Acquisition and loss of amplified genes

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DISSERTATION

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by

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To my
Family,
Friends
and
Teachers
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LIST OF ABBREVIATIONS

DHFR, dihydrofolate reductase

dhfr, dihydrofolate reductase gene

TS, thymidylate synthase

SHMT, serine hydroxymethyl transferase

THF, tetrahydrofolate

DHF, dihydrofolate

f-MTX, fluorescein methotrexate

α-globin, alpha-globin

dTMP, deoxythymidine monophosphate

dTTP, deoxythymidine triphosphate

dUMP, deoxyuridine monophosphate

Ser, serine

Gly, glycine

Glu, glutamic acid
CHAPTER I
INTRODUCTION

PURPOSE

Normal growth and differentiation of mammalian cells are highly regulated processes. Breakdown of cellular regulatory mechanisms can occur as a result of inappropriate expression of cellular oncogenes or loss of tumor suppressor genes. Both benign and malignant tumors can result from the loss of normal cellular regulation. In comparison to normal tissues and benign tumors, malignant tumors show greater cell population heterogeneity with respect to growth rate, morphology, cell surface antigens and other aspects of cellular phenotype. The tumor cell heterogeneity is thought to be due to genetic instability associated with the transformed phenotype. This genetic instability is the basis for tumor progression and leads to increasing malignancy associated with invasiveness, metastasis and drug resistance (Nowell, 1982; Heppner, 1984; Fiedler and Hart, 1982; Dexter and Calabresi, 1982). Facile drug resistance is thus thought to be one aspect of genetic instability. Although, the relationship between the expression of specific oncogenes and tumor progression is poorly understood (Nicholson, 1987; Mareel and Roy, 1986; Barbacid, 1986), the possible effects of oncogene expression on drug resistance in tumor cells has been an area of significant interest. Genetic instability in progressing tumors is also evidenced by chromosomal changes, including rearrangements and sequence...
amplifications involving cellular proto-oncogenes (Yunis, 1983). There is evidence that expression of some oncogenes can increase genetic instability (Cerni, Mogneau and Cuzin, 1987).

Methotrexate is one of the most useful antineoplastic drugs in use today (Calabresi and Parks, 1985). However, cancer cell resistance to methotrexate is a common occurrence. Resistance to cytotoxic drugs, like metastatic potential and invasiveness, is considered a part of spectrum of tumor progression and may be a result of genetic instability in the evolving tumor cells (Ling, et al, 1985; Nowell, 1976; Clifone and Fidler, 1981). Since amplification of the dihydrofolate reductase (dhfr) gene is one of the major mechanisms of resistance to methotrexate (Sirotnak et al, 1981; Barsoum and Varshavsky, 1983; Hamlin et al, 1984; Tlsty et al, 1982), the frequency of methotrexate resistance is generally taken as a measure of genetic instability (Sager et al, 1985; Cillo et al, 1987; Chambers, Harris and Grundy, 1988). Based on these reports, one of the purposes of this study was to explore the possibility that oncogene expression in mammalian cells might influence resistance to anticancer drugs by increasing genetic instability.

NIH 3T3 cells expressing three different polyoma virus oncogenes (polyoma T antigens) were studied for their ability to develop resistance to methotrexate. The experimental results detailed in this study, indicate that expression of these oncogenes can have a great influence on the frequency of methotrexate resistance. Mechanisms other than gene amplification could be the exclusive basis of both high and low frequencies of methotrexate resistance in cells expressing polyoma oncogenes. The observations, made in this study, suggest that expression of specific oncogenes may have profound effects on
drug resistance, but these effects may be mediated by epigenetic changes involving cellular regulatory pathways rather than by global changes involving genome plasticity.

Resistance to anticancer drugs can arise due to amplification of genes coding for their respective target enzymes. A well known example for this is dihydrofolate reductase gene \( (dhfr) \) amplification in the case of methotrexate resistance (Stark and Wahl, 1984; Hamlin, 1988; Schimke, 1988). Phosphonoacetyl-L-aspartate, an inhibitor of aspartate transcarbamylase is cytotoxic to mammalian cells because it blocks the pyrimidine biosynthesis pathway (Swyryd et al, 1974). Resistance to this drug occurs as a result of overexpression of a trifunctional protein known as CAD (carbamyl-phosphate synthetase, dihydroorotase and aspartate transcarbamylase) (Kempe et al, 1976). Experimental results have demonstrated that overexpression of CAD protein results from amplification of the CAD gene complex in the resistant cells (Padgett et al, 1979; Wahl et al, 1982; Ardeshir et al, 1983). Resistance to inhibitors of asparagine synthetase is also known to arise due to amplification of the asparagine synthetase gene (Stark and Wahl, 1984; Schimke, 1982). Amplification of the gene for p-glycoprotein can confer resistance to a wide variety of unrelated drugs; a phenomenon known as multidrug resistance (Roninson et al, 1984; Robertson, Ling and Stanners, 1984; Fojo et al, 1985; Gerlach et al, 1986).

Activation of various oncogenes is also known to occur by amplification and this is a common early step in carcinogenesis (Stark and Wahl, 1984; Hamlin et al, 1988; Schimke, 1988; Alitalo and Schawab, 1986). A role for gene amplification in tumor progression is likely based on the cytological evidence of amplified genes in advanced tumors (Cowell, 1982; Barker, 1982). Amplification of proto-oncogene N-\text{myc} occurs in one third of neuroblastomas (Brodeur et al, 1986). Different oncogenes like c-\text{myc},
N-myc, c-Ki-ras, c-Ha-ras, c-myb and c-abl have been shown to be amplified in cell lines derived from several types of human tumor cell lines or in primary human tumors (Heisterkamp et al, 1983: Collins and Goudine, 1982: Montgomery et al, 1983; Little et al, 1983; Alitalo et al, 1983; McCoy et al, 1983; Filmus et al, 1985; Pelicci et al, 1984).

Because gene amplification is involved in cancer etiology and drug resistance, it is a potential target for cancer chemotherapy. It has been shown that mouse cells carrying unstably amplified dhfr genes lose them very rapidly after exposure to subcytotoxic doses of hydroxyurea (Snapka and Varshavsky, 1983). Another aim of the present study was to discover the mechanism of drug-induced loss of amplified genes. The results presented in this study show that low level stress due to a wide variety of cytotoxic agents can induce rapid loss of unstably amplified dhfr genes. Our studies indicate that hypersensitivity to low level cytotoxic stress is associated with gene amplification. This is a likely basis for drug-induced loss of amplified genes.
GENERAL INTRODUCTION

Thymidylate synthesis

For efficient DNA synthesis, a cell must maintain an adequate supply of thymine nucleotides. An important step for such a supply, is methylation of deoxyuridine 5'-monophosphate (dUMP) to give rise to deoxythymidine 5'-monophosphate (dTMP). Further phosphorylation of dTMP then leads to formation of deoxythymidine 5'-triphosphate (dTTP), an essential precursor for DNA synthesis. The conversion of dUMP to dTMP is catalyzed by the enzyme, thymidylate synthase (TS). The methyl transfer requires a co-factor, N5,N10-methylene tetrahydrofolate, which is derived from folic acid. Folic acid, also called as pteroylglutamic acid (Fig. 1), consists of a pteridine ring linked by a methylene bridge at C6 to the amino group of p-aminobenzoic acid. The carboxyl group of p-aminobenzoic acid in turn is linked to L-glutamic acid by an α-amide bond. Inside the cells, folates exist predominantly as polyglutamates, containing from one to seven L-glutamyl residues (in Fig. 1, a triglutamyl derivative is shown), and to make the peptide linkage, the gamma-carboxyl group of glutamic acid is used. These polyglutamates are not merely storage forms but are the natural co-factors of one carbon enzyme reactions (Coward et al, 1979, Coward et al, 1974).

The cells have developed a complex set of reactions for regeneration of methylene tetrahydrofolate. This sequence of reactions (Fig. 2) is known as the thymidylate synthesis cycle (Huennekens, 1968). The chemical details of thymidylate synthesis are summarized in Figure 3. The major changes occur in the pteridine ring and the region from C6 to N10. The cycle is necessary as the co-factor, (N5,N10-methylene THF) is used stoichiometrically rather than catalytically, i.e., one methylene transfer produces one
Figure 1. Folic Acid Structure
Figure 2. The Thymidylate Synthesis Cycle
Figure 3. The Essential Chemical Transformation of the Thymidylate Synthesis Cycle
dTMP molecule. During the one carbon transfer process, $N^5,N^{10}$-methylene THF is oxidized to dihydrofolate (DHF). Stereospecific reduction of dihydrofolate to tetrahydrofolate is carried out by dihydrofolate reductase (DHFR), an NADPH-dependent enzyme. Regeneration of $N^5,N^{10}$-methylene THF is effected by a pyridoxal-phosphate dependent enzyme, serine hydroxymethyl-transferase.

**Methotrexate, an Inhibitor of Dihydrofolate Reductase (DHFR)**

Dihydrofolate reductase (DHFR) is essential for regeneration of reduced folates. This enzyme is one of the key enzymes in thymidylate synthesis and is the target of the widely used anticancer drug, methotrexate. Methotrexate, a folate analog, has been used for the treatment of several human neoplastic diseases as well as arthritis. It is also an immunosuppressive agent (Chu and Whitley, 1977; Johns and Bertino, 1973). It is structurally similar to dihydrofolate, the substrate for DHFR (Fig. 4), and is a stoichiometric inhibitor (Bertino et al, 1964; Werkheiser, 1961). The binding to DHFR although extremely tight, is reversible, and at an alkaline pH, substrate dihydrofolate has been shown to compete for binding (Bertino et al, 1964). The $K_i$ for methotrexate binding to L5178 cell DHFR is $2.6 \times 10^{-9}$ M (Freisham and Mathews, 1984) while the $K_m$ for folic acid, is $1.72 \times 10^{-5}$ M (Freisham and Mathews, 1984) indicating much higher binding by the inhibitor.

Methotrexate was one of the first folate antagonists to be synthesized (Seeger et al, 1949), and it has become one of the most useful anticancer drugs. It was designed entirely empirically, with no knowledge of the target enzyme, dihydrofolate reductase. However, details of the binding were understood after the crystal structure of the
Figure 4. Comparison of Structures of Dihydrofolate and Methotrexate
methotrexate:dihydrofolate reductase complex was achieved (Mathews et al, 1977; Mathews et al, 1978; Mathew et al, 1979; Volz et al, 1982).

**Mechanism of Cell Death by Methotrexate**

Thymidine is essential for DNA synthesis. Thus, blocking the synthesis of dTMP, by methotrexate, leads to unbalanced growth (Cohen, 1971) in which DNA synthesis is selectively blocked while RNA and protein synthesis continue. Eventually the cells die, probably from extensive DNA damage resulting from thymine starvation. This type of cell death has been referred to as, "thymineless" death (Cohen, 1971).

Although it is established that in conventional doses, methotrexate kills cells primarily via inhibition of DHFR causing thymineless and purineless death, transport studies have shown that high levels of methotrexate can block uptake of reduced folates into the cells as well as facilitate their efflux (Bertino et al, 1977; Goldman, 1971; Goldman, 1975). Thus an additional mechanism of methotrexate cytotoxicity, especially at high doses, is folate transport inhibition.

**Transport of Methotrexate**

In order to prevent cellular proliferation, methotrexate must enter the cell. At relatively low drug levels, cellular uptake of methotrexate is an active process mediated by a specific transport system for the reduced folates, 5-formyltetrahydrofolate and 5-methyltetrahydrofolate (Sirotnak et al, 1984; Goldman, 1975; Sirotnak, 1980). Regulation of this transport system seems to involve cyclic AMP (Henderson et al, 1978). The transport system, like many others, appears to involve a membrane associated binding protein (Henderson et al, 1980; Henderson et al, 1977). A 56,000-dalton methotrexate binding protein has been isolated from the plasma membrane of L1210 cells.
(McCormick et al, 1979). At high concentrations, methotrexate can pass through cell membranes by passive diffusion (Sirotnak et al, 1984).

