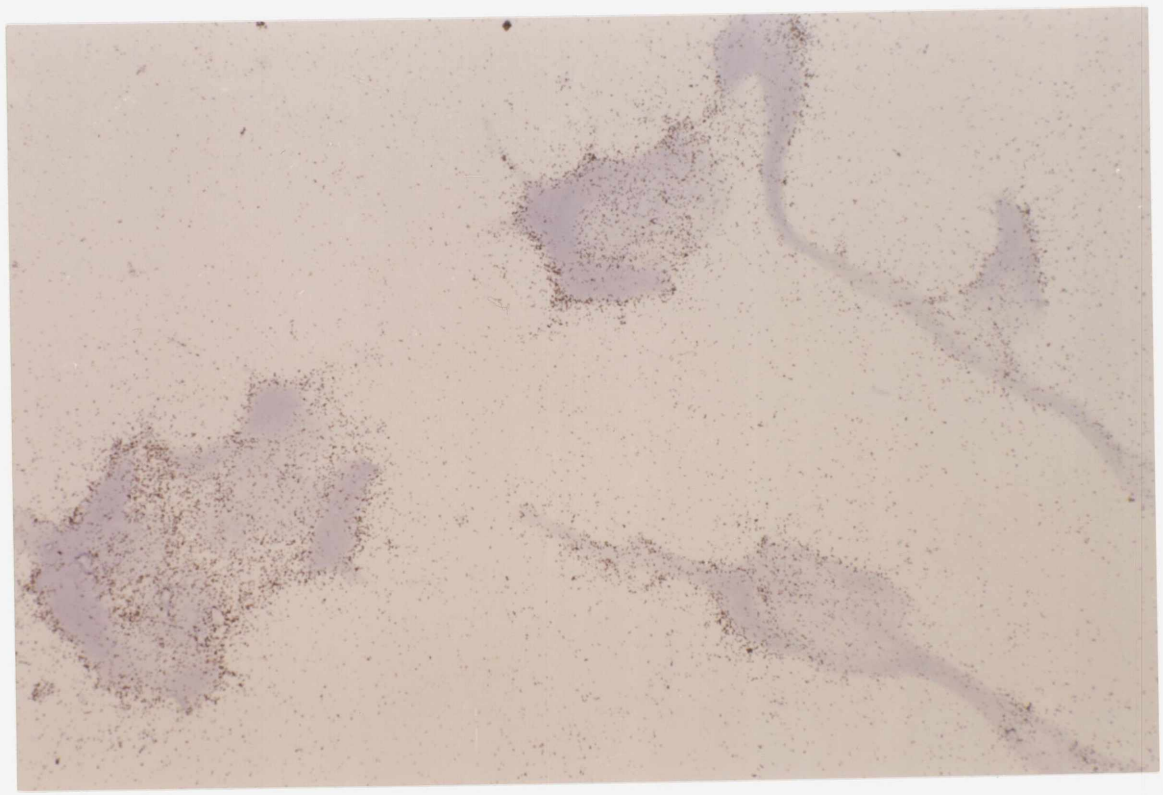


Figure 6.1 **In Situ Hybridisation of P-glycoprotein Anti - sense Riboprobe to $CHR^{C}5$ Cells.**

Positive hybridisation of P-glycoprotein anti-sense (T7 polymerase) riboprobe (^{32}P) to multidrug resistant Chinese hamster ovary cells. Cells grown on glass slides and fixed in 10% buffered formalin.

Autoradiograph (14 days) counterstained with Haematoxylin.
Magnification x 100.

Figure 6.2 **In Situ Hybridisation of P-glycoprotein Anti - sense Riboprobe to AUXB1 Cells.**

Positive hybridisation of P-glycoprotein anti-sense (T7 polymerase) riboprobe (^{32}P) to sensitive Chinese hamster ovary cells. Cells grown on glass slides and fixed in 10% buffered formalin.

Autoradiograph (14 days) counterstained with Haematoxylin.
Magnification x 64.

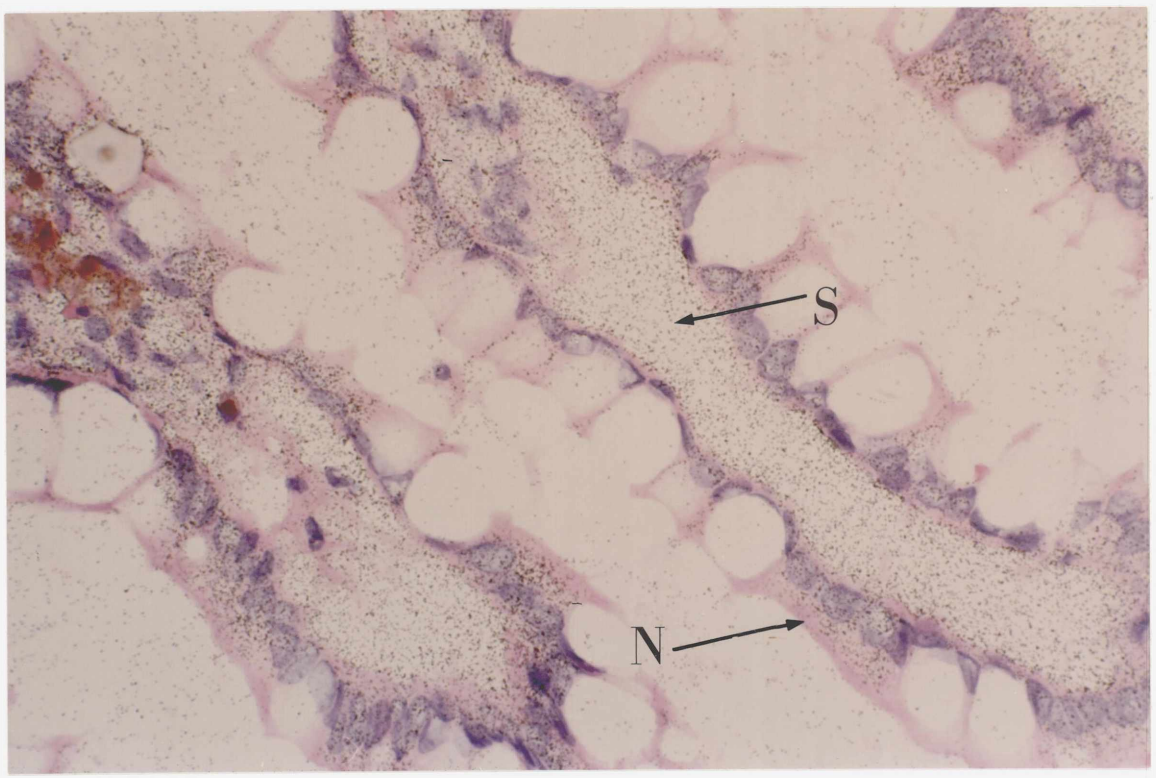


Figure 6.3 **In Situ Hybridisation of P-glycoprotein Anti - sense Riboprobe to Non-neoplastic Section.**

Section showing hybridisation of P-glycoprotein riboprobe (^{32}P) hybridised to non-neoplastic colonic mucosa, adjacent to tumour. Note background labelling of cytoplasm in goblet cells (N) and non-specific hybridisation to stromal elements (S).

Autoradiograph (14 days) counterstained with Haematoxylin and Eosin.
Magnification x 100.