**Metabolism of Methotrexate**

Once inside the cell, methotrexate is metabolized. The products of metabolism in man include 7-hydroxymethotrexate (Goldman et al, 1983), 2,4-diamino-\(\text{N}^{10}\)-methylpterioic acid and methotrexate polyglutamates (Fig. 5). At high to medium doses of methotrexate, 7-hydroxymethotrexate is rapidly formed. This compound is a weaker inhibitor of DHFR than the parent compound. However, it strongly inhibits methotrexate transport. The enzyme carboxypeptidase G1, a cellular enzyme, cleaves the peptide linkage of methotrexate to produce 2,4-diamino-\(\text{N}^{10}\)-methylpterioate (McCullough, Chabner and Bertino, 1971) (Fig. 5), and therefore, inactivates it. Consequently it has been used as a rescue agent to reduce methotrexate toxicity. Folylpolyglutamates can be synthesized enzymatically by all organisms (McGuire et al, 1983). Once formed within a cell they are better retained because of their polyanionic nature. After methotrexate is transported into the cell it is rapidly polyglutamated (Whitehead, 1977). Polyglutamation contributes to a longer intracellular retention time and thus prolongs methotrexate exposure to its intracellular target (Balinska et al, 1982). Between one to seven glutamate residues are condensed with folyl substrates, including methotrexate. The polyglutamation does not decrease the binding of methotrexate to the target enzyme, DHFR (Goldman et al, 1983). Indeed, methotrexate polyglutamates are better inhibitors of DHFR from many sources (Covey, 1980). The enzyme which catalyzes polyglutamation of folates and folate analogue, is known as folylpolyglutamate synthetase (Fig. 5). Lack of this enzyme in cells results in poor retention of folates within the cells leading to
Figure 5. Metabolism of Methotrexate
cell death (McBurney and Whitmore, 1974). Specific inhibitors of this enzyme can be therapeutically useful.

**Resistance to Methotrexate**

Methotrexate is effective against many cancers. However rapid emergence of a resistant subpopulation of tumor cells is a major obstacle to the clinical utility of this drug (Farbers et al, 1948). Cells may possess an intrinsic resistance based on: (i) poor methotrexate influx, (ii) elevated DHFR levels, (iii) rapid synthesis of DHFR, (iv) utilization of salvage pathways (thymidine and hypoxanthine), and competitive levels of intracellular folates. Lowered ability to synthesize methotrexate polyglutamates (Curt et al, 1983; Cowan and Jolivet, 1984) can also account for intrinsic resistance to methotrexate. Acquired resistance to methotrexate arises when a tumor cell population is exposed to and mostly killed by methotrexate. Any remaining cells represent resistant forms able to survive and grow in the presence of methotrexate.

There are diverse biochemical alterations associated with the acquired resistance to methotrexate. These include alterations at the level of membrane transport (Dembo and Sirotnak, 1984), in dihydrofolate reductase the primary intracellular target (Bertino et al, 1983), and polyglutamation of methotrexate inside the cell. The most striking genetic phenomenon associated with acquired resistance to methotrexate is the amplification of the gene coding for DHFR in tumor cell variants overproducing this enzyme (Beidler and Spengler, 1976; Beidler et al, 1980; Johnston et al, 1983; Schimke et al, 1979; Schimke, 1984). Most of the studies on methotrexate resistance have been done with cultured cell lines.
Various mechanisms through which cells in culture become resistant to methotrexate are as follows:

(i) Amplification of the *dhfr* gene, resulting in overproduction of the target enzyme, dihydrofolate reductase.

(ii) Altered transport of methotrexate across the cell membrane, leading to reduced intracellular levels of the drug.

(iii) Mutations in *dhfr* gene, resulting in an altered enzyme with lower affinity for methotrexate.

(iv) Reduced polyglutamylation of methotrexate, with reduced retention of the drug within the cells.

Amplification of the Dihydrofolate Reductase Gene and Methotrexate Resistance

As mentioned earlier, one mechanism through which cultured cells become resistant to methotrexate is overproduction of the target enzyme, dihydrofolate reductase (Alt et al, 1976). Overproduction of this enzyme is the result of amplification of the *dhfr* gene (Alt et al, 1978; Bostock and Tyler-Smith, 1981; Melera et al, 1980). Early studies on methotrexate resistance of mouse and hamster cells have shown that resistance due to *dhfr* gene amplification develops gradually. In such studies, populations of cells were subjected to a concentration of methotrexate that kills most of the cells, the survivors were then pooled and subjected to a slightly higher methotrexate concentration, and the process was repeated. Over several months cultures emerged that were resistant to methotrexate concentrations many orders of magnitude greater than that which would be toxic to wild type cells. Clones or populations removed from the selective conditions at intermediate
concentrations of the drug exhibited resistance only to those intermediate levels (Fischer, 1961; Littlefield, 1969; Courtenay and Robbins, 1972; Biedler et al, 1978). Single step selection of cells resistant to high concentrations of methotrexate, is rarely successful. Single step selection experiments for methotrexate resistant clone with \textit{dhfr} gene amplification can be done at very low methotrexate concentrations (Barsoum and Varshavsky, 1983). Cells resistant to the highest concentrations of methotrexate, generally 0.5-1.0 mM, achieve DHFR levels more than 100 times that of wild type; in these cases the proportion of total soluble protein synthesis devoted to dihydrofolate reductase can be several percent (Alt et al, 1976; Hanghii and Littlefield, 1976; Nunberg et al, 1978; Melera et al, 1982). This protein in overexpressing cells was indistinguishable from the enzyme found in wild type drug-sensitive cells (Nakamura and Littlefield, 1972).

Increased levels of the protein were not due to decreased degradation of protein (Alt et al, 1976; Hanghii and Littlefield, 1976) but were a result of increased levels of translatable dihydrofolate reductase mRNA in resistant cells (Chang and Littlefield, 1976; Kellems et al, 1976). After it became clear that high levels of methotrexate resistance could be traced to high levels of dihydrofolate reductase mRNA, it was shown that high \textit{dhfr} mRNA content in methotrexate resistant cells was due to the presence of high \textit{dhfr} gene content (Alt et al, 1978). Apparently, at high intracellular dihydrofolate reductase levels, there is sufficient methotrexate-free enzyme for one carbon metabolism.

The overproduction of DHFR enzyme was often accompanied by the appearance of expanded regions on certain chromosomes in resistant cells which were not present in sensitive cells (Biedler and Spengler, 1976). These expanded regions of chromatin did not give rise to the irregularly banded pattern typical of trypsin-Giemsa-stained metaphase
chromosomes and were thus called a "homogeneously staining regions" or HSRs. Interestingly, the length of the HSR in given clones was roughly proportional to the degree of DHFR overproduction. It was thus suggested that HSRs contained amplified copies of \textit{dhfr} genes. Direct evidence that \textit{dhfr} genes were amplified in methotrexate resistant cells was provided after isolated dihydrofolate reductase cDNA was used as a probe to quantitate \textit{dhfr}f mRNA and DNA sequences in methotrexate-resistant and methotrexate-sensitive cells (Alt et al, 1978). In their study it was demonstrated that \textit{dhfr} gene sequences were present in high copy number in resistant cells but not in sensitive cells and that there was a strong correlation between levels of DHFR activity, the rates of DHFR synthesis, the abundance of \textit{dhfr} mRNA and degree of \textit{dhfr} gene amplification. The amplified \textit{dhfr} genes were shown to be physically located in the HSRs by \textit{in situ} hybridization (Nunberg et al, 1978), using isolated \textit{dhfr} cDNA as a probe. Besides their presence on HSRs, amplified genes can also be located on small extrachromosomal minichromosomes known as double minute chromosomes (DMs). These are very small paired chromatin structures that stain like normal chromosomes but lack functional centromeres (Cowell, 1982; Hamlin et al, 1983). The number of DMs per cell can range from one to several hundred in any given cell. As resistance to methotrexate increases during emergence of drug resistance, the number of amplified genes and number of DMs per cell increase in parallel (Brown et al, 1981). Although HSRs and DMs can both be present at the same time in drug resistant population, the two forms do not co-exist within the same cell (Cherif et al, 1989).

In vitro selections for gene amplification are done by increasing the drug concentration in discrete steps. The primary events of gene amplification take place in
single cells and thus are difficult to study. Secondary changes, not necessarily associated with drug resistance, may pre-exist or develop during clonal expansion to a population sufficiently large for analysis. Analyses of the structure of amplified DNA reveal that regions far larger than the selected gene are always involved. These regions vary in size from a few hundred to as many as 10,000 kilobases long. Tandemly arranged amplified genes bear different DNA sequences at their flanking regions. These sequences, called novel joints, are not present at the normal gene locus. Amplified novel joints, a common feature of highly amplified DNA, can arise by secondary amplification of a single-copy joint formed in the an earlier step (Debatisse et al, 1986; Debatisse et al, 1988; Giuloto et al, 1986; Saito et al, 1989; Hyrien et al, 1988; Ford et al, 1985). The varied structures and locations of amplified genes are consistent with the idea that more than one mechanism may be involved in gene amplification. Different mechanisms may operate in different situations. It is also possible that a single basic mechanism could lead to different outcomes, depending on how unstable structures generated in a primary event are resolved. It is unclear whether DMs and HSRs represent different outcomes of the same type of primary event or result from fundamentally different processes. DMs can integrate to generate HSRs at random chromosomal locations (Carroll et al, 1988). Evidence from human and mouse cells, involving amplification of various genes, support the idea that DMs arise from submicroscopic precursors known as episomes, that enlarge as the cells are passaged (Wahl, 1989).

Depending on the location of the amplified gene, the associated drug resistance phenotype can be either stable or unstable. Unstable resistance is due to extrachromosomally located amplified genes in DMs (Kaufman et al, 1979). Double
minutes vary in size between different cell lines or within the same cells. They replicate during S phase of cell cycle, but contain no centromeres (Levan et al, 1977; Barker et al, 1980). As a result of unequal distribution, the cell population carrying these DMs become very heterogenous with respect to number of DMs per cell. When unstably methotrexate resistant cells are grown in the absence of methotrexate the amplified genes located in DMs are lost from the population slowly (Schimke et al, 1981). The loss of amplified genes may occur in a week or may require months, depending on the cell line and level of gene amplification. On the otherhand amplified genes located in HSRs are much more stable because of their intrachromosomal location (Nunberg et al, 1978; Dolnick et al, 1979). HSRs seem to replicate by the same mechanisms that govern replication of the remainder of the genome. Since they are carried on chromosomes with centromeres, the daughter HSRs are distributed equally to the two daughter cells at mitosis. Some cell lines, like chinese hamster cells, tend to generate HSRs (Biedler and Spengler, 1976; Nunberg et al, 1978) whereas highly aneuploid lines, like mouse cell lines, generally generate DMs (Kaufman et al, 1979). The majority of cells, when first selected for methotrexate resistance, contain unstably amplified \textit{dhfr} genes, and when selection is maintained for sometime, the emerging population shows stably amplified genes (Dolnick et al, 1979).

The frequency of gene amplification varies depending on the cell type, the nature of the selection agent, and the stringency of the selection. Generally, frequencies of gene amplification range from $10^{-4}$ to $10^{-6}$ and in single step selections the number of amplified genes ranges from five to ten copies per cell (Brown et al, 1983; Wahl et al, 1979).

Spontaneous amplification of \textit{dhfr} genes has also been shown to occur in the absence of
methotrexate (Johnston et al, 1983). Cells with dhfr gene amplification as high as 50 fold amplification were sorted from a methotrexate sensitive cell line, using a fluorescine conjugate of methotrexate and cell sorting by Fluorescence Activated Cell Sorter. Since methotrexate and its fluorescent derivatives bind very tightly to DHFR, cellular retention of these drugs is proportional to the intracellular enzyme levels (Kaufman et al, 1978) Therefore, for the cells with amplified dhfr genes, retention of fluorescent-methotrexate is an indirect measure of gene amplification (Jonston et al, 1983; Mariani and Schimke, 1984). These methods have been used to sort spontaneous amplifiers from methotrexate sensitive cells (Johnston et al, 1983), as well as to evaluate the distribution of dhfr gene dosage in stably and unstably methotrexate resistant cell lines (Brown et al, 1981; Schimke et al, 1980). Various DNA damaging agents have been also tested for their effect on the frequency of methotrexate resistance and dhfr gene amplification. The frequency with which cells become resistant to methotrexate during single step selection can be dramatically increased by various treatments before exposure of the cells to methotrexate. Transient inhibition of DNA synthesis with hydroxyurea, before methotrexate selection, enhances the frequency of methotrexate resistance as well as that of dhfr gene amplification (Brown et al, 1983). Also, prior treatment of cells with methotrexate itself can increase the frequency of methotrexate resistance by 10-fold (Tlsty et al, 1982). Inclusion of the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), either at the time of initial methotrexate treatment or during clonal selection (Varshavsky, 1981a) increases the frequency of methotrexate resistant colony-forming 3T6 cells by 100-fold. Phorbol (a non-promoting analog of TPA), thymidine and dimethylsulfoxide each fail to produce any TPA like effect in these
cells (Varshavsky 1981a). Besides these agents, exposure of cells to ultra-violet radiation
and the carcinogen N-acetoxy-N-acetyl-aminofluorene, greatly increases frequency of
methotrexate resistance and presence of TPA further increases such frequency (Tlsty et al,
1984). From these studies, it is apparent that any number of agents that cause transient
inhibition of DNA synthesis may facilitate gene amplification. Based on such
observations a hypothesis was put forth that a variety of cell treatments can result in
misfiring of replicons, resulting in the generation of an "onionskin" structure which can
then be resolved into amplified DNA sequences (Varshavsky, 1981b).
Loss of Unstably Amplified Genes

As discussed above double minutes are acentric extrachromosomal DNA sequences and thus segregate randomly into the daughter cells during cell division. The random distribution of DMs during mitosis generates a substantial cell to cell variation in amplified gene dosage (Schimke et al, 1980; Barker, 1982; Cowell, 1982), and the number and size of the DMs (Barker, 1982; Cowell, 1982; Hamkalo et al, 1985; Trent et al, 1986). Cells carrying amplified genes either as HSRs or as DMs often grow more slowly in the absence of the selecting drug than the corresponding drug-sensitive parental cells. Thus, in the absence of methotrexate, cells with higher numbers of amplified genes are at a replicative disadvantage and are continuously overgrown by the cells with fewer amplified genes (Kaufman, Brown and Schimke, 1981). In addition to the statistical likelihood that DMs are distributed unequally between the nuclei of two daughter cells during mitosis, with subsequent selection (in the absence of drug) against cells carrying higher number of DMs, loss of DMs to the cytoplasm may also occur during mitosis (Cowell, 1982). Rapid degradation of these DMs within the cytoplasm through formation of micronuclei may follow. Loss of unstably amplified genes from drug resistant cell populations has been studied by flow cytometry and in situ hybridization techniques (Kaufman et al, 1981; Kaufman and Schimke, 1981; Shen et al, 1988). All of these studies show that normal loss of unstably amplified genes is very heterogenous as expected for a process that is based on the random distribution of DMs at each mitosis. This normal loss of unstably amplified genes can be greatly accelerated by subcytotoxic concentrations of hydroxyurea (Snapka and Varshavsky, 1983).
Since amplified genes contribute to the development of cancer and also to drug resistance during cancer chemotherapy, it would be of interest to find ways to minimize the emergence or persistence of cells with amplified genes. In the present study, we show that exposure of cells to subcytotoxic concentrations of various structurally unrelated drugs induces rapid loss of unstably amplified dhfr genes from methotrexate resistant cells. We also show that a major mechanism of drug induced loss of unstably amplified genes is hypersensitivity to cytotoxic stress of cells which carry unstably amplified genes.
CHAPTER II
MATERIALS AND METHODS

MATERIALS

Reagents

Methotrexate (+amethopterin), hydroxyurea, guanazole, proflavine, salmon sperm DNA, tRNA, RNase and protease were purchased from Sigma chemicals. Camptothecin was obtained from the National Cancer Institute, Division of Cancer Treatment, Natural Products Branch. Alpha difluoromethylornithine (DFMO) was a gift from Merrel Dow Research Institute, Cincinnati, Ohio. A fluorescent derivative of methotrexate, fluorescin methotrexate triammonium salt (f-MTX), was purchased from Molecular Probes, Inc. Restriction enzymes were obtained from Bethesda Research Laboratories. Random oligo primers, dATP, dCTP, dGTP, dTTP, Klenow fragment of DNA polymerase and DNAse free bovine serum albumin were purchased from Pharmacia Chemicals. $^{32}$P-alpha deoxycytidine triphosphate, triammonium salt was from Amersham. All other reagents were standard laboratory items.

Cell Lines and cell culture supplies

Dulbecco’s modified Eagle’s medium, fetal bovine serum (Certified grade), trypsin, penicillin-streptomycin and glutamine were obtained from Gibco. All the plastic ware for cell culture was purchased from Corning.

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NIH3T3 cells expressing various polyoma virus oncogenes from the retroviral cloning vector pZIPNeoSV(X)1 (Cepko et al, 1984) were provided by Van Cherington (Cherington et al, 1986). These cell lines had undergone an estimated 15-20 population doublings from the time of their isolation to the point of our experiments. LT11 cells express large T antigen, and ST1-5 cells express small T antigen, each at roughly 10-20% of the levels found in lytically infected cells. MT12 cells express middle T antigen at 50% of the level in lytically infected cells. CC1 cells are a polyclonal population of NIH3T3 cells carrying the cloning vector without any oncogene. This line was made by pooling numerous G418-resistant colonies. R500 cells, derived from NIH3T3 cells by gradual selection in methotrexate were a gift from Robert Schimke. These cells are resistant to 250 μM methotrexate and carry, on average, approximately 200 copies of the dihydrofolate reductase gene per cell (Hamkalo et al, 1985). R.3 cells were derived from 3T6 cells by a single step methotrexate selection. R.3 cells are resistant to 300 nM methotrexate and carry, on average, approximately 13 copies of the dihydrofolate reductase gene per cell.

Plasmids and Cloned DNA Probes

The plasmid p1.3 contains a 0.6 kb gene fragment with the first two 5' exons and the first intron of the mouse alpha globin gene (Vidya Rao and Elio F. Vanin, personal communication). This fragment was used as single copy probe. The mouse dhfr gene probe was a 0.73 kb cDNA cloned in pSV2-dhfr (Subramanium, Mulligan and Berg, 1981) and was purchased from the American Type Culture Collection. The 6.4 kb ras gene probe in plasmid pbc-N1 (Pulciani et al, 1982) was available in the laboratory. Gel
electrophoresis of plasmid DNAs and DNA fragments was performed in electrophoretic grade agarose from Seakam.

**Bacteriological Supplies**

Escherichia coli (E. coli) host bacteria (HB101 strain) was obtained from American Type Culture Collection. Difco Laboratories agar, bacto-peptone, bacto-tryptone and yeast extracts were used in the bacteriological media preparations, (Maniatis et al, 1982).

**Growth and Selection for Methotrexate Resistance**

**Cell Culture**

CC1, LT11, MT12, ST1-5, 3T6 and NIH3T6 Cells were grown in Dulbecco’s modified Eagle’s medium (pH 7.4), supplemented with sodium bicarbonate (37 g/l), 10% fetal bovine serum and 0.5% penicillin-streptomycin. R500 and R.3 cells were also grown in the same media except that fetal bovine serum was dialyzed before use. For R500 and R.3 cells, methotrexate was always used in the medium (250 μM for R500 and 300 nM for R.3). Cell cultures were maintained at 37°C under a 5% CO₂ atmosphere in a humidified, water jacketed incubator.

**Selection of cells for Methotrexate Resistance**

For selections of methotrexate resistant cells, cultures just reaching confluence were detached with 0.05% trypsin in phosphate buffered saline (PBS; 0.2 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.5 mM KH₂HPO₄, 0.5 mM MgCl₂.6H₂O and 1 mM CaCl₂) and suspended in warm fresh media. The cells were counted with a haemocytometer, diluted appropriately in warm media and replated, at various cell densities in 10 cm plates containing CO₂ equilibrated medium, 10% dialyzed fetal bovine
serum and methotrexate at different concentrations. The plates were transferred to a 37°C incubator, left undisturbed for three weeks, without any media changes, to avoid the production of any artifactual satellite colonies, by dislodging cells from nascent drug resistant colonies. This procedure also allows media conditioning and build up of growth factors which would be otherwise removed with frequent media changes. In order to fix and stain drug resistant colonies on plates, media was poured off the plates and the colonies were rinsed gently in warm phosphate-buffered saline, fixed with 7% formaldehyde in isotonic saline for 30 minutes and then stained with 1% crystal violet for 5 minutes. The stain was drained and the plates were gently rinsed with tap water before drying. Resistant colonies were counted in each plate and the frequency of methotrexate resistant colony-forming cells was calculated as the number of colonies with 50 or more cells divided by the number of cells plated initially. Plating efficiency of the cells was used to correct for frequency of methotrexate resistant colony forming cells. Since the plating efficiency of the cells was close to 50% for each cell line used in selection experiments, the relative frequencies of methotrexate resistance for the different lines was not significantly changed by this correction.

In order to isolate and grow methotrexate resistant cells from various cell lines, clones of methotrexate resistant cells derived from the CC1, LT11, MT12 or ST1-5 lines were isolated with glass cloning cylinders, removed by trypsin digestion, and replated in multiwell culture plates in media at the selecting concentration of methotrexate. Drug resistant clones derived from MT12 cells required plating in drug-free media overnight before re-exposure to methotrexate. The resistant clones from the multiwell plate were then expanded to larger cultures for further experiments.
Plating Efficiency

In order to determine the plating efficiency of each cell line, 500 viable cells were plated with $1 \times 10^6$ irradiated cells. The irradiated cells were prepared by exposing the cells to 5000 rads by $^{137}$Cs gamma-irradiation in a Gamma cell 40 unit. The irradiated feeder cells in plating efficiency experiments were used to mimic methotrexate selection conditions in which most of the plated cells attach and metabolize but are eventually killed by the drug. Ten days after plating, colonies were fixed, visualized and counted as described above. Control plates containing only irradiated cells did not develop any colonies either with or without added methotrexate. Plating efficiency was also determined at a non-selective concentration of methotrexate (10 nM).

Growth Curves

Growth curves for CC1, LT11, MT12 and ST1-5 were performed by plating $1 \times 10^5$ cells, in duplicate plates, in drug free medium. After a 12 hour attachment period, cells were harvested by trypsinization and counted using a haemocytometer. Cells were counted at four hour intervals.

In gene loss experiments, $1 \times 10^5$ R500 cells were seeded per 10 cm plate in medium without methotrexate and either with or without a test drug. For each test a set of four 10 cm plates were used. At confluence, cells were trypsinized and counted from one 10 cm plate in each experimental set. The second plate from each set was passed to four new 10 cm plates at the original cell density. Cells from the remaining two 10 cm plates were harvested for isolation of genomic DNA which was then used for slot blot experiments.
Plating efficiency of R500 cells was determined by counting the number of attached cells 12 hours after plating. R500 cells were plated in 10 cm plates at a density of 1x10^5 cells either in the absence of any drug or in the presence of various test drugs. After 12 hours of attachment, the plates were rinsed with warm PBS. Cells were then trypsinized and counted by a haemocytometer.

**Measurement of cell viability by Trypan Blue Exclusion**

Cells were trypsinized, suspended in fresh media, centrifuged and resuspended gently in fresh media before staining. To 0.9 ml of cell suspension in a test tube, 0.1 ml of 0.5% trypan blue was added. After 5 minutes of staining the sample was applied to a haemocytometer and the numbers of clear cells and blue cells were determined. Since the viable cells exclude the stain and appear clear under microscope compared to non-viable blue cells, the percentage of viability was determined from total number of cells counted and the number of viable cells.