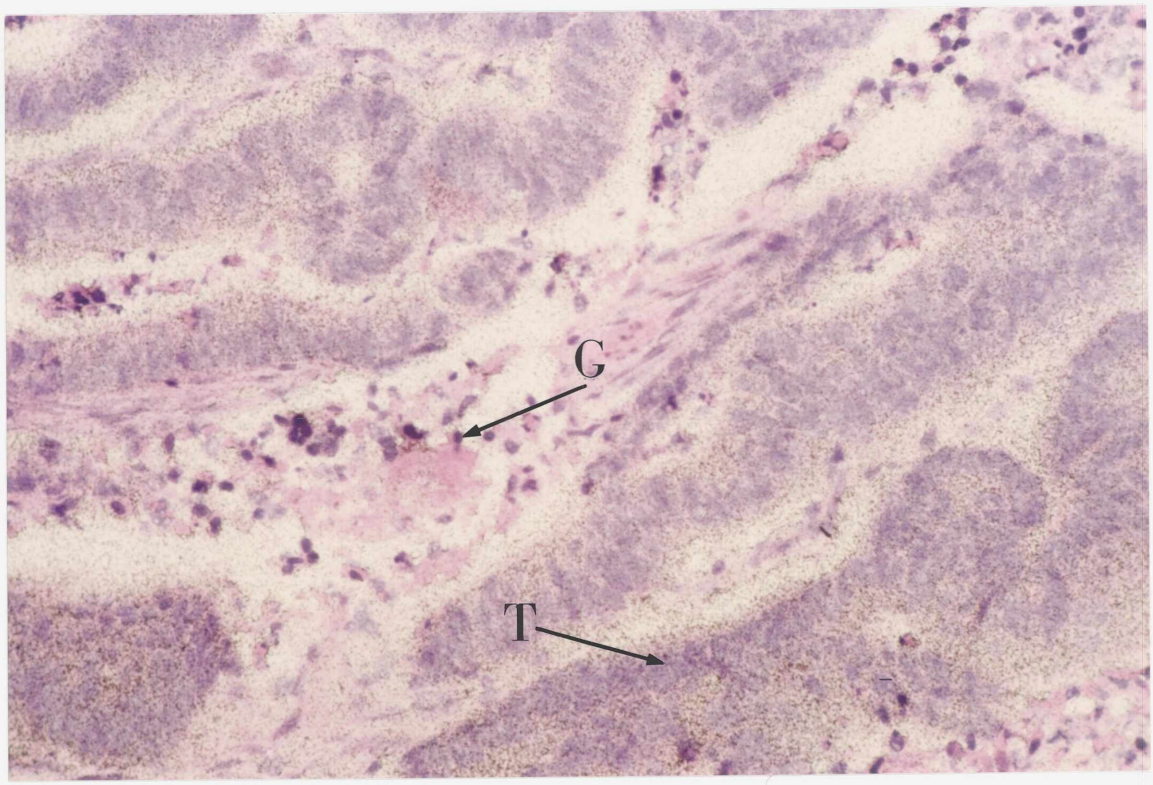


Figure 6.4 **In Situ Hybridisation of P-glycoprotein Anti - sense Riboprobe to Colonic Tumour Section.**

Section showing high intensity signal of P-glycoprotein riboprobe (^{32}P) hybridised to colonic adenocarcinoma cells (T). Note absence of signal in necrotic glandular debris (G).

Autoradiograph (14 days) counterstained with Haematoxylin and Eosin.
Magnification x 64.

CHAPTER 7. GENERAL DISCUSSION.

This thesis presents work on two tumour types, choriocarcinoma and colon carcinoma, using cell lines and biopsy material. Methotrexate resistant choriocarcinoma sublines have been produced which are associated with low level gene amplification. The resistant sublines show a proportional increase in DHFR gene copy numbers, DHFR mRNA expression levels and dihydrofolate reductase enzyme activity with the concentration of methotrexate to which they are resistant. No methotrexate resistant colonic tumour cells were produced. Analysis of colon tumour biopsies, which had not been exposed to chemotherapy before excision, indicated elevation of P-glycoprotein mRNA in some of the biopsies. There was a significantly greater proportion in the number of Dukes' grade C tumours found in the sub-population of tumours with elevated P-glycoprotein mRNA, suggesting that such studies may be of prognostic value.

7.1 VALIDITY OF THE CELL CULTURE MODEL.

Within the last 20 years human tumour continuous cell lines have become an invaluable model for the study of human cancer. The detailed characterisation of new cell lines coupled with information about the origin of the tumour from which they have been derived make the in vitro model a valuable source of material for experimental study. One immediate problem is that cells with low passage numbers are

often preferable to lines that have been long established (Hill, 1986). Stable cell lines, if regarded within their limitations, as models, are useful for establishing methods, wherein the cell line consistency can be guaranteed. Thereafter the methods can be applied to other lines. Both JAR and MAWI cell lines used in this study were established several years ago and were both used at high passage numbers. No attempt was made to establish fresh primary cell lines from choriocarcinoma or colonic adenocarcinoma biopsies. Problems arising from changes in the properties of human tumour cells with time include increases in population doubling times and/or colony-forming efficiencies, changes in DNA content (Engelholm et al., 1985) and positive selection of cells containing HSR's with simultaneous loss of DM containing cells (Trent et al., 1984). For example, a human small cell carcinoma of the lung (SCLC) was established as a cell line in vitro (Engelholm et al., 1985). Cloned tumour cell lines were set up from a lymph node metastasis from an untreated patient with SCLC. Flow cytometric analysis of DNA indicated only one tumour cell population in the early in vitro passages and in the parent tumour. By passage six, two different populations were present with disparate DNA content. Chromosome analysis of the two cloned populations indicated further genetic changes which had occurred between passages 7 and 19. Within individual populations chromosome number varied between cells. Growth rates between the two clonal populations also differed. When the two cell lines were transplanted into nude mice, tumours were produced, expressing genetic

instability. These data support the theory of clonal evolution, which suggests that tumour progression may result from sequential selection of variant subpopulations within a clonal population which enhances the genetic instability within the tumour cell population (Nowell, 1986).

There have also been reports of karyotypically heterogeneous tumours where cell populations arising from these tumours have responded inconsistently to chemotherapeutic agents and the cultured cells have exhibited variable stability (Shapiro and Shapiro, 1985). Human malignant gliomas were karyotypically heterogeneous with chromosome numbers per cell varying from 35-57 for tumours which were primarily diploid (Shapiro and Shapiro, 1985). Others were found, which were hyperdiploid, with up to several hundred chromosomes per cell. Hyperdiploid cells were unstable in culture and sensitive to chemotherapeutic agents compared to the near-diploid population which was more stable and more resistant to these agents. No karyotype analysis was performed on the parental or resistant cell lines. This would have been of interest check the consistency in the cells' ploidy especially in the resistant sublines with may have carried extrachromosomal elements.

Cloned continuous cell lines may also exhibit genetic instability and heterogeneity (Brattain et al., 1984). This presents an advantage and a disadvantage of cell culture as a human tumour model. On the positive side these cells may mirror the natural heterogeneity of tumours, but on the

other hand the responses in vitro of a selected subpopulation may not reflect the situation in the patient.

Another problem is that if a tumour is considered to be inherently heterogeneous, containing more than one cell type, then a clonal cell population may not reflect this. Either only one cell type may be cloned or if more than one cell type is selected only one may preferentially grow in culture and take over the cell population.

Cell lines may be used in disease orientated screening although the value of this is yet to be proven. Establishment of a cell line from a patient suffering with small-cell lung carcinoma showed drug resistance by gene amplification (Curt et al., 1983). The patient had been treated with methotrexate and initial analysis of the tumour cell line indicated a 2.4-fold amplification of the dihydrofolate reductase gene and an elevation in the target protein. The amplified genes were present in the form of double minute chromosomes and were lost as the cell line was cultured in drug free medium (Curt et al., 1983). The implication from this study is that prolonged exposure to methotrexate may have led to stable resistance in the form of homogeneously staining regions. Stopping methotrexate treatment would lead to loss of DM's and tumour reversion to methotrexate sensitivity. It may be worthwhile to monitor the length of time required in drug free medium before cells revert to sensitivity. These data may be useful in predicting how long a patient need be without methotrexate

once resistance to the drug has occurred, before treatment can recommence. If this time period is too long this approach may be unethical as the tumour would regrow without treatment, but the information may be used to direct combination chemotherapy. Combining drugs in cycles of treatment attempts to prevent the tumour from becoming resistant to any single agent as a result of continual exposure.

An alternative to the cell culture model is to use xenografts of human tumours in rodents (Hill, 1986). This is more expensive than tissue culture but encompasses features such as drug delivery and complicated treatment regimens. There are, however, fresh problems with the xenograft model, such as interpreting results of human tumours implanted into other species with interspecies complications such as drug transport, targeting, metabolism, absorption, distribution and excretion which may not necessarily reflect the in vivo situation in a human host. Xenograft models were not used in this study as once resistant sublines were available for xenografts time was insufficient. The xenograft model would, however, provide important data concerning methotrexate resistance in choriocarcinoma, using the resistant JAR sublines to establish the xenografts. Due to closer similarities in the animal model as regards drug delivery it may have been more informative to have attempted to produce resistant xenografts rather than in vitro resistant cell lines.

Realistically, the cell culture model is probably the best and most cost effective model presently available for the study of drug resistance in cancer. Ideally, results obtained in vitro should be compared with parallel experiments set up using xenografts where possible. Another model worth studying would be the analysis of cells from biopsies removed before and after treatment, although this is difficult practically in the case of choriocarcinoma. If this material was available primary cultures could be developed from the biopsies and these would be closer to the clinical situation than long established cell lines.

7.2 IN VITRO SELECTION OF RESISTANCE.

The selection of drug resistant cells in vitro is artificial because the methods used are not directly comparable to the clinical situation. Selection in vitro occurs either by intermittent or continuous drug exposure.

Intermittent drug exposure, as attempted with MAWI and JAR cells, did not produce drug resistant cells. Low risk choriocarcinomas are treated clinically with a five day course of methotrexate with weekly gaps between treatment (Bagshawe et al., 1973). The intermittent drug exposure protocol was an attempt to mimic the clinical situation. This intermittent treatment is designed to avoid bone marrow and other normal tissue toxicity, whilst killing as many tumour cells as possible. The in vitro protocol involved

exposing the cells to methotrexate and then allowing them to recover before retreatment. Clearly this approach, specifically the wait for tumour cell recovery, is vastly different from the true clinical situation and the only similarity may be the non-continuous exposure to methotrexate. Attempts to mimic clinical treatment schedules in vitro are largely impossible as drug concentrations and exposure durations are difficult to match (Hill, 1986). There is still relatively little human tumour pharmacokinetic data available for chemotherapeutic agents. Delivery, transport, absorption and excretion of drugs are extremely difficult to model in vitro.