**Cell sorting and flow cytometry**

Methotrexate and its derivatives bind very tightly to dihydrofolate reductase. As a result cellular retention of these drugs is proportional to the intracellular enzyme levels (Kaufman et al, 1978). Thus, for cells with amplified dihydrofolate reductase genes, retention of f-MTX is an indirect measure of gene amplification (Johnston et al, 1983; Mariani and Schimke, 1984). Fluorescent derivatives of methotrexate have been used to study the distribution of dihydrofolate reductase gene dosage in methotrexate resistant cells (Schimke, 1980; Brown et al, 1981). In this study R500 cells were sorted on the basis of their bound f-MTX.
For uptake of f-MTX, cells were grown to 90% confluence, and placed in methotrexate free media for 24 hours (three media changes) in order to remove all methotrexate. The cells were then incubated for an additional 24 hours in medium containing 25 μM f-MTX. Just before cell sorting or flow cytometry, the cells were removed by gentle trypsin digestion, and suspended in fresh drug-free medium. A cytofluorograph 50H flow cytometer was used for flow cytometry and cell sorting. Based on the amount of green fluorescence exhibited, R500 cells were sorted into two subpopulations, one with lower fluorescence lower than 16 units (R500⁻) and the other with fluorescence higher than 16 units (R500⁺). The success of the sorting was confirmed by performing flow cytometry on samples of these sorted populations. Flow sorted populations were placed in 35 mm tissue culture dishes and grown to confluence. The low fluorescence population was grown to confluence in methotrexate free media while the high fluorescence population was maintained in 250 μM methotrexate. After reaching confluence, the cells in each of the 35 mm plates were passed to one 10 cm tissue culture plate. When these plates reached confluence, the cells were used for the experiments.

**Cloning efficiency of the sorted populations**

Cells were seeded in 10 cm plastic tissue culture plates at a density of 3x10³ cells per plate in the presence or absence of the test drugs. The plates were then placed in the incubator and left undisturbed for two weeks. Colonies were stained and counted as described above. The colonies containing 50 or more cells were scored in order to determine the cloning efficiency. The cloning efficiency was expressed as the number of colonies formed from total number of the cells seeded.
Quantitation of Gene Dosage

DNA was isolated from cells and quantitated by the Hoechst Dye assay. The DNA samples were immobilized on a nitrocellulose filter using a vacuum slot blot apparatus. DNA was loaded in duplicate or in triplicate and were probed separately with cloned $^{32}$P-labelled DNA probes for dhfr, α-globin and ras genes. Cloned probe sequences were separated from vector sequences by restriction digestion and agarose gel electrophoresis before labelling. Labelling of DNA was performed by random primer method (Feinberg and Vogelstein, 1983). Autoradiographic exposures were made on Kodak X-AR5 film, and the autoradiograms were quantitated with a LKB 2222-010 ultrascan XL soft laser densitometer. Peaks were integrated electronically with the LKB 2400 GelScan XL software package and an IBM AT computer.

Isolation of genomic DNA from cells

The cells for isolation of genomic DNA were grown in 10 cm plates. At confluence, media from the plates was removed and the cell monolayer was rinsed with wash buffer (5 mM Tris.HCl, pH 7.5; 0.14 M NaCl). The cells were then covered with ice cold cell lysis buffer (10 mM Hepes pH 7.5; 5 mM EDTA; 0.5% Nonidet 40). Following this cells were gently scraped off with a rubber policeman and collected in a 15 ml polypropylene centrifuge tube. They were then centrifuged in a table top centrifuge at 4°C at 1500 rpm for 5-10 minutes. The pellet was again resuspended in cell lysis buffer in order to ensure complete lysis of the cells and the nuclei were checked under microscope. If necessary cells were also Dounce homogenized. Nuclei were then pelleted and suspended in a small volume of Hepes buffer (1mM Hepes, pH 7.5; 1mM EDTA). To this 1/10 volume of 10% SDS (Sodium dodecyl sulphate) and 2/10 volume of 5M NaCl
were added. As a result of SDS and high salt the nuclei lysed and the lysate was extracted with an equal volume of chloroform:isopropanol (24:1). Extraction with chloroform:isopropanol was repeated two more times and the extract was dialyzed against dialysis buffer (1 mM Hepes, pH 7.5; 1 mM EDTA) with several changes of dialysis buffer. Dialyzed DNA was collected and quantitated using the DNA Hoechst assay as described below.

Quantitation of DNA by the Hoechst Assay

This assay was used to quantitate cellular DNA for slot blots. The assay is very sensitive and can be used to quantitate nanogram amounts of DNA. First a stock of calf thymus DNA (5 μg/ml) solution was prepared in TE (10 mM Tris.HCl, pH 8.0; 1 mM EDTA). Different standard concentrations of this DNA (25ng, 50ng, 100ng, 150ng, 200ng, 250ng and 300ng) were prepared in a total volume of 100μl of TE. The isolated DNA samples with appropriate dilutions (100 and 200 fold) were also made in TE in a total volume of 100 μl. All samples, standards as well as test samples, were prepared in duplicate. To each tube 50 μl of 4X Hepes solution (200 mM Hepes, 60 mM MgCl2, 800 mM NaCl, pH 7.4) and 50 μl of Hoechst dye 33258 solution (4 μg/ml in distilled water) was added. Each tube was completely vortexed and the absorbance was measured using a Fluorometer-A4 (Farrand Optical Co.). A standard curve was made from the DNA standards the DNA concentrations in the test samples was determined.

Preparation of cytoplasm for cytodot to measure gene expression

RNA levels in cytoplasm were determined by the cytodot method as described in Methods in Enzymology, Volume 152 (pp 585). The cells were grown as monolayers in 10 cm culture dishes. At confluence, cells were scraped and collected in a 15 ml
polypropylene centrifuge tube and the total number of cells was counted by
haemocytometer. The cells were then centrifuged at 1500 rpm for 5 minutes in a table
top centrifuge tube. The pellet was resuspended and washed once in PBS and centrifuged
again at 1500 rpm for 5 minutes. Finally the cell pellet was resuspended at $10^7$-$10^8$
cells/ml in ice cold buffer (10 mM Tris.HCl, pH 7.0; 1 mM EDTA; 10 mM Vanadyl
complex. Vanadyl complex was used as a ribonuclease inhibitor. To the cell suspension
0.1 volume of 5% Nonidet 40 was added and then left on ice for 5 minutes. The nuclei
were pelleted by centrifuging at 15,000xg for 2-3 minutes and the supernatant was
collected. After incubating the supernatant for 15 minutes at 60°C a mixture of freshly
made 37% formaldehyde (0.3 volumes) and 20X SSC was added to each sample. The
samples were stored at -70°C till they were used.

**DNA-DNA Hybridization**

The 72 well Slot-blot apparatus (S&S) was used to blot different DNA samples
onto the nitrocellulose paper. The nitrocellulose paper was soaked in 1 M ammonium
acetate and put in between the two halves of the slot blot apparatus. Two GB001 filter
papers, also soaked in 1 M ammonium acetate, were placed under the nitrocellulose in
order to avoid drying of the nitrocellulose during vacuum suction. The DNA samples
were prepared in TE buffer and were adjusted to 0.3 N NaOH by adding 5 M NaOH in a
total volume of 200 µl. The samples were left overnight to ensure complete denaturation
of DNA and hydrolysis of any RNA. The samples were chilled before use. Next 200 µl
of cold ammonium acetate was added to each sample just before loading into the wells of
the slot blot apparatus. After all the samples were loaded, they were passed slowly
through the nitrocellulose filter by applying slow vacuum to the apparatus. Once all the
samples were sucked through, 400 µl of 1 M ammonium acetate was put in each well and then filtered as above. The filter was removed from the apparatus and washed with buffer (3 M NaCl; 10 mM EDTA, pH 7.5). The filter was air dried and baked at 80°C under vacuum for two hours in a vacuum oven. The baked filter was then processed for hybridization with the labelled probe. All the solutions were filtered before use.

Prehybridization and Hybridization

All solutions used in these experiments were filtered before use and were prepared as described below.

20X SSPE: 7.4 gram of EDTA, 24 grams of Na₂H₆PO₄ and 174 gram of NaCl were weighed to make 1 liter of 20X SSPE. A solution of EDTA and phosphate was first made and the pH was adjusted to 7.0 with 10 N NaOH. Next sodium chloride was added. The solution was stirred until dissolved and total volume was adjusted to 1 liter. 1X, 2X, 5X and 10X SSPE solutions were made by diluting 20X SSPE solution 1:20, 1:10, 1:4 and 1:2 respectively.

Prehybridization Solution (50 ml): 25 ml of 100% deionized formamide, 5 ml of 50X Denhardt’s Solution, 12.5 ml of 20X SSPE, 0.5 ml of 10% SDS, 0.5 ml of 10 mg/ml sonicated salmon sperm DNA and 6.5 ml of TE buffer. The mixture was boiled for 15 minutes and chilled on ice before use.

100% deionized formamide: 10 grams of mixed-bed ion-exchange resin, AG 501-X8 (Bio-Rad) was added to 100 ml of formamide and stirred at 4°C for 1 hour. The mixture was filtered through Whatman filter paper No.1 twice and the filtrate was frozen at -20°C as 100% deionized formamide.
**50X Denhardt's Solution:** 1 gram of Ficoll, 1 gram of polyvinylpyrrolidone, 1 gram of BSA (Pentax Fraction V) were dissolved in 100 ml of distilled water. The components were completely dissolved and the solution was stored at -20°C.

The filter was prehybridized in a sealed polypropylene plastic bag containing prehybridization mixture (50% deionized formamide, 5X SSPE, 5X Denhardt's solution, 0.1% SDS and 100 µg/ml of sonicated salmon sperm DNA). Prehybridization was performed at 55°C for 3 hours. After 3 hours, the prehybridization solution was discarded and hybridization was done by putting the filter in a sealed polypropylene plastic bag with fresh prehybridization solution containing $^{32}$P-labelled DNA probe. Hybridization was done for 24-36 hours, at 55°C with occasional shaking in a heating water bath. The filter was removed and washed in 100 ml of 2X SSPE at room temperature. The rest of the washes were performed at 55°C in the sealed plastic bag containing 0.1% SDS, 0.1% SSPE. Each wash was for 30-40 minutes, and they were repeated until no radioactivity was detected in the washes. The filter was air dried and exposed to Kodak X-omat film at -70°C in a cassette with an intensifying screen. The exposures were scanned to quantitate the intensity of different bands.

**Radiolabelling of DNA with $^{32}$P**

The following stock solutions were made for labelling DNA. Different chemicals to make these stocks were purchased from Pharmacia.

**Solution O:** 1.25 M Tris pH 8.0, 0.125 M MgCl$_2$. This solution was stored at 4°C.

**dATP Stock:** 10 mg of dATP was dissolved in 0.17 ml of 3 mM Tris, 0.2 mM EDTA pH 7.0 and was stored at -20°C.
dGTP Stock: 10 mg of dGTP was dissolved in 0.164 ml of 3 mM Tris, 0.2 mM EDTA pH 7.0, and was stored at -20°C.

dTTP Stock: 10 mg of dTTP was dissolved in 0.17 ml of 3 mM Tris, 0.2 mM EDTA pH 7.0 and was stored at -20°C.

Solution A: To 1 ml of solution "O" 18 μl of 2-mercaptoethanol and 2.5 μl each of dATP, dGTP and dTTP stocks were added and the solution was stored at -20°C.

Solution B: 2 M Hepes, pH 6.6. stored at 4°C.

Solution C: 50 units of hexamer random primer oligonucleotide were dissolved in 0.556 ml of 3 mM Tris, 0.2 mM EDTA, pH 7.0.

OLB Stock: In order to make oligolabelling (OLB) stock, 100 μl of solution A, 250 μl of solution B and 150 μl of solution C were mixed together and stored at -20°C.

DNA gene fragments were isolated from the plasmid vector sequences as described below and were labelled by random primer oligonucleotide labelling (Feinberg and Vogelstein, 1983). In order to label the DNA, 50 ng of standard DNA in a volume of 30 μl was denatured by boiling for 5 minutes and then chilled on ice for 5 minutes. To the denatured sample, 10 μl of OLB stock, 2 μl of DNase free BSA, 2 μl of Klenow fragment of DNA polymerase and 5μl of $^{32}$P-dCTP was added. The reaction was carried out in a total volume of 50 μl behind plexiglass beta shielding and was left for overnight for completion. The labelled DNA was purified from unincorporated nucleotides using a G50 Sephadex spin column from Boehringer Mannheim Biochemicals. In brief the spin columns were centrifuged first for 2 minutes to elute excess buffer from the column. The labelling reaction mixture was then added and the column was again centrifuged at 3000 g for 4 minutes. The labelled DNA comes out in the void volume whereas the
unincorporated nucleotides are retained in the column and discarded. An aliquot of the labelled DNA was counted by scintillation counter and the specific activity of the DNA was determined.

**Preparation of DNA for radiolabelling**

**Growth of bacteria for transformation with plasmids containing specific gene fragments**

The host bacteria, E.coli (strain HB101) was first grown by streaking a loop of bacterial culture on a Bacto nutrient agar plate. After streaking, the plates were incubated at 37°C overnight. A single bacterial colony was picked with a sterilized loop and inoculated into about 10 ml of nutrient broth. In order to make a single colony culture stock, the isolate was incubated at 37°C in an incubator with vigorous shaking. This culture was used for preparation of competent cells for transformation with plasmid DNA. Some of the single colony culture was stored as stock at -70°C.

**Preparation of competent cells**

Luria broth (LB) bacterial medium (10 ml) was inoculated with about 100 μl of freshly made HB101 bacterial culture and was incubated overnight at 37°C. A 0.5 ml aliquot of this culture was then added to 55 ml of LB media in a 250 ml Erlenmeyer flask and was again incubated at 37°C overnight with vigorous shaking. When the cells reached 0.6 O.D. (λ=600 nm), they were harvested by centrifugation at 5500 rpm in a 50 ml polypropylene centrifuge tube for 15 minutes at 4°C. The cell pellet was resuspended in 10 ml of ice cold sterile MgCl₂ and then recentrifuged at 5500 rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 1.7 ml of ice cold sterile 50 mM CaCl₂. To this 25 ml of 50 mM calcium chloride was added.
and left on ice for 30 minutes. The cells were then centrifuged (5000 rpm for 15 min., 4°C) and the supernatant was removed with a pipette. Finally cells were suspended in 2.3 ml of ice cold 50 mM calcium chloride and left undisturbed on ice for about one hour. The cells settled during this time and just before the transformation procedure, they were resuspended by gently inverting the tube.

Transformation of Competent Cells with Plasmid DNA

Calcium treated competent cells (0.1 ml) prepared as described above, were aliquoted into a 1.5 ml sterile polypropylene microcentrifuge tube. The cells were resuspended completely before aliquotting. The microfuge tube containing competent cells was left on ice for several minutes, and 100 ng of plasmid DNA in about 5μl was added to the cells. The cells with plasmid DNA were then left on ice for 10 minutes before heat shocking (quick transfer to a 37°C water bath for 2 minutes). LB media (0.5 ml without antibiotics) was added to the tube. The tube was closed, the contents mixed by gentle inversion and then the tube was incubated at 37°C for 45 minutes. Next a 50 μl aliquot of treated cells was plated on an LB agar plate, containing penicillin, by spreading the cells with a sterile glass spreader. The plate was transferred to a 37°C incubator overnight and individual transformed bacterial colonies were picked and grown for large scale isolation of plasmid DNA (see below).

Isolation of Plasmid DNA from Bacterial Host

Ten ml of LB medium containing penicillin was inoculated with a single bacterial colony and incubated at 37°C overnight with vigorous shaking. The next morning 50 ml of medium was inoculated with 0.2 ml of the overnight culture and incubated at 37°C with vigorous shaking. Cell growth was monitored by optical density (λ=600nm). The
culture was grown to late log phase. One liter of LB media was then inoculated with 50 ml of the late log culture. The flask containing the inoculated culture was incubated at 37°C and when the OD660 of the culture reached 0.4, chloramphenicol was added to the culture (170 μg/ml). The flask was again incubated for a further 12-16 hours in order to amplify plasmid DNA. The bacterial cells were harvested by centrifugation at 4000 x g for 10 minutes at 4°C. The supernatant was discarded and the bacterial cell pellet was washed with ice cold STE solution (0.1 M NaCl, 10 mM Tris.HCl, pH 7.8 and 1 mM EDTA). Cells were disrupted by alkaline lysis (Maniatis et al, 1982). The bacterial cell pellet from 500 ml culture was resuspended in 10 ml of solution I (50 mM glucose, 25 mM Tris.Cl, pH 8.0 and 10 mM EDTA) containing 5 mg/ml lysozyme. Lysozyme was added to the solution just before use. The cells were transferred to a polyallomer tube and left at room temperature for 5 minutes. To this tube, 20 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added, the tubes were covered and the contents were mixed gently by inverting the tubes several times. The tubes were then left on ice for 10 minutes. Next, 15 ml of 5 M potassium acetate solution was added. The contents were mixed thoroughly by sharply inverting each tube several times before leaving it on ice for 10 more minutes. The lysate was centrifuged (13,000 rpm, 30 minutes, 4°C) in order to pellet the cell debris and cellular DNA. About 60 ml of the supernatent, containing plasmid DNA, was transferred to four 30 ml glass corex tube and 0.6 volumes of isopropanol was added to each tube. Contents were mixed and left at room temperature for 15 minutes to precipitate plasmid DNA. Plasmid DNA was recovered by centrifugation at 13,000 rpm for 30 minutes at room temperature. The supernatant was discarded and the DNA pellet was washed with 70% ethanol at room temperature. The
DNA pellet was then dissolved in TE buffer and purified by centrifugation to equilibrium in a cesium chloride-ethidium bromide density gradient as described below.

To every milliliter of DNA solution, 1 g of solid cesium chloride was added. All of the salt was dissolved by gentle mixing. Next 0.8 ml of ethidium bromide solution (10 mg/ml) was added for every 10 ml of cesium chloride solution. The contents of the tube were mixed well and transferred to a plastic tube to the top. The tubes were plugged and centrifuged at 45,000 rpm for 36-40 hours in a Beckman ultracentrifuge at 20°C. After centrifugation the plasmid DNA was visible as two distinct bands. The upper band was nicked circular relaxed DNA and the lower band was covalently closed supercoiled plasmid DNA. The lower band was collected into a glass tube with a #21 hypodermic needle inserted into the side of the tube. Ethidium bromide was removed from DNA by extraction with water saturated butanol. Extraction was repeated 4-6 times until all of the pink color was removed from the aqueous solution. The aqueous solution was dialyzed against several changes of TE buffer. Dialyzed DNA was quantitated, aliquotted into small tubes and frozen at -20°C.

Isolation and Elution of DNA fragments from agarose gel

The plasmid DNA was cut with restriction enzymes in order to release the specific gene fragments from the vector sequences. About 5 μg of plasmid DNA was restriction digested and the DNA fragments were separated by electrophoresis using 0.7% agarose gel (2 volts/cm). The gel was stained with ethidium bromide (1 μg/ml) for 30 minutes and destained under tap water for 20 minutes in order to visualize DNA bands on an ultraviolet transilluminator. Pieces of the gel containing specific DNA fragments were cut out and dissolved in a saturated sodium iodide solution. The DNA in solution was bound
to glass powder and then eluted from the glass by of low ionic strength buffer (Tris.HCl, 10 mM; EDTA, 1 mM). The following detailed procedure for isolation of DNA fragments from agarose gels was used.

The gel band was cut and weighed. About 2-3 volumes of saturated solution of sodium iodide was added to it. For a gel slice of less than 0.4 gram, 1.5 volumes of sodium iodide was used. It was then incubated at about 45°C-55°C to dissolve the gel completely. To this, 5µl of 50% glass powder suspension was added and the suspension was left on ice for about 5 minutes to allow DNA binding to the glass powder. The glass powder was centrifuged for 5 seconds. The supernatant was discarded and the pellet was resuspended in diluted sodium iodide solution (1:3) in order to remove traces of agar. The glass powder was again centrifuged at high speed and the pellet was washed in 10-20 volumes of wash buffer (0.1 m NaCl, 10 mM Tris, 1 mM EDTA, 50% ethanol) to remove sodium iodide. Washing was done three times and after the final wash the washing buffer was completely removed from the glass pellet. DNA was then eluted from the glass powder by suspending the glass powder pellet in about 10 µl of TE buffer. The suspension was left at 45°C-55°C for 2-3 minutes. The suspension was then centrifuged and the DNA recovered in the supernatant. The elution was repeated two more times to recover all of DNA from glass powder. A sample of the recovered DNA was checked for purity by gel electrophoresis before use as a probe for ³²P labelling.
CHAPTER III

RESULTS

Effect of Polyoma T antigens on Methotrexate Resistance in NIH 3T3 Cells

For studies on drug resistance in transformed cells, a set of polyoma oncogene expressing cell lines derived from a common parent cell line was used. The cell lines expressed immortalizing and transforming viral oncogenes at known levels. Cherington and coworkers (Cherington et al, 1986) have developed NIH 3T3 cell lines expressing each of the polyoma virus oncogenes individually. Since the polyoma transforming functions are strong, yet separated from one another in these lines, they were chosen for this study. These lines express polyoma oncogenes at levels of 10-20% (LT11, large T antigen and ST1-5, small T antigen) and 50-100% (MT12, middle T antigen) relative to levels in lytically infected cells (Cherington et al, 1986). The control cells (CC1) are a heterogenous population of NIH 3T3 cells carrying the cloning vector without an oncogene.

Clonal Variation in Frequencies of Methotrexate Resistance in CC1 cells

Since the CC1 line is not monoclonal but a heterogenous population of cells, clonal isolates were obtained by plating these cells at high dilution followed by isolation of individual colonies arising from single cells. The frequency of methotrexate-resistant
TABLE 1

Comparison of frequencies of methotrexate-resistance colony-forming cells in CC1 and four subclones. Four subclones (CC1-1, CC1-2, CC1-3 and CC1-4) were derived from the CC1 cell line. These subclones and their parent cell line were compared for their frequencies of methotrexate-resistant colony-forming cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Frequency of MTX-resistant colony-forming cells ($\times 10^{-4}$)</th>
<th>Frequency of MTX-resistance relative to CC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1</td>
<td>5.2 ± 0.4</td>
<td>1.0X</td>
</tr>
<tr>
<td>CC1-1</td>
<td>10.5 ± 0.8</td>
<td>2.0X</td>
</tr>
<tr>
<td>CC1-2</td>
<td>7.0 ± 0.2</td>
<td>1.3X</td>
</tr>
<tr>
<td>CC1-3</td>
<td>7.2 ± 0.0</td>
<td>1.4X</td>
</tr>
<tr>
<td>CC1-4</td>
<td>9.4 ± 0.8</td>
<td>1.8X</td>
</tr>
</tbody>
</table>

aSingle step selection for resistance to 50 nM MTX, $5 \times 10^5$ cells per plate.

bMean ± standard deviation.
colony-forming cells was then determined for four of these cloned lines and the heterogenous parental cell population (Table 1). Although all four clonal lines showed slightly higher frequencies of methotrexate resistance than the parental CC1 population, none was more than twice that of CC1. Thus for frequencies of methotrexate resistance, the range of clonal variation in NIH 3T3 cells infected with the retrovirus vector appears to be narrow.

**Frequencies of Methotrexate Resistance in Cells Expressing Polyoma Oncogenes**

In single-step selections for methotrexate resistance, cell lines expressing the individual polyoma T antigens showed dramatically different frequencies of methotrexate-resistant variants. As shown in Figure 6, different cell lines varied by approximately 800-fold when the frequency of methotrexate-resistant colony-forming cells was compared over a range of methotrexate concentrations. The ST1-5 and LT11 cell lines both showed greatly increased frequencies of methotrexate resistance compared to the CC1 population. The difference in frequency of methotrexate resistance for middle T antigen expressing MT12 cells and the small T antigen expressing ST1-5 cells was approximately 800 fold. The large T antigen expressing LT11 cells and the CC1 cells that carry the cloning vector alone fell between these extremes. As expected, the frequency of resistance dropped off sharply with increasing concentrations of methotrexate for all the cell lines studied. These lines keep their relative order of resistance over the full range of selecting methotrexate concentrations.