Continuous exposure to methotrexate with stepwise increments in drug concentration can be used to produce methotrexate resistant cells (Schimke, 1988). Cells are exposed to initially low methotrexate concentrations until their growth rate is approaching that of the parental cell line and then the drug concentration is raised (typically doubled), and the process repeated until the required level of resistance is reached (Nunberg et al., 1978). If the increase in drug concentration is too great the frequency of amplification is dramatically lowered (Schimke, 1986). This procedure successfully produces methotrexate resistant cells, but little comparison can be made with the development of clinical resistance, because the initial treatment aims are opposite.

A more representative model which incorporates the advantages of in vitro cell lines and tumour biopsies is the use of multicellular spheroids grown directly from biopsy material (Wibe et al., 1984).

Selection of resistance should also be performed at a range of drug concentration as different degrees of resistance may be mediated by different resistance mechanisms (Frei et al., 1984).

7.3 DEGREE OF RESISTANCE.

Using stepwise increments in drug concentrations highly methotrexate resistant cell lines have been produced with up to 200-fold increases in dihydrofolate reductase in a murine Sarcoma 180 cell line (Schimke et al., 1977). These cells were considered to be 3000-fold resistant to methotrexate (Alt et al., 1978). Murine lymphoblastoid cells have been developed with >100,000-fold resistance to methotrexate containing a 300-fold elevation in dihydrofolate reductase activity compared to the parental cell line (Dolnick et al., 1979). The methotrexate resistant cell lines produced in this study were 2.5-, 3.6-, 6.8- and >8.3-fold resistant and 1.12-, 1.68-, 1.82- and 2.68-fold DHFR gene amplified respectively for the B^0, B^1, B^2 and B^3 sublines. Clearly there is a vast difference between the different sets of data.

Where gene amplification has been studied as a mechanism of drug resistance the highest possible levels have been produced (Schimke et al., 1977; Alt et al., 1978; Dolnick et al., 1979). This may not reflect the true clinical situation. Data on gene amplification in drug resistant human tumours is limited. A cell line produced from a small-cell lung carcinoma treated with methotrexate was found to have 2.4-fold amplified DHFR genes (Curt et al., 1983). This biopsy was from a patient who had initially responded to methotrexate but who had relapsed. The implication from both this and my study is that the low levels of resistance and amplification may be more clinically relevant than the massively resistant and amplified murine models. This implies that to advance the understanding of clinical resistance using in vitro cell lines low level methotrexate resistance and its mechanisms may be more informative. It would have been interesting to obtain resistant choriocarcinoma biopsies to monitor the level, if any, of DHFR gene amplification and mRNA expression in patients treated with methotrexate who have relapsed.

7.4 STABILITY OF RESISTANCE.

Methotrexate resistant cells with amplified dihydrofolate reductase genes may be stable, with the amplified genes located chromosomally as homogeneously staining regions, or unstable with extrachromosomally located genes known as double minutes (Schimke et al., 1981). Both forms may be

present in the same cell population but are mutually exclusive to individual cells (Cowell, 1982). Initial resistance due to gene amplification is normally an unstable event coupled with double minutes which are converted to homogeneously staining regions with continued selection (Warr and Atkinson, 1988). Double minutes, indicative of unstable resistance, are rapidly lost, along with the drug resistance, without continued selection (Kaufman et al., 1981). It was found that the growth rate of MTX-resistant S-180 cells was inversely proportional to the number of double minute chromosomes. Consequently in the absence of drug cells containing fewer DM's were positively selected. DM's lacking centromeres and segregating randomly during mitosis are therefore readily lost from the population. Stability is, however, a relative term and when stably resistant cells containing amplified DHFR genes in the form of HSR's are grown out of methotrexate the amplified genes are slowly lost (Biedler et al., 1980). The rate of loss is much slower than in DM containing cells.

Karyotype analysis was not performed on the methotrexate resistant JAR sublines. This would have allowed the location of amplified genes, but would not have told us anything about RNA stability. No information was obtained on the stability of resistance, either by monitoring the presence of DM's and/or HSR's, or by looking at gene amplification and expression in the resistant cells when they are grown in methotrexate-free medium. The resistant sublines could have been grown in a methotrexate-free environment for several

months and DNA and RNA samples extracted at regular intervals. Any loss of mRNA expression or gene amplification could then be quantified and a time scale attached to this loss. Such a study would elucidate whether elevated RNA or gene copy number was the more stable by monitoring and comparing the rate, and order, at which both were lost.

7.5 FUTURE PROSPECTS.

The stability of resistance of the derived methotrexate resistant choriocarcinoma cells would be of interest and prolonged growth of the resistant cells in methotrexate-free medium coupled with regular DNA and RNA extraction would help to monitor the progressive loss of the amplified genes and loss of mRNA expression. Similarly further analysis of these cell lines would be useful to determine whether or not gene amplification is the only mechanism of resistance present. Although no uptake defect was found there may be defective polyglutamate formation present. Another possibility is that the increased dihydrofolate reductase protein isolated from these cells may be altered in some way from the parental protein. This could be analysed using SDS polyacrylamide gel electrophoresis. Other mechanisms might include altered drug metabolism, drug binding and the degree of DNA repair.

Polymerase chain reaction (PCR) would allow much lower cell numbers to be required for detection, but as yet the

technique is not sufficiently advanced for low level quantitative analysis of gene copy numbers (Rothenberg and Ling, 1989). The RNA protection assay would also allow more sensitive detection of mRNA levels and avoid any possible cross-contamination from structurally related genes, either known or unknown (Goldstein et al., 1990; Ueda et al., 1989).

Further analysis of P-glycoprotein levels in colonic tumours would also be worthwhile. Although no conclusive results can be drawn from the small sample size studied here the implication of a link between P-glycoprotein mRNA expression and Dukes' grading may well be relevant. Dukes' grade C carries a poor prognosis for patients with adenocarcinoma of the colon and response to therapy and survival may be linked to the P-glycoprotein levels, making these levels of prognostic value for survival. It would be interesting to study the uptake of multidrug resistance drugs, such as adriamycin and actinomycin D whose impaired cytotoxicity has been correlated with elevated expression of the P-glycoprotein, in the Dukes' grade C tumours, or even look at uptake in primary cultured cell lines from the biopsies with elevated mRNA expression. Similarly further analysis of P-glycoprotein levels in individual patients and in their normal colonic tissue may aid the understanding of why colonic tumours are generally insensitive to most chemotherapy.

7.6 SUMMARY.

The main conclusions from the work outlined in this thesis are:

(1) Low level continuous and increasing exposure to methotrexate induces resistance in JAR choriocarcinoma cells in vitro more readily than intermittent exposure, which more closely resembles the clinical regimen.

(2) A similar exposure to methotrexate did not induce resistance in MAWI colorectal cancer cells beyond that initially present (in relation to untreated JAR cells).

(3) A low level of amplification of dihydrofolate reductase may be sufficient to have caused the resistance induced experimentally. Defective uptake was excluded, but changes in polyglutamation were not formally excluded. The increase at mRNA and protein level were consistent with the amplified DNA. This implies that there was no further enhancement beyond the DNA level. If the resistance is indeed solely attributable to minor increases (1.12 - 2.68-fold) of DHFR at the gene level, the clinical relevance of such small increases should be explored in biopsy material.

(4) In 31 biopsy specimens from colonic adenocarcinoma and adjacent normal colonic tissue (31 pairs), there was a statistically significant correlation between elevated P-glycoprotein mRNA expression and Dukes' grading. This

observation, if maintained in a larger collection of samples, may be pertinent in identifying, by in situ hybridisation, patients who are least likely to benefit from treatment with drugs associated with the multidrug resistant phenotype. The factors which predispose tumours of colonic origin to evade chemotherapy may emerge from a close study of small elevations of P-glycoprotein mRNA expression and the regulatory processes responsible for these.

APPENDIX 1. QUALITY CONTROL.

A1.1 Plasmid restriction mapping.

The plasmid, pSVDHFR, did not map as expected from previous data and consequently further restriction mapping was required.

Digests of the plasmid with one restriction endonuclease were performed to determine the number of sites for each enzyme and the position of these sites relative to one another. Double digests using two enzymes together, were performed such that the sites from each endonuclease could be mapped relative to one another. In order to determine fragment size, restriction markers with known molecular weights were also run on the agarose gels. The distance migrated under electrophoresis was plotted against molecular weight to generate a standard curve (Figure A1.1; Table A1.1). From this curve all restriction bands on that gel could be sized.

Single digests indicated that all enzymes, apart from Bcl I and Bgl II, cleaved the DNA at one or more sites (Figure A1.2, Table A1.2a).

A possible reason for the apparently undigested plasmid samples obtained from incubation with Bcl I and Bgl II was due to the homology shown between the sites recognised by these two restriction endonucleases, Table A1.3. Digestion

by each enzyme produces the same 5' and 3' sticky ends and this may cause problems in digestion. Further evidence that this was the case that the two sites had ligated effectively destroying them both could be provided by sequence analysis of the site in question.