Although ST1-5 cells exhibited higher frequencies of methotrexate resistance than LT11 cells, the average size of the methotrexate resistant colonies was smaller for ST1-5 cells than LT11 cells (Fig. 7, compare LT11 and ST1-5 at 100 nM and 150 nM
Figure 6. Frequencies of methotrexate resistant colony forming cells for NIH3T3 cell lines expressing different polyoma virus oncogenes. The cells were seeded at a density of $1 \times 10^6$ per plate in the presence of methotrexate and the selection was done for a period of 3 weeks. At the end of selection, methotrexate resistant colonies were stained with crystal violet and then counted to determine the frequency of methotrexate-resistant colony-forming cells.

- Open circles: ST1-5 cells
- Closed circles: LT11 cells
- Open triangles: CC1 cells
- Closed triangles: MT12 cells
FIGURE 6

Incidence Of MTX-Resistant, Colony-Forming Cells

MTX Concentration (μM)
methotrexate). This suggests that the LT11 cells achieved methotrexate resistance earlier in the selection than the ST1-5 cells. This observation was supported by comparison of LT11 and ST1-5 colonies grown out in the absence of methotrexate (Fig. 8). As shown in this Figure, in the absence of methotrexate, ST1-5 cells form larger colonies than LT11 cells. The MT12 cell line shows markedly lower frequencies of methotrexate resistance in comparison to CC1 cells. Although the MT12 cell line exhibited the lowest frequencies of drug resistance at all concentrations of methotrexate, an occasional drug resistant colony was found even at 150 nM methotrexate (Fig. 7).

**Plating Efficiencies of Polyoma Virus Oncogene Expressing cell Lines**

Since the frequencies of methotrexate-resistant cells were determined by the ratio of colonies formed to cells plated, significant variation in plating efficiency would result in apparent variation in methotrexate resistance. Thus, plating efficiency experiments were carried out for each of the cell lines studied. To mimic the conditions of the drug selections viable cells were mixed with large numbers of "feeder" cells prepared from the same lines by gamma-irradiation. As shown in Table 2, plating efficiencies in the absence of methotrexate were similar for these lines. The plating efficiencies were not significantly changed by the presence of a non-selective concentration of methotrexate (10 nM). Thus, the differences in frequencies of methotrexate resistance in these lines were not a result of differences in plating efficiencies.

**Growth Curves of Polyoma Virus Expressing Cell Lines**

Growth curves of three polyoma oncogene expressing cell lines and the control CC1 cells were compared to see if any aspect of growth kinetics correlated with the frequencies of methotrexate resistance. As depicted in Figure 9, the order of maximum
TABLE 2

Determination of plating efficiency$^a$ of NIH3T3 cells expressing different polyoma virus oncogenes and the control cell line.

<table>
<thead>
<tr>
<th>Cells</th>
<th>No MTX</th>
<th>10 nM MTX$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1</td>
<td>0.55 ± 0.01</td>
<td>0.50 ± 0.01</td>
</tr>
<tr>
<td>MT12</td>
<td>0.50 ± 0.01</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>LT11</td>
<td>0.54 ± 0.02</td>
<td>0.55 ± 0.01</td>
</tr>
<tr>
<td>ST1-5</td>
<td>0.41 ± 0.0</td>
<td>0.42 ± 0.01</td>
</tr>
</tbody>
</table>

$^a$Number of colonies divided by number of cells plated.

$^b$A non-selective concentration of MTX was used to determine if MTX affects plating efficiency.
Figure 7. Simultaneously selected methotrexate resistant colonies of each of the four cell lines derived from NIH3T3 expressing various polyoma virus oncogenes. These plates are part of the raw data used to construct figure 6. Although four plates were at each methotrexate concentration, only two are shown in the figure.
FIGURE 7
Figure 8. Comparison of plating efficiency results for ST1-5 and LT11 cells. In the absence of methotrexate, ST1-5 cells form larger colonies than LT11 cells.
growth rate in mid log phase was ST1-5 > MT12 > LT11 = CC1. The order of lag in the beginning of growth was MT12 > ST1-5 > CC1 > LT11, and the order of maximum cell density was MT12 > ST1-5 > LT11 > CC1. It is clear that the differences in the maximum cell density were substantial, although this might not be readily apparent because of the use of a log scale in Figure 9. The frequencies of methotrexate resistance in these cell lines did not appear to be related to growth kinetics in any simple way.

**Evaluation of Dihydrofolate Reductase and ras Gene Copy Number in Methotrexate Resistant Cells**

To evaluate the role of *dhfr* gene amplification in methotrexate resistance, colonies obtained from each cell line in a one-step selection at 100 nM methotrexate were isolated and grown in medium with the same concentration of methotrexate. DNA was isolated from each of the methotrexate-resistant subclones and was blotted onto nitrocellulose filters with a slot-blot apparatus. After drying and prehybridization, the filters were hybridized to $^{32}$P-labelled probes for the *dhfr*, *ras*, and alpha-globin genes. The alpha globin gene probe was used as a single copy and loading control, since it is very unlikely that this gene would undergo any change in its copy number during methotrexate selection. The relative *dhfr* and *ras* gene dosages were estimated by comparison to that of alpha-globin gene in each case (Fig. 10). Densitometric scans of the slots were done with a soft laser densitometer. The results for the *dhfr* gene are shown in Figure 11. Only values greater than 2 were regarded as evidence of gene amplification.

None of the 19 methotrexate subclones derived from ST1-5 cells showed evidence of *dhfr* gene amplification. Two of the 20 LT11 derived methotrexate subclones exhibited
Figure 9. Growth curves of NIH3T3 cell lines expressing various polyoma virus oncogenes and control cells in methotrexate free media.

Open squares: ST1-5
Open circles: LT11
Closed triangles: CC1
Closed circles: MT12
FIGURE 9
low level (less than three fold) $dhfr$ gene amplification. The distribution of $dhfr$ gene dosage values was shifted slightly upwards for the LT11 methotrexate resistant subclones. This might indicate a tendency, to increase $dhfr$ gene copy number slightly by a nonrandom change in chromosome complement. A high frequency of significant $dhfr$ gene amplification was seen in CC1 cells. In this cell line, ten of the 15 methotrexate-resistant subclones exhibited $dhfr$, and levels of amplification ranged up to 17.4-fold. One-third of the CC1-derived methotrexate resistant clones showed no evidence of $dhfr$ gene amplification. The range and distribution of $dhfr$ gene dosage in the methotrexate resistant subclones of CC1 cells is of special interest. Although there were three subclones showing a tenfold amplification, there was no periodicity or pattern to the level of amplification. The distribution of $dhfr$ gene dosage in MT12 derived methotrexate resistant cells centered near the value of one, indicating no amplification of dihydrofolate reductase gene (Fig. 11).

Since ras gene dosage has been reported to change in human ataxia telangiectasia cells undergoing $dhfr$ gene amplification (Lucke-Huhle, Hinrichs and Speit, 1987), the ras gene dosage was also determined in the methotrexate resistant sublines (Fig. 12). No evidence of ras gene amplification or deletion was found in any of the methotrexate resistant subclones.
Figure 10. Analysis of relative dihydrofolate reductase and ras gene dosage in methotrexate resistant subclones of ST1-5, LT11, and CC1 cells. Each DNA sample was blotted in triplicate, and each blot was then hybridized with $^{32}$P-labelled dihydrofolate reductase, ras and alpha-globin probes. The alpha-globin gene was used as a single copy control and dihydofolate reductase and ras gene dosages were measured relative to the alpha-globin signal. Methotrexate resistant subclones are indicated by "r" prefix. Controls include the unselected methotrexate-sensitive parent lines in each case, NIH3T3 cells, R500 cells, and dilutions of R500 cells DNA with NIH3T3 DNA (1/2 R500, 1/4 R500 and 1/8 R500).
FIGURE 10
Figure 11. Distribution of relative dihydrofolate reductase gene copy number in methotrexate resistant subclones. The relative dihydrofolate reductase gene dosage was plotted against the number of methotrexate resistant subclones of each cell line showing that dosage.
FIGURE 11
Figure 12. Distribution of relative ras gene copy number in methotrexate resistant subclones. The ras gene dosage relative to alpha-globin was plotted against the number of subclones of each cell line showing that dosage. The distribution of relative dihydrofolate reductase gene dosage for these methotrexate-resistant subclones also are shown in Figure 11.
FIGURE 12

NUMBER OF MTX-RESISTANT SUBCLONES

RELATIVE RAS GENE DOSAGE

ST1-5     LT11     CC1     MT12
Quantitation of Dihydrofolate Reductase Gene Expression in Methotrexate Resistant Cells

Several methotrexate-resistant subclones derived from each cell line were used to evaluate levels of dhfr mRNA (Table 3). The methotrexate-resistant CC1 subclones with dhfr gene amplification exhibited clearly elevated levels of dihydrofolate reductase message. None of the methotrexate resistant ST1-5, LT11, or MT12 subclones exhibited levels of dihydrofolate reductase gene expression significantly higher than those of the corresponding methotrexate-sensitive cells. The levels of dhfr gene expression for the unselected methotrexate sensitive ST1-5 and MT12 lines were slightly higher relative to CC1 cells. This can be attributed to the fact that cells for this experiment were harvested at confluence. Both of these cell lines were still in log phase growth at confluence, and it is known that dhfr gene expression is slightly elevated in growing cells as compared with quiescent cells (Johnson et al, 1978; Collins et al, 1983).

Accelerated Loss of Amplified Dihydrofolate Reductase Genes by Various Treatments

Loss of Dihydrofolate Reductase (dhfr) Gene Dosage

When R500 cells were grown in the absence of methotrexate (Fig. 13, upper right), the dhfr gene dosage decreased with biphasic kinetics (Fig. 13, upper left). A loss of about 40% of the original dihydrofolate reductase gene dosage occurred in the first passage of cells grown without methotrexate. This was followed by a slow constant decline in dhfr gene dosage through the remaining passages. The data shown are from four different experiments, none of which were concurrent. The data points from
TABLE 3

Expression of dihydrofolate reductase gene in methotrexate resistant subclones of

CC1 and three polyoma oncogene expressing cell lines.a

<table>
<thead>
<tr>
<th>Cells</th>
<th>dhfr gene expression</th>
<th>dhfr gene copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1d</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>CC1-R1</td>
<td>7.7</td>
<td>10.2</td>
</tr>
<tr>
<td>CC1-R2</td>
<td>9.6</td>
<td>11.7</td>
</tr>
<tr>
<td>CC1-R3</td>
<td>6.5</td>
<td>14.4</td>
</tr>
<tr>
<td>CC1-R4</td>
<td>12.6</td>
<td>14.1</td>
</tr>
<tr>
<td>CC1-R5</td>
<td>4.0</td>
<td>10.2</td>
</tr>
<tr>
<td>LT11d</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>LT11-R16</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td>LT11-R17</td>
<td>2.3</td>
<td>1.5</td>
</tr>
<tr>
<td>LT11-R18</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>LT11-R19</td>
<td>0.4</td>
<td>1.4</td>
</tr>
<tr>
<td>LT11-R20</td>
<td>1.9</td>
<td>2.8</td>
</tr>
<tr>
<td>ST1-5d</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td>ST1-5-R16</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>ST1-5-R17</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>ST1-5-R18</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>ST1-5-R19</td>
<td>2.2</td>
<td>1.3</td>
</tr>
<tr>
<td>ST1-5-R20</td>
<td>0.6</td>
<td>ND*</td>
</tr>
<tr>
<td>MT12d</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td>MT12-R1</td>
<td>2.7</td>
<td>1.1</td>
</tr>
<tr>
<td>MT12-R2</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>MT12-R3</td>
<td>0.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>

aCells harvested at confluence.

b dhfr gene expression measured relative to MTX-sensitive CC1 cells.

c dhfr gene copy number relative to the α-globin gene.

d Unselected, MTX-sensitive cells.

*Not determined.
these different experiments were all fitted to a single graph (dashed line). Several of the
nineteen points plotted from different experiments are coincident with one another. From
this it is concluded that both growth and dhfr gene loss kinetics were very reproducible
for the R500 cell line. The rate of gene loss was only slightly different from that
observed for this line earlier (Snapka and Varshavsky, 1983).

Inhibitors of Ribonucleotide Reductase

As observed earlier (Snapka and Varshavsky, 1983), low concentrations of
hydroxyurea can accelerated the rate of loss of unstably amplified dhfr genes (Fig. 13,
middle). Here, 50 and 75 μM hydroxyurea had only slight effects on the growth of R500
cells in the absence of methotrexate, but both caused a rapid loss of about 70% of the
original dhfr gene dosage. Again there was a rapid initial loss of dhfr gene dosage,
followed by a slow second phase.

Since there are reports that hydroxyurea may have targets other than
ribonucleotide reductase (Wawra and Wintersberger, 1983; Masuda et al, 1982), another
inhibitor of this enzyme, guanazole, (Moore and Hurlbert, 1985) was used. Three
concentrations of guanazole were chosen so that a wide range of growth inhibition from
slight (0.3 mM guanazole) to marked (1.0 mM guanazole) was obtained (Fig. 13, bottom).
The kinetics of dhfr gene loss were very similar at all three guanazole concentrations.
The first passage resulted in a loss of 70-80% of the original dhfr gene dosage and was
followed by a slow decline to about 10% of the original levels during the remaining
passages. The rate of dihydrofolate reductase gene loss in this second phase in the
presence of guanazole was similar to the rate of loss in the second phase
Figure 13. Loss of unstably amplified genes in the presence of ribonucleotide reductase inhibitors. The top left and top right graphs show the loss of dihydrofolate reductase gene dosage and growth, respectively, for four separate and nonconcurrent experiments in which R500 cells were grown in the absence of methotrexate. The dihydrofolate reductase gene loss and growth curves for cells grown in the absence of methotrexate and in the presence of two concentrations of either hydroxyurea or guanazole are shown in the middle and bottom graphs. In each graph, the control gene loss from the top graphs is represented by a dashed line.
without added drugs (Control). Guanazole thus accelerated \textit{dhfr} gene loss only in the first phase.

**Topoisomerases Inhibitors**

Two topoisomerase inhibitors were studied for their ability to induce rapid \textit{dhfr} gene loss. Camptothecin is an inhibitor of type I topoisomerase (Hsiang et al, 1985; Kjelsen et al, 1988; Andoh et al, 1987) which breaks moving replication forks in mammalian cells (Snapka, 1986). At a concentration of 20 nM, camptothecin caused a moderate reduction in growth and only a slight increase in the rate of dihydrofolate reductase gene loss (Fig. 14, top). At 50 nM camptothecin, growth was still slower, and loss of \textit{dhfr} gene dosage was pronounced. The dihydrofolate reductase gene dosage dropped 93% in the first passage at this concentration and remained at this level for the next four passages.

Intercalators and inhibitors of DNA gyrase (bacterial topoisomerase II) can efficiently eliminate extrachromosomal DNAs from bacteria at concentrations which are not toxic to the cells (Hahn and Ciak, 1976; Wolfson et al, 1982). Proflavine has been shown to inhibit type II topoisomerase and interfere with the separation of circular viral chromosomes in mammalian cells (Snapka, Powelson and Strayer, 1988). Thus the DNA intercalating drug, proflavine, was tested for its ability to cause rapid loss of amplified \textit{dhfr} genes. In 0.2 \textmu M proflavine, the rate of loss of amplified \textit{dhfr} genes was not significantly different from that of the controls (Fig. 14, bottom). In 0.5 \textmu M proflavine, the \textit{dhfr} gene dosage dropped to about 25% in the first passage, then remained constant through the remaining passages. The pattern was similar to that of camptothecin, with a pronounced initial drop, followed by a plateau region in which no further gene loss was
observed. For both camptothecin and proflavine, the induction of accelerated \textit{dhfr} gene loss was strongly concentration dependent and all of the gene loss occurred in the first passage with exposure to the drug.

\textbf{Cytostatic Treatments}

Since the drugs studied above are cytotoxic, cytostatic treatments were also studied for their ability to cause accelerated loss of unstably amplified \textit{dhfr} genes. DFMO is an inhibitor of ornithine decarboxylase, a key enzyme in polyamine biosynthesis. This drug has been reported to have both cytotoxic and cytostatic effects (Pegg, 1988). In this study DFMO was found to cause accelerated loss of unstably amplified \textit{dhfr} genes (Fig. 15, top), but the dose dependence of the effect was complex. As expected, DFMO slowed cell growth in a concentration-dependent manner. However, the dose dependence of the rate of \textit{dhfr} gene loss did not parallel that of slowed growth. At 0.1 mM, the initial loss of \textit{dhfr} gene dosage was identical to that of the control. In the second phase the rate of loss was greater than that of the control. There was an initial loss of about 80\% of the original \textit{dhfr} gene dosage in the first passage in 0.25 mM DFMO, and no further decline in the remaining passages at this concentration. This pattern was similar to that of proflavine. At 0.5 mM DFMO, the rate of \textit{dhfr} gene loss actually decreased, returning to a pattern similar to that of the control. This indicates that there is no simple relationship between the kinetics of \textit{dhfr} gene loss and slowing of growth. For DFMO, there appears to be an optimal concentration for the gene loss effect. To further test for any possible relationship between the kinetics of \textit{dhfr} gene loss and slowed growth,
Figure 14. Loss of unstably amplified dihydrofolate reductase genes in the presence of topoisomerase inhibitors. The control curves from figure 13, top, are indicated by the dashed line in each graph. R500 cells were seeded in several 10 cm plates at a density of $1 \times 10^5$ per plate either in the absence of any drug or in the presence of a test drug. At confluence the cell number was counted to determine the population doublings. Two of the plates from each set were used to isolate genomic DNA for hybridization experiments in order to determine gene copy number.
Figure 15. Effects of cytostatic treatments on the loss of unstably amplified dihydrofolate reductase genes. Controls; dashed line (from figure 13, top).
we studied the effect of growth in low serum. When R500 cells were grown in 2.5% serum, their growth was slowed substantially (57% of the control, Fig. 15, bottom). However, the kinetics of dhfr gene loss were almost identical to those of the control.

**Indicators of Cell Stress**

For the control and all of the treatments, the decrease in dhfr gene dosage was greatest in the first passage in the absence of any methotrexate, and it was in this passage that most drug treatments had their greatest effect (Fig. 16). No correlation was found between rates or extents of first passage dhfr gene loss and reduced growth rates resulting from the treatments.

Cells exposed to cytotoxic drugs show an increase in size as a result of unbalanced growth (Ross, 1981; Sinclair, 1967; Ross, 1985). This is reflected in a lower cell density at confluence in cultured cells exposed to antimetabolites such as hydroxyurea (Snapka and Varshavsky, 1983). The decrease in cell density at confluence correlates well with forward scattered light in the flow cytometer, another measure of average cell size (Shapiro, 1985). As seen in Figure 17, all of the treatments employed in this study reduced the cell density at confluence. However, the reduction in the cell density for treatments having little or no effect on dhfr gene loss (Fig. 17, upper points) was almost identical to that seen for treatments which caused pronounced gene loss (Fig. 17, lower points). The lowest point in the figure represents 50 nM camptothecin.

Although drug treatments employed were sufficient to slow cell growth, they were not cytotoxic. The plating efficiency for R500 cells in the absence of any drug was 93%
(Table 4) and trypan blue positive cells were 2.6% (Table 5) of the population in the late log phase (80% confluence). The corresponding values for these cells undergoing the drug treatments described here were not significantly different.
### TABLE 4

Effect of various drugs on plating efficiency of R500 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plating Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>93%</td>
</tr>
<tr>
<td>50μM Hydroxyurea</td>
<td>92%</td>
</tr>
<tr>
<td>75μM Hydroxyurea</td>
<td>91%</td>
</tr>
<tr>
<td>0.3 mM Guanazole</td>
<td>95%</td>
</tr>
<tr>
<td>0.5 mM Guanazole</td>
<td>88%</td>
</tr>
<tr>
<td>1.0 mM Guanazole</td>
<td>85%</td>
</tr>
<tr>
<td>20 nM Camptothecin</td>
<td>81%</td>
</tr>
<tr>
<td>50 nM Camptothecin</td>
<td>81%</td>
</tr>
<tr>
<td>0.2 μM Proflavine</td>
<td>88%</td>
</tr>
<tr>
<td>0.5 μM Proflavine</td>
<td>80%</td>
</tr>
<tr>
<td>0.1 mM DFMO</td>
<td>95%</td>
</tr>
<tr>
<td>0.25 mM DFMO</td>
<td>91%</td>
</tr>
<tr>
<td>0.5 mM DFMO</td>
<td>89%</td>
</tr>
<tr>
<td>2.5 % serum</td>
<td>88%</td>
</tr>
</tbody>
</table>
TABLE 5

Determination of cell viability of R500 cells, by trypan blue exclusion, after exposure to hydroxyurea and camptothecin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>97%</td>
</tr>
<tr>
<td>250 µM Methotrexate</td>
<td>95%</td>
</tr>
<tr>
<td>50 µM Hydroxyurea</td>
<td>98%</td>
</tr>
<tr>
<td>20 nM Camptothecin</td>
<td>98%</td>
</tr>
<tr>
<td>50 nM Camptothecin</td>
<td>97%</td>
</tr>
</tbody>
</table>
Figure 16. First passage decrease in relative dihydrofolate reductase gene dosage with cytotoxic and cytostatic treatments. R500 cells were grown in the absence and presence of various test drugs. At confluence DNA from each test was isolated and blotted on nitrocellulose filter. The filters were hybridized to $^{32}$P-labelled dihydrofolate reductase and alpha-globin genes.
FIGURE 16
Figure 17. Cell density at confluence and first passage loss of dihydrofolate reductase gene dosage for treatments employed in the gene loss study.

Closed triangle: Camptothecin
Open square: Proflavine
Closed square: DFMO
Open triangle (inverted): 2.5% serum
Closed circle: Hydroxyurea
Open triangle: Guanzole
Open circle: Controls
PERCENT DHFR GENE DOSAGE
AFTER FIRST PASSAGE

FIGURE 17
Effect of Low Level Cytotoxic Stress on Cells with High Levels of Dihydrofolate Reductase (dhfr) Gene Amplification

Sorting of the R500 Cell Population

Methotrexate resistant cells with amplified dhfr genes overproduce the enzyme, dihydrofolate reductase (Alt et al, 1978; Flintoff, 1982). Since methotrexate and methotrexate derivatives bind very tightly to DHFR, cellular retention of these drugs is proportional to the intracellular enzyme levels (Kaufman, Bertino and Schimke, 1978). Thus, for cells with amplified dhfr genes, retention of fluorescin methotrexate (f-MTX) is an indirect measure of gene amplification (Johnston, Beverley and Schimke, 1983; Mariani and Schimke, 1984). Fluorescent derivatives of methotrexate have been used to evaluate the distribution of dhfr gene dosages in cells of stably and unstably methotrexate resistant cells (Brown, Beverley and Schimke, 1981; Schimke et al, 1980).

R500 cells have an average dhfr gene dosage of about 200 copies per cell (Hamkalo et al, 1985). Flow cytometry (Fig. 18-A) shows that cells of the highly methotrexate resistant R500 cell line retain, on average, much higher levels of f-MTX than do cells of the methotrexate sensitive NIH 3T3 line. It is also clear that the cells of the R500 line are very heterogenous with respect f-MTX retention. At the low end of the distribution, a few cells retain no more f-MTX than NIH 3T3 cells. The distribution also has a long tail at the high end, suggesting a small population of cells with very high levels of intracellular dihydrofolate reductase. These results are in agreement with earlier cytometric studies on similar methotrexate resistant cell lines (Brown, Beverley and Schimke, 1981; Schimke et al, 1980).
Cells of the R500 population were separated into high and low f-MTX retention sub-populations by flow sorting. Flow cytometry of these populations immediately after sorting (Fig. 18-B) demonstrated that the average f-MTX retention of the low f-MTX population R500− was lower than that of unsorted R500 cells, but higher than that of methotrexate sensitive NIH 3T3 cells. The average f-MTX retention of the high f-MTX subpopulation R500+ was higher than that of the unsorted population of R500 cells. Slot blot analysis of dhfr gene dosage relative to the mouse alpha-globin gene showed that the R500− population had an average of 132 copies of the dhfr gene per cell, and R500+ population had an average of 240 copies of the dhfr genes per cell relative to the R500 level of 200 copies per cell.