Double restriction digestion was performed twice: One digest was electrophoresed through a 1% agarose gel to isolate fragments greater than 500 bp in size and another through 2% agarose to isolate fragments below 500 bp in size (Figure A1.3, Table A1.2b).

From this restriction endonuclease analysis it was possible to generate an extensive restriction map of the plasmid pSVDHFR, Figure A1.4. From this map it was decided, on the basis of size, to isolate the 500 bp fragment from the Sac I / Hind III digest for use as a cDNA probe using techniques previously described (Section 2.3.6).

Confirmation of the restriction map was provided by southern blot analysis. Double restriction endonuclease digests of the plasmid were probed using the 500 bp isolated cDNA fragment as a probe. In each case the probe showed preferential hybridisation to the fragment containing this insert thus confirming the previously constructed plasmid map, Figure A1.5.

Similar techniques were used to construct a restriction map of the plasmid pCHP1 (Figure A1.6). The whole insert

isolated from this plasmid was the 600 bp Eco RI / Eco RI site and this was isolated for further use as a cDNA probe.

A1.2 Slot Blotting.

The degree of hybridisation is directly proportional to gene copy number when a suitable gene specific cDNA or riboprobe is used for hybridisation. This relationship allows the direct quantification of relative gene copy number from autoradiographic signals using a densitometer once the samples have been normalised using an internal control.

Human β-cytoskeletal actin genes are present in multiple copies in the human genome and are highly conserved. There are in excess of 20 genes and pseudogenes throughout the genome (Ponte et al., 1983). The β-actin specific cDNA probe was used to indicate the relationship between hybridisation signal and DNA. The linearity of this relationship is shown in figure A1.7 and the mean from five experiments calculated (Table A1.4). When the results are expressed graphically, figure A1.8, there is a clear direct relationship between DNA quantity and intensity of hybridisation signal, with intensity being proportional to quantity. This relationship allows the quantification of gene copy numbers from unknown samples to gene specific probes by duplicate filter hybridisation using the β-actin probe.

A1.3 Southern Blot Analysis.

Southern blotting provides information not available from slot blot analysis. Hybridisation to DNA applied and fixed to solid support by slot blot provides no reference to actual gene size within the genome. This was a problem in that although the probes may be specific slot blot data do not provide any evidence of specificity of hybridisation. Southern blot analysis was performed as confirmation of the specificity of hybridisation.

There are certain inherent limitations in the use of Southern blot analysis. Unlike slot blot analysis the DNA is not applied directly to the filter and therefore Southern blots are not as comparable between different experiments as slot blots are. Although transfer of DNA is maximised under correct conditions the degree of transfer always varies slightly. Large fragments, above 10 kb, do not transfer efficiently out of agarose gels. At the other end of the scale, small fragments, under 200 bp, may not bind efficiently either. Southern blot analysis is also laborious, expensive and time consuming and is not suitable for processing large numbers of samples.

A1.3.1 Dihydrofolate Reductase Gene Analysis.

The dihydrofolate reductase gene contains a minimum of 5 intervening sequences (introns) and spans a minimum of 32 kilobase pairs in the human genome (Bostock and Clark, 1983).

Before agarose gel electrophoresis the genomic DNA was restriction endonuclease digested with Eco RI. Hybridisation was evident when DNA extracted from L1210 and L1210 R7A cell lines was blotted and probed with no differences noted between the organisation of the gene between the sensitive and highly methotrexate resistant cell lines, Figure A1.9. This implies that the organisation of the gene seen was that of the wild type gene and slot blot hybridisation was to the correct species. This allows the slot blot results to be accepted as a genuine indication of the copy number of the DHFR gene.

Southern blot analysis of methotrexate resistant L1210 R7A cells indicated the presence of 5 bands which hybridised to the pSVDHFR probe. Their respective sizes were 18.5kb, 15.0kb, 8.6kb, 6.3kb and 5.8kb. DNA from sensitive L1210 cells and MAWI and JAR cells showed poor comparative hybridisation and only the lower molecular weight bands were visible even when the quantity of DNA loaded was raised and the autoradiography time increased.

The Southern blot technique was not found to be sufficiently sensitive for the low gene copy numbers present in MAWI and JAR cell lines unless approximately 20μg DNA was transferred onto the solid support. Although a positive signal using 10μg DNA could be detected it involved autoradiography times in excess of 10 days and this made the procedure extremely slow. This was also true with the low levels of DHFR gene copy number expressed in the tumour samples and consequently it was not viable to repeatedly blot these samples by this method when reliable results were obtained from slot blot analysis where only 1 μg of limited material was required.

Previous reports of amplified DHFR genes have indicated the presence of up to 5 pseudogenes which are not all amplified during selection, together with one functional gene that is always amplified (Hahn et al., 1987). This may be reflected in the hybridisation pattern seen in the methotrexate resistant JAR cells which may also differ slightly due to the probe being from a non-human (rodent) source. A similar pattern of hybridisation was seen in sensitive and methotrexate resistant JAR cells, although the sensitive lines required higher concentrations of DNA and longer exposure times for visualisation. This confirmed that hybridisation was to the correct species and no gene rearrangement had occurred in the transformation from JAR cell sensitivity to methotrexate resistance.

A1.3.2 P-glycoprotein Gene Analysis.

The P-glycoprotein is encoded by a multigene family of which not all are co-amplified in multidrug resistant cells (Deffie et al., 1988). DNA extracts from CH^RC5 and AUXB1 cells, multidrug resistant and sensitive cell lines respectively, as well as DNA extracted from MAWI cells, JAR cells and tumour extracts was restriction endonuclease digested with Hind III prior to agarose gel electrophoresis.

Hybridisation of the pCHP1 probe was seen to 7 separate fragments in the multidrug resistant cell line, CH^RC5, but low level hybridisation was only seen in the sensitive parental cell line when increasing the amount of DNA electrophoresed, Figure A1.10. The fragments identified were approximately 12kb, 6kb, 5.5kb, 3.5kb, 2.3kb, 1.9kb and 1.0kb in size (Riordan et al., 1985; Assaraf et al., 1989).

The autoradiograph shows the fact that not all bands were equally co-amplified in the multidrug resistant cells.This reflects the P-glycoprotein being coded for by a multigene family, not all of which are vital to the multidrug resistant phenotype. No evidence of the P-glycoprotein gene was seen in DNA extracted from MAWI or JAR cells.

These Southern blots confirmed that the hybridisation seen in the corresponding DNA slot blots was specific and related to P-glycoprotein gene copy number.

A1.4 Northern Blot Analysis.

Northern blot analysis, for RNA extracts, was performed for similar reasons as that for Southern blot analysis: to overcome the inherent limitations of slot blot analysis. Northern blot analysis was required to confirm the specificity of hybridisation seen in slot blot analysis.

When RNA was transferred to a solid support and hybridised at low stringency DHFR and P-glycoprotein cDNA probes hybridised to the 28S and 18S ribosomal RNA bands. This was invariably removed by further stringent washes; however because the bands are of consistent size their migration when electrophoresed was clear and they were distinct from positive hybridisation to either probe.

A1.4.1 Dihydrofolate Reductase mRNA Analysis.

Northern blot analysis of RNA extracted from L1210, sensitive, and L1210 R7A, methotrexate resistant, cells indicated hybridisation to two species, approximately 1.5 and 0.8 kb in size, Figure A1.11. The two bands were clearly present in higher copy number in the drug resistant cells but the messenger RNA organisation within the two cell lines was clearly the same. This confirmed that the hybridisation seen between the pSVDHFR cDNA probe and RNA extracts on slot

blot analysis was specific and the blots were a measurement of dihydrofolate reductase mRNA copy numbers.

A1.4.2 P-glycoprotein mRNA Analysis.

P-glycoprotein has been shown to be encoded by a mRNA species of approximately 4.5 kb in size (section 1.6.5.2) Northern blotting analysis of RNA extracted from AUXB1 and CH^RC5 showed specific hybridisation to a 4.5 kb RNA species, Figure A1.12. The degree of hybridisation reflected the elevated levels of gene copy and mRNA copy number which exist in the multidrug resistant cells, CH^RC5, when compared with the sensitive parental cell line, AUXB1. This confirms the hybridisation results from slot blot analysis of RNA samples using the pCHP1 cDNA probe.

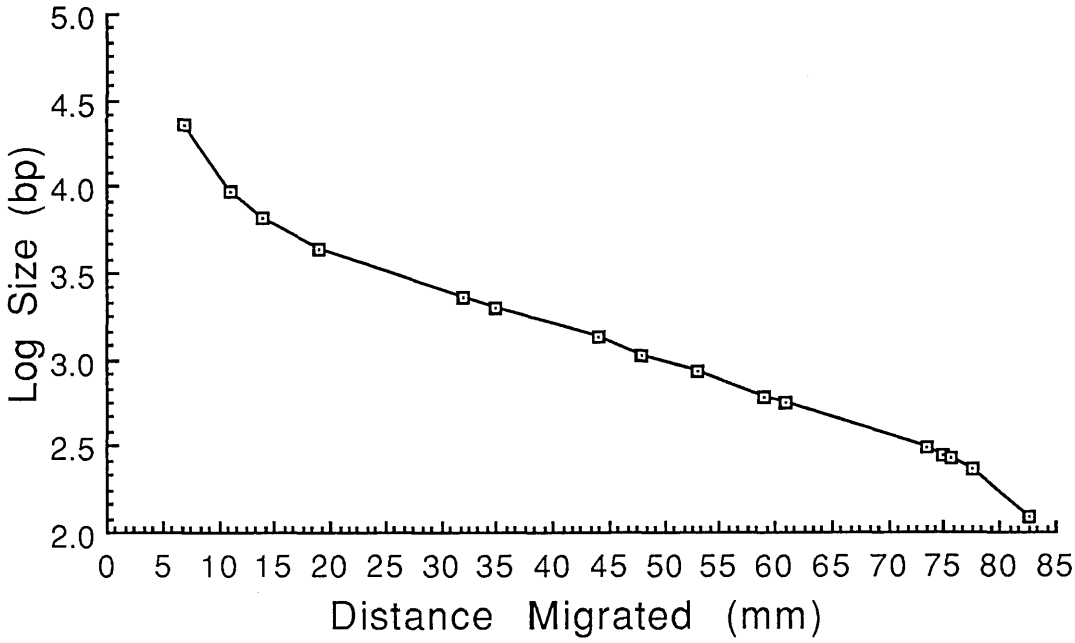
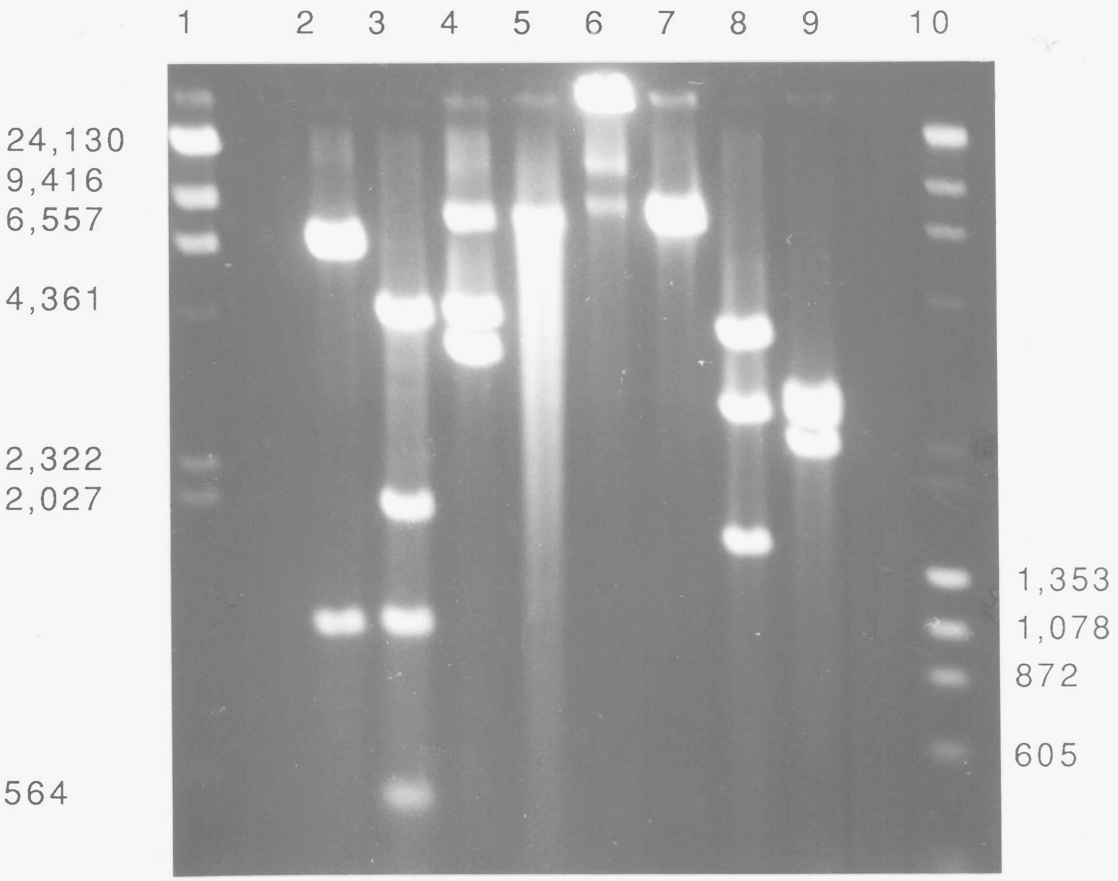


Figure A1.1 Standard curve of migration of DNA markers.

Lambda-Hind III Fragment siz (bp)	Phi X174-Hae III Fragment size (bp)	Distance migrated (mm)
23,130		7.0
9,416		11.0
6,557		14.0
4,361		19.0
2,322		32.0
2,027		35.0
	1,353	44.0
	1,078	48.0
	872	53.0
	605	59.0
564		61.0
	310	73.5
	281	75.0
	271	75.5
	234	77.5
125		82.5

Table A1.1 DNA Markers - fragment size and distance migrated.



Lane 1: Lambda Hind III markers.

Lane 2: pSVDHFR Digested With Bam HI.
Lane 3: pSVDHFR Digested With Hind III.
Lane 4: pSVDHFR Digested With Eco RI.
Lane 5: pSVDHFR Digested With Sac I.
Lane 6: pSVDHFR Digested With Bcl I.
Lane 7: pSVDHFR Digested With Bgl II.
Lane 8: pSVDHFR Digested With Pst I.
Lane 9: pSVDHFR Digested With Bgl I.

Lane 10: Lambda Hind III + PhiX174 Hae III markers.

Figure A1.2 Single Restriction Endonucease Mapping of pSVDHFR.

Restriction Endonuclease	Fragments isolated (bp)
Bam HI	6,800; 1,200
Hind III	4.400; 1,800; 1,100; 450; 220
Eco RI	4,360; 3,650
Sac I	8,000
Bcl I	--------
Bgl II	--------
Pst I	4,200; 3,800
Bgl I	2,800; 2,700; 2,300; 250

Table A1.2a **Single Restriction Endonuclease Digestion Fragment Size Analysis**

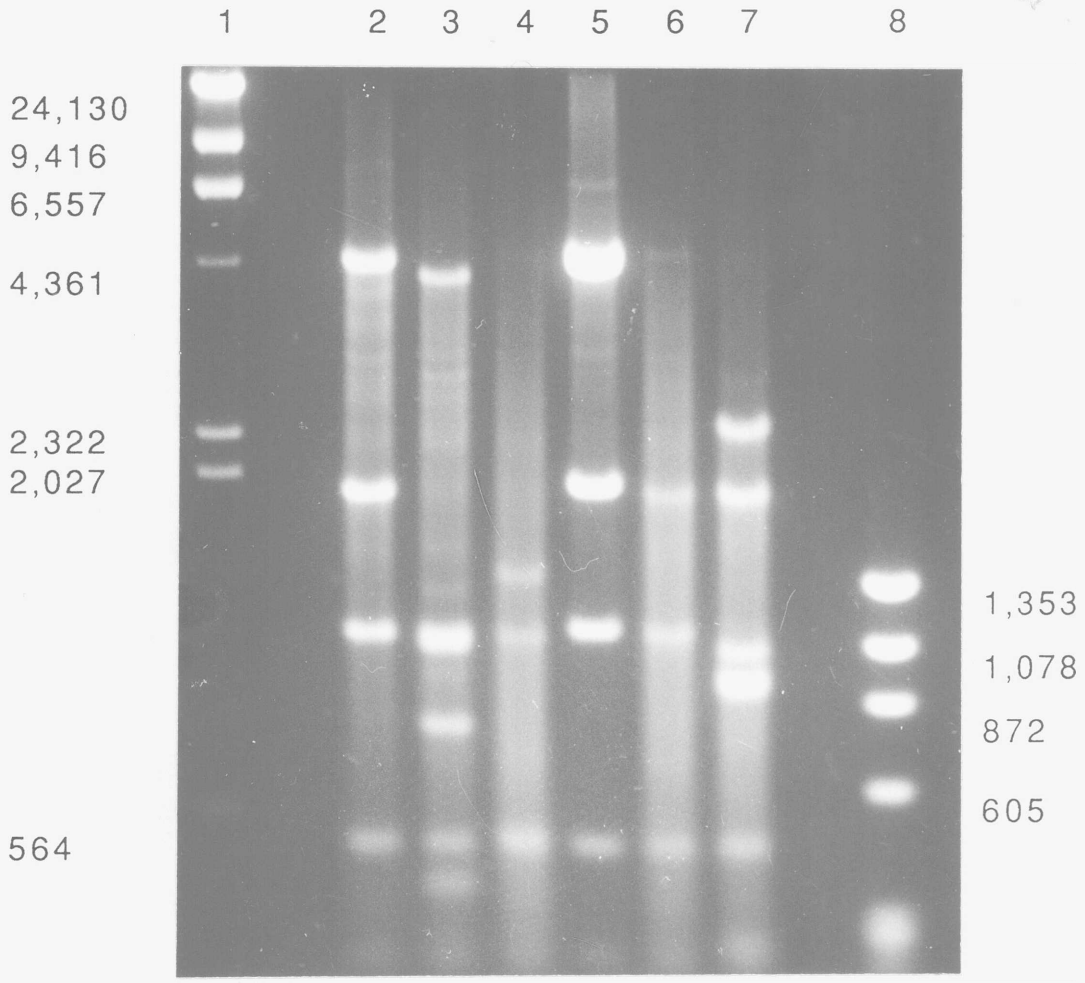
Restriction Endonucleases	Fragments isolated (bp)
Hind III + Hind III	4.400; 1,800; 1,100; 450; 220
Bam HI + Hind III	4,100; 1,100; 1,000; 800, 450; 350; 220
Sac I + Hind III	4,400; 1,300; 1,100; 500; 450; 220
Bcl I[a] + Hind III	4.400; 1,800; 1,100; 450; 220
Bgl II[a] + Hind III	4.400; 1,800; 1,100; 450; 220
Bgl I + Hind III	2,300; 1,800; 1,050; 950; 900; 450; 250; 220

(a) Restriction endonuclease did not cut.

Table A1.2b **Double Restriction Endonuclease Digestion Fragment Size Analysis**

Enzyme	Isolated from..?	Recognition site
BamHI	Bacillus amyloliquefaciens	5'...G*GATCC...3' 3'...CCTAG*G...5'
BclI	Bacillus caldolyticus	5'...T*GATCA...3' 3'...ACTAG*T...5'
BglI	Bacillus globigii	5'...GCCNNNN*NGGC...3' 3'...CGGN*NNNNCCG...5'
BglII	Bacillus globigii	5'...A*GATCT...3' 3'...TCTAG*A...5'
EcoRI	Escherichia coli	5'...G*AATTC...3' 3'...CTTAA*G...5"
HaeIII	Haemophilus aegyptius	5'...GG*CC...3' 3'...CC*GG...5'
HindIII	Haemophilus influenza	5'...A*AGCTT...3' 3'...TTCGA*A...5'
NarI	Nocardia argentinensis	5'...GG*CGCC...3' 3'...CCGC*GG...5'
NdeI	Neisseria denitrificams	5'...CA*TATG...3' 3'...GTAT*AC...5'
PstI	Providencia stuarti	5'...CTGCA*G...3' 3'...G*ACGTC...5'
SacI	Streptomyces achromogenes	5'...GAGCT*C...3' 3'...C*TCGAG...5'

Table A1.3 Restriction Enzyme Recognition Sequences.



Lane 1: Lambda Hind III markers.

Lane 2: pSVDHFR Digested With Hind III + Hind III.
Lane 3: pSVDHFR Digested With Bam HI + Hind III.
Lane 4: pSVDHFR Digested With Sac I + Hind III.
Lane 5: pSVDHFR Digested With Bcl I + Hind III.
Lane 6: pSVDHFR Digested With Bgl II + Hind III.
Lane 7: pSVDHFR Digested With Bgl I + Hind III.

Lane 8: PhiX174 Hae III markers.

Figure A1.3 Double Digest Restriction Endonuclease Mapping of pSVDHFR.

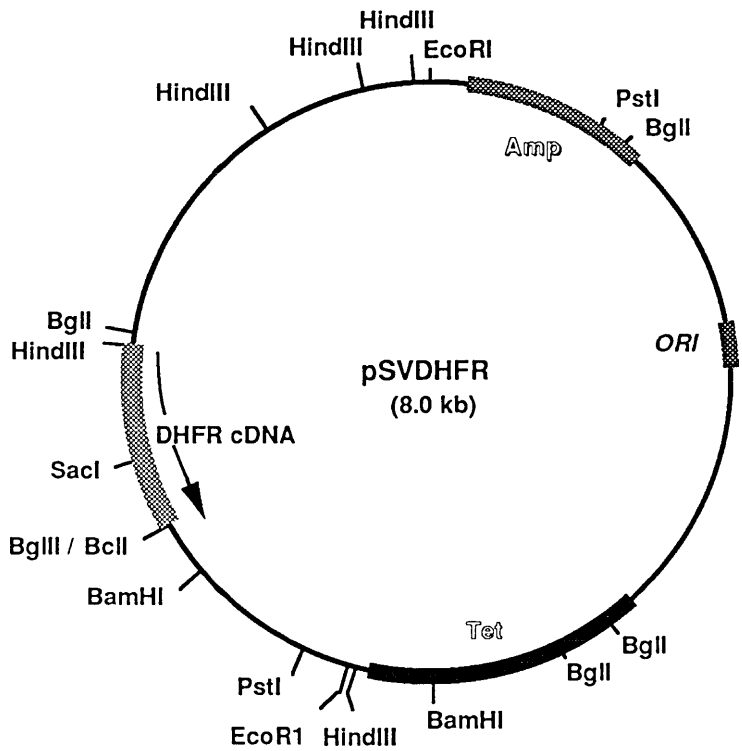
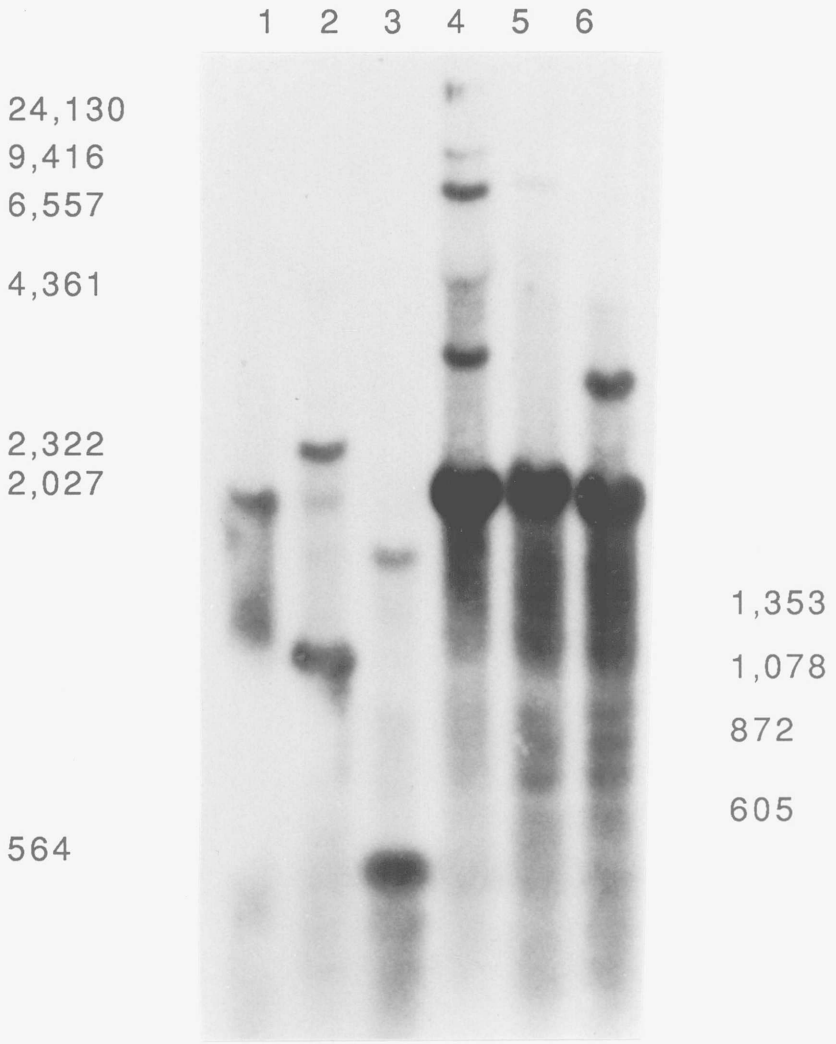


Figure A1.4 Plasmid Map - pSVDHFR.



Lane 1: pSVDHFR Digested With Hind III + Hind III.
Lane 2: pSVDHFR Digested With Bam HI + Hind III.
Lane 3: pSVDHFR Digested With Sac I + Hind III.
Lane 4: pSVDHFR Digested With Bcl I + Hind III.
Lane 5: pSVDHFR Digested With Bgl II + Hind III.
Lane 6: pSVDHFR Digested With Bgl I + Hind III.

Figure A1.5 Southern Blot of Double Restriction Endonuclease Digest of pSVDHFR.

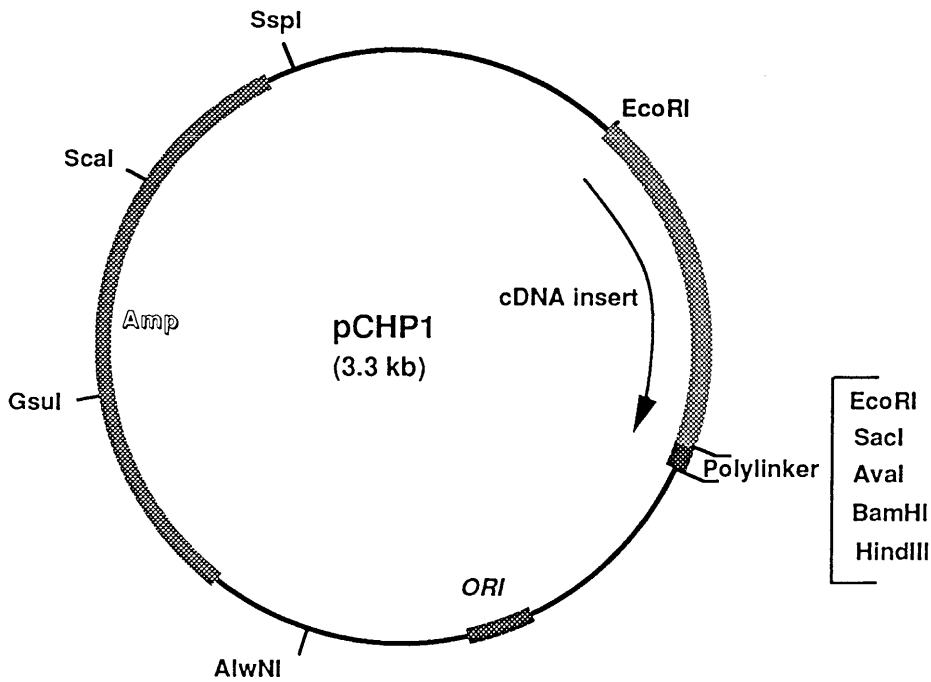


Figure A1.6 Plasmid Map - pCHP1.

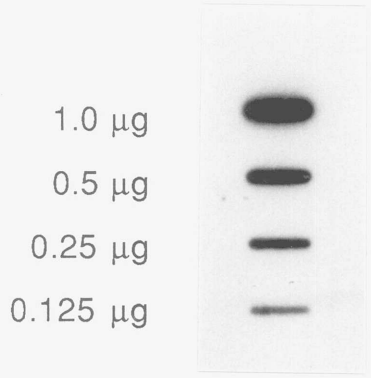


Figure A1.7 **Positive Human DNA Controls, Hybridised to Actin; Exposed for 12 Hours.**

	DNA (µg)			
	1.0	0.5	0.25	0.125
INTENSITY (O.D. units)	12,125	7,100	4,005	2,055
	12,080	6,800	3,215	1,640
	13,070	6,235	3,790	1,835
	12,005	6,475	3,380	1,470
	11,995	7,005	3,445	1,685
Mean	12,255	6,723	3,567	1,737
St.Dev.	410	325	288	197

Table A1.4 **Relative Intensity of Hybridisation Compared to Quantity of DNA Loaded, Probed with Actin.**

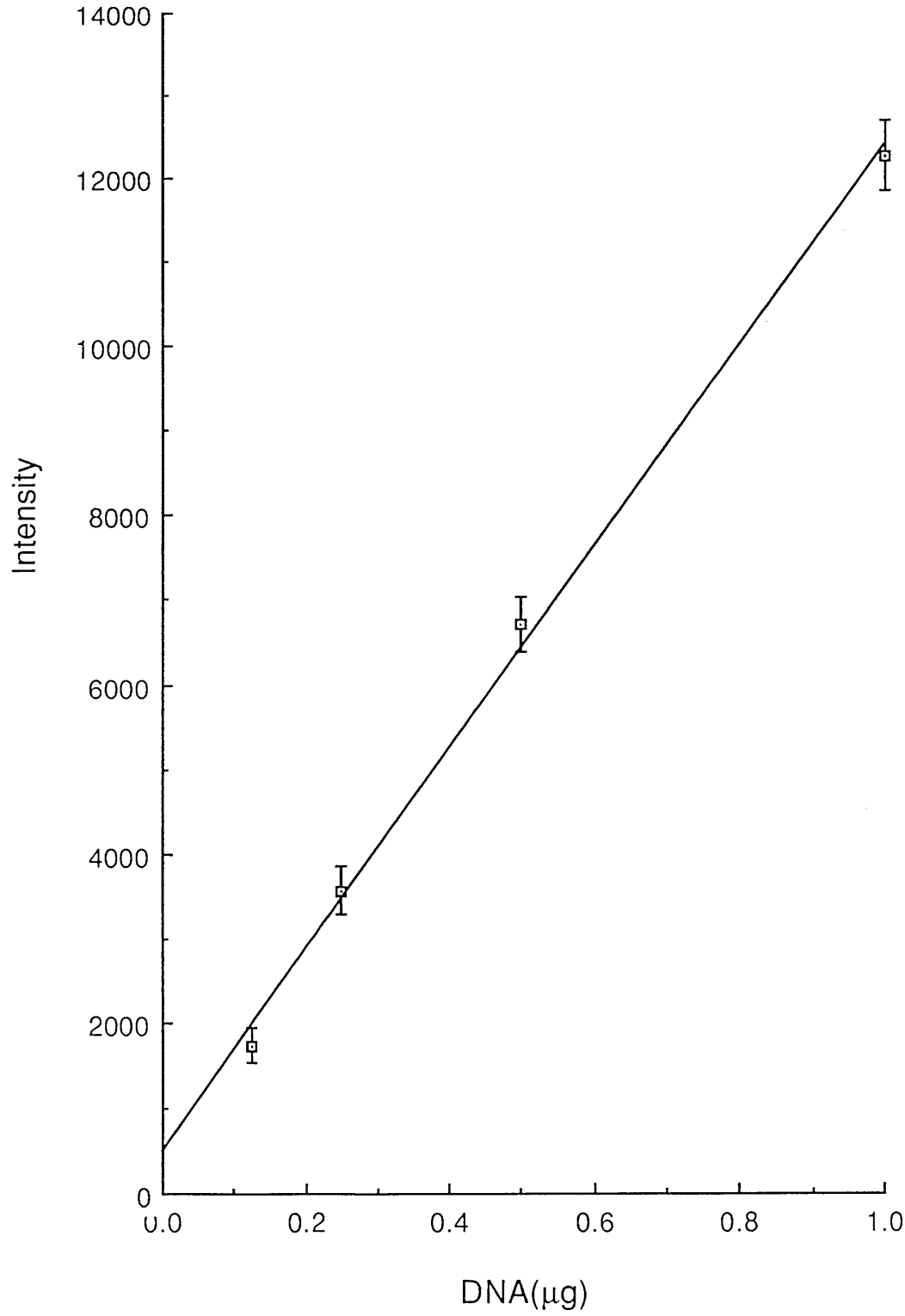
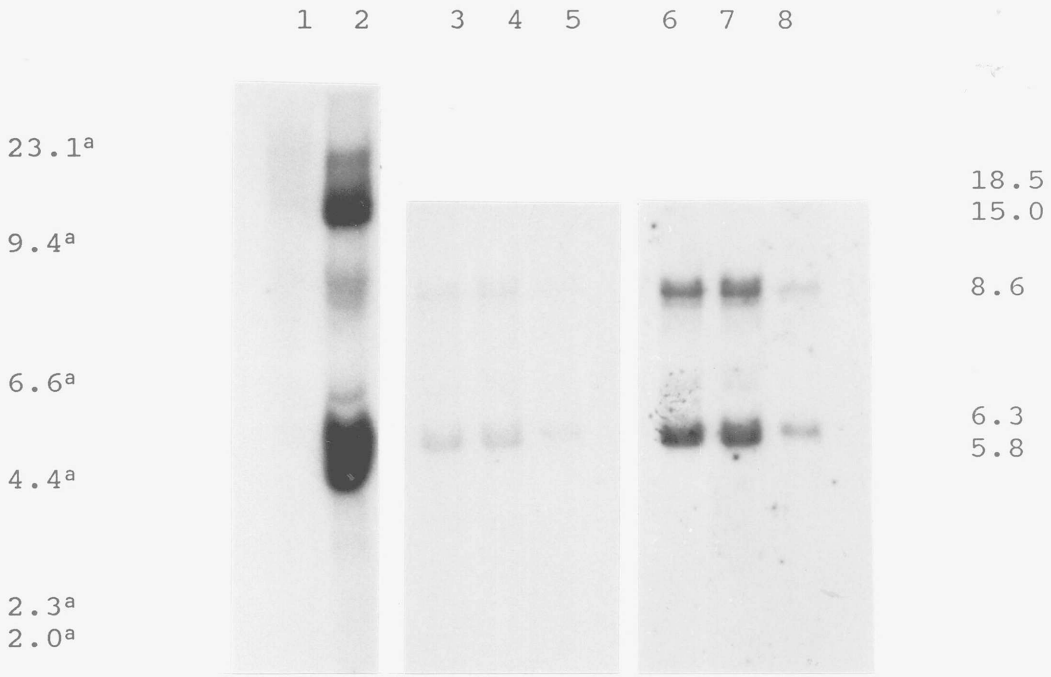


Figure A1.8 Linear Relationship Between Hybridisation Signal Intensity and DNA Loaded.



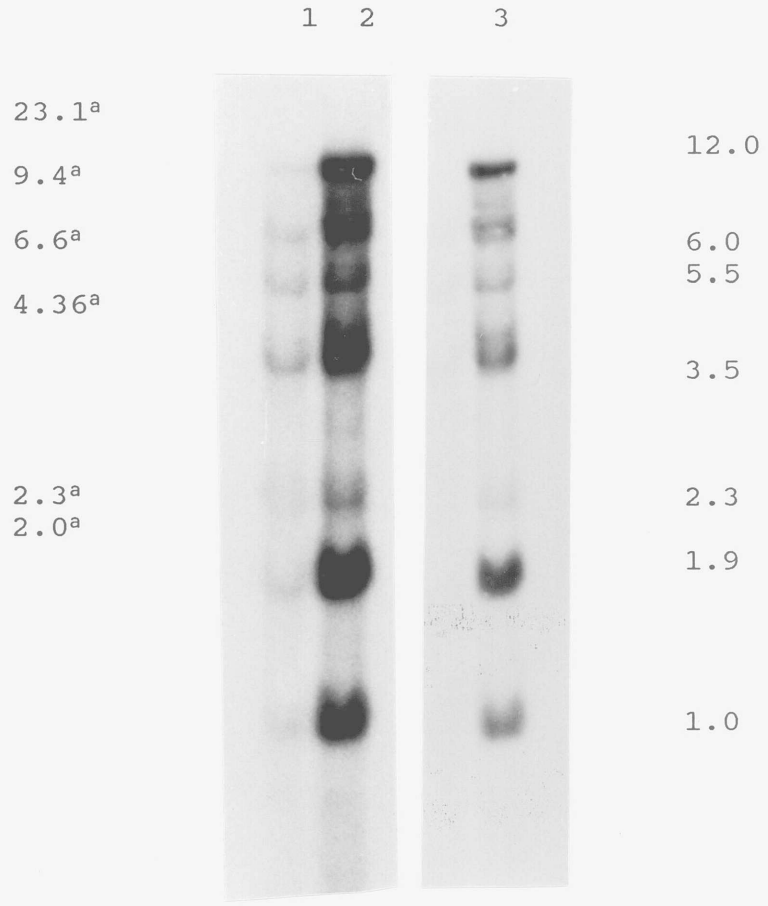
[a] = Molecular weight markers

Figure A1.9 **Southern Blot Analysis of the Dihydrofolate Reductase Gene.**

Lane 1: L1210
Lane 2: L1210 R7A
Lane 3: JAR subline B^3
Lane 4: JAR subline B^2
Lane 5: JAR subline B^1
Lane 6: JAR subline B^3
Lane 7: JAR subline B^2
Lane 8: JAR subline B^1

Lanes 1-5: 1 hour exposure at -70°C.
Lanes 6-8: Overnight exposure at -70°C.

10μg DNA digested with EcoR1 and electrophoresed on a 1% agarose gel before transfer by capillary action onto Hybond-N membrane. DNA fixed to membrane by u.v. irradiation and baking. Prehybridistion and hybridisation to radiolabelled pSVDHFR probe at 65°C, followed by washing at 65°C in 0.5 x SSC / 0.1% SDS.



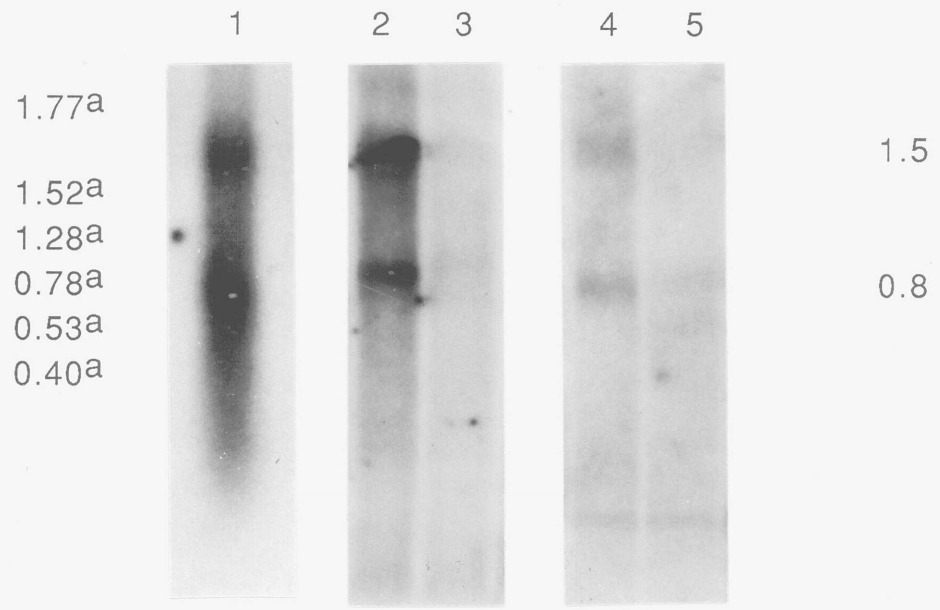
[a] = Molecular weight markers

Figure A1.10 **Southern Blot Analysis of the P-glycoprotein Gene.**

Lane 1: 20μg AUXB1
Lane 2: 10μg CH^RC5
Lane 3: 10μg CH^RC5

Lanes 1&2: 48 hour exposure at -70°C.
Lane 3: 16 hour exposure at -70°C.

DNA digested with HindIII and electrophoresed on a 1% agarose gel before transfer by capillary action onto Hybond-N membrane. DNA fixed to membrane by u.v. irradiation and baking. Prehybridistion and hybridisation to radiolabelled pSVDHFR probe at 65°C, followed by washing at 65°C in 0.5 x SSC / 0.1% SDS.



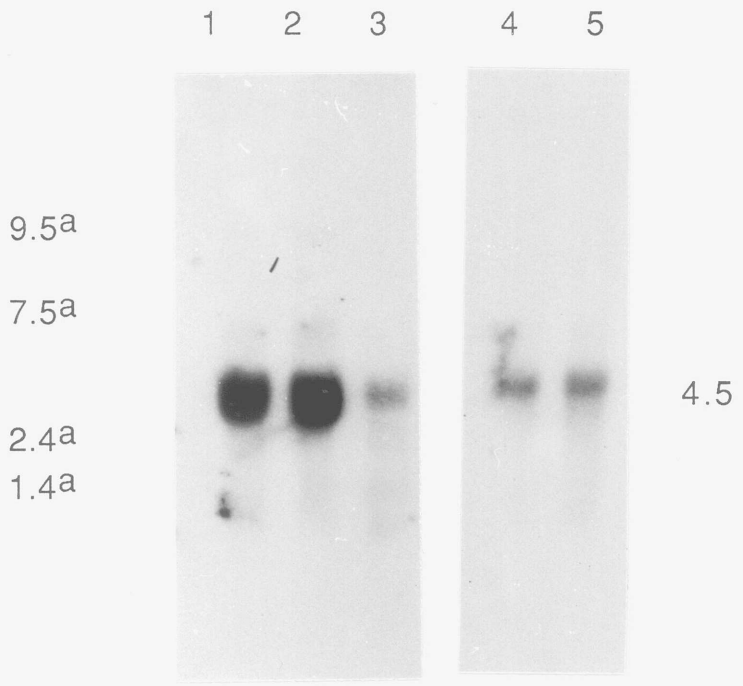
a: RNA molecular weight markers.

1: 10 µg RNA, extracted from L1210 R7A cells, glyoxylated.
2: 10 µg RNA, extracted from L1210 R7A cells, glyoxylated.
3: 10 µg RNA, extracted from L1210 cells, glyoxylated.
4: 20 µg RNA, extracted from L1210 cells, glyoxylated.
5: 15 µg RNA, extracted from L1210 cells, glyoxylated.

1: Autoradiographed at -70°C, 72 hours.
2-5: Autoradiographed at -70°C, 24 hours.

Figure A1.11 **DHFR Northern Blot Analysis.**

RNA glyoxylated and run on a 1% agarose gel in circulating phosphate buffer (pH 6.8), run at 100V for 3 hours. Transfer by capillary action was on to Hybond-N paper which was uv irradiated and baked after nucleic acid transfer. Filter prehybidised, hybridised and washed at 65°C.



a: RNA molecular weight markers.

1: 10 µg RNA, extracted from CH^RC5 cells, glyoxylated.
2: 15 µg RNA, extracted from CH^RC5 cells, glyoxylated.
3: 10 µg RNA, extracted from AUXB1 cells, glyoxylated.
4: 15 µg RNA, extracted from AUXB1 cells, glyoxylated.
5: 20 µg RNA, extracted from AUXB1 cells, glyoxylated.

1-5: Autoradiographed at -70°C, 24 hours.

Figure A1.12 **P-glycoprotein Northern Blot Analysis.**

RNA glyoxylated and run on a 1% agarose gel in circulating phosphate buffer (pH 6.8), run at 100V for 3 hours. Transfer by capillary action was on to Hybond-N paper which was uv irradiated and baked after nucleic acid transfer. Filter prehybidised, hybridised and washed at 65°C.

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