Growth curves

The three methotrexate resistant populations (R500, R500− and R500+) were compared for growth in 50 μM hydroxyurea. This concentration of hydroxyurea slightly slows the growth of unstably methotrexate resistant mouse cell lines and causes a very rapid drop in dhfr gene dosage (Snapka and Varshavsky, 1983). The slowed growth is due in part to increased cell size and early cell to cell contact in the presence of the drug (Snapka and Varshavsky, 1983). Under these conditions, there is no significant increase in cell death as determined by trypan blue exclusion and plating efficiency. Growth in 50 μM hydroxyurea slows the R500 population and leads to confluence at a lower cell density (Fig. 19, top). The same growth conditions slowed the growth of R500+ population significantly, but had no effect on the R500− population (Fig. 19, bottom and middle). This result suggests selective action by hydroxyurea against
Figure 18. Distribution of f-MTX in R500 and related cell lines.

(A) Retention of f-MTX (25 μM) by NIH3T3 cells (solid line) and R500 cells (dashed line). (B) Retention of f-MTX by R500⁻ (solid line) and R500⁺ (dashed line) subpopulations derived from R500 cells. The R500⁻ and R500⁺ populations were obtained by flow sorting the R500 population into cells with less than or more than 16 green fluorescent units respectively.
FIGURE 18
Figure 19. Growth curves of cells and subpopulations with lower (R500') and higher (R500+) average dihydrofolate reductase gene dosage, in 50 µM hydroxyurea. 5x10^5 cells were plated in 60 mm plates. After 24 hours of attachment, plates were removed in groups of four at regular intervals and the cells from each plate were harvested by trypsin digestion and then counted.
FIGURE 19
those cells in the R500 cell population with the highest levels of \textit{dhfr} gene amplification.

\textbf{Cloning Efficiency}

Although 50 \textmu{}M hydroxyurea does not significantly decrease plating efficiency (number of cells attached 24 hours after plating at high cell density) or trypan blue exclusion in R500 cells, it is possible that these methods might miss selective killing of a small subpopulation. The long tail in the distribution of f-MTX retention by R500\textsuperscript{*} and R500 cells suggests that there are small subpopulations of cells with very high levels of \textit{dhfr} gene amplification within the larger population. In addition, the complex kinetics of drug induced \textit{dhfr} gene loss in R500 cells could be a manifestation of differential targeting of subpopulations by different drugs. These considerations led to the testing of these cell lines for cloning efficiency in the presence of low levels of cytotoxic drugs known to accelerate the loss of unstably amplified dihydrofolate reductase genes.

When R500 cells were compared to an NIH 3T3 cell population for cloning efficiency in 50 \textmu{}M hydroxyurea, there was only a small difference in reduction of cloning efficiency (40\% reduction for R500 and 30\% reduction for NIH 3T3 when plated at 3 \times 10^3 cells/plate in 50 \textmu{}M hydroxyurea). While this might suggest that gene amplification has little effect on sensitivity to cytotoxic agents, the interpretation of such a result is complex. The R500 cell line is highly heterogenous with respect to numbers of double minutes (DMs) per cell (Hamkalo et al,1985), and numbers of \textit{dhfr} genes per cell. These cells are also likely to show clonal variation in a number of characteristics not associated with \textit{dhfr} gene amplification. NIH 3T3 cells also become heterogenous during growth in culture, and this heterogeneity might include sensitivity to cytotoxic agents. The R500 line was isolated from NIH 3T3 cells approximately ten years ago (Brown,
Beverley and Schimke, 1981), and it is possible that substantial population drift has taken place over the years. Thus, a more rigorous way of conducting the experiment would involve comparison of a large number of clonal isolates of NIH 3T3 cells with a large number of clonal isolates of R500 cells for cloning efficiency in low levels of cytotoxic drugs. In order to do that, a comparison of at least 100 isolates of each line would be required for such a study. As an alternative, the sorted R500+ and R500- populations were compared for cloning efficiency under a variety of cytotoxic conditions. Any variation in the R500 population not associated with dihydrofolate reductase gene amplification should be distributed randomly between the two subpopulations during flow sorting.

As shown in Figure 20, the cloning efficiency of R500- and R500+ populations was the same in the absence of any drugs. In the presence of 250 μM methotrexate, the R500- population showed a slightly reduced cloning efficiency relative to the R500+ population. In low levels of hydroxyurea and camptothecin, the situation was dramatically reversed. Both of these drugs reduced the cloning efficiency of R500+ cells to a much greater extent than that of the R500- cells. For both cell populations and both drugs, the reduction in the cloning efficiency was concentration dependent.

The effect of these drugs in combination with methotrexate was also examined in cloning efficiency experiments (Fig. 21). Even in the presence of 250 μM methotrexate, the R500+ cells were much more sensitive to low level cytotoxic stress. Thus, cytotoxic
Figure 20. Cloning efficiency of R500+ and R500− subpopulations in media containing methotrexate, hydroxyurea or camptothecin. For each pair of duplicate plates, the average number of colonies is shown with the percentage of the "no drug" control being given in the parentheses.
Figure 21. Cloning efficiency of R500* and R500* subpopulations in media containing methotrexate with either hydroxyurea or camptothecin. Average number of colonies for each pair of duplicate plates is shown, with the percentage of the "no drug" control (see Figure 20) being given in parentheses.
<table>
<thead>
<tr>
<th>Methotrexate (µM)</th>
<th>250</th>
<th>250</th>
<th>250</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyurea (µM)</td>
<td>50</td>
<td>75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Camptothecin (nM)</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>50</td>
</tr>
</tbody>
</table>

**LOW DHFR**

- (132 copy/genome)

- 326 [75%]
- 258 [60%]
- 203 [47%]
- 43 [10%]

**HIGH DHFR**

- (240 copy/genome)

- 231 [54%]
- 143 [33%]
- 125 [30%]
- 6 [1.5%]
stress can act selectively against cells with the highest levels of \textit{dhfr} gene amplification even in the presence of the selecting drug, methotrexate.
CHAPTER IV
DISCUSSION

Methotrexate Resistance in NIH 3T3 cells Expressing Different Polyoma Oncogenes

NIH 3T3 cells expressing three different polyoma oncogenes showed large differences in their frequencies of methotrexate resistance, when compared to each other and the control cell line. These differences were several orders of magnitude over a range of drug concentrations. The two cell lines showing the highest frequencies of methotrexate resistance were ST1-5 and LT11, which express polyoma small T antigen and large T antigen respectively. Both of these T antigens are located in the nucleus (Zhu et al, 1984; Noda et al, 1986) and both can complement polyoma middle T antigen to transform rodent cells to tumorigenicity in vivo (Asselin et al, 1983; Asselin et al, 1984). The experimental results shown in this study suggest that polyoma virus small and large T antigens have similar but somewhat different effects on resistance to methotrexate. Although ST1-5 cells show higher frequencies of methotrexate resistance than LT11 cells, the LT11 cells appear to achieve resistance more rapidly than ST1-5 cells. Middle T antigen is considered to be the transforming gene of polyoma since it can transform established cell lines by itself (Treisman et al, 1981) or can transform early passage primary cells when fused to a strong promoter (Spandios and Riggio, 1986) or assisted by
an immortalizing gene such as large T antigen (Land et al, 1983; Rassoulzadegen et al, 1982). The frequency of methotrexate resistance in the MT12 cell line was actually lower than that in the control CC1 population.

Although control cells (CC1), carrying the cloning vector without any oncogene showed intermediate frequencies of methotrexate resistance, two thirds of the methotrexate resistant subclones derived from these cells showed clear dihydrofolate reductase gene (dhfr) amplification, a fraction typical of mouse fibroblasts undergoing single step selections for methotrexate resistance (Sirotnak et al, 1981; Barsoum and Varshavsky, 1983; Hamlin et al, 1984; Tlsty et al, 1982). This result indicates that integration of the cloning vector alone does not significantly alter methotrexate resistance. Although cell lines expressing polyoma oncogenes showed frequencies of methotrexate resistance that were both lower (MT12) and much higher (ST1-5 and LT11) than those seen in CC1 cells, these lines became resistant to methotrexate almost entirely by mechanisms other than dhfr gene amplification. Only two of the LT11 subclones among 20 methotrexate resistant subclones showed marginal dhfr gene amplification. Studies on clonal variation in frequency of dhfr gene amplification have been done (Brown, Tlsty and Schimke, 1983) and the lowest frequency of amplification in any subclone was 30%. No normal mouse fibroblasts have been reported that do not show dhfr gene amplification as a major mechanism of methotrexate resistance. From the present studies, it is clear that there is no dhfr gene amplification in methotrexate resistant subclones derived from the ST1-5 and MT12 cell lines. There is also little if any dhfr gene amplification in methotrexate resistant LT11 derived subclones. These results suggest that expression of the polyoma oncogenes may be responsible for this unique pattern of drug resistance.
Expression of immortalizing oncogenes (polyoma large T antigen, c-myc and v-myc) but not transforming genes (polyoma middle T antigen and ras) increase sister chromatid exchange and aneuploidy in early passage rat fibroblasts (Cemi et al, 1987). These authors also noted that cells expressing both immortalizing genes and transforming genes had normal levels of sister chromatid exchange, suggesting that transforming oncogenes could offset the genome destabilizing effects of the immortalizing oncogenes. Immortalizing oncogenes such as large T antigen of polyoma virus and SV40 have been reported to increase genetic instability as evidenced by increased rate of recombination (Cemi et al, 1987), and genetic instability has been linked to increased rates of dhfr gene amplification (Sager, 1985). The treatments that are thought to increase genetic instability in cultured cells (Zdzienicka, 1985) also increase the frequency of dhfr gene amplification (Tlsty, 1982). In the present studies the large T antigen expressing cell line LT11 showed both a much higher frequency of methotrexate resistance as well as much lower levels of dhfr gene amplification compared with CC1 cells that do not express an oncogene. NIH 3T3 cells are already immortalized and expression of polyoma large T antigen does not greatly alter the NIH 3T3 phenotype (Cherington et al, 1986). Thus the decreased gene amplification in LT11 cell line was unexpected. Resistance to methotrexate can arise through several mechanisms which include altered transport (Sirotnak et al, 1981; Hakala, 1964; Sirotnak, Kuriata, and Hutchinson, 1968; Flintoff et al, 1976; Hill et al, 1986), mutations resulting in altered DHFR with decreased binding to methotrexate (Flintoff et al, 1976; Hill et al, 1986), or amplification of the dhfr gene (Alt et al, 1978; Bostock and Tyler-Smith, 1981; Melera, Lewis et al, 1980), resulting in overproduction of DHFR. These are the only established mechanisms of acquired methotrexate resistance, although
several other mechanisms are possible including altered methotrexate polyglutamate formation (Fry et al, 1983; Poser, Sirotnak and Chello, 1986; Curt et al, 1983; Cowan and Jolivet, 1984) and changes in nucleotide pools. Methotrexate resistance due to up-regulation of dhfr gene transcription in the absence of amplification of its gene, has not been observed. However, the large T antigen of DNA tumor viruses can increase transcription from some cellular genes (Nevins, 1986). The data from this study show that significant overexpression of the dhfr gene is seen only in the cells which show amplification of the dhfr gene.

Cells expressing polyoma small T antigen exhibited the highest frequencies of methotrexate resistance. Resistance due to either altered methotrexate transport or to gene amplification both occur at high frequencies relative to mutation rates. Since none of the resistant subclones derived from this cell line showed any amplification of dhfr gene, amplification of this gene as a major possible mechanism in these cells is thus ruled out. It is also unlikely that a greatly increased frequency of methotrexate resistance in ST1-5 cells relative to CC1 cells is due to mutation since there is no evidence that oncogene expression increases mutation rates. Altered transport is thus the most likely mechanism for methotrexate resistance in this cell line. If the ST1-5 cells are predisposed to alteration or inactivation of the folate transporter, resistance by this mechanism would occur before the cells would acquire dhfr gene amplification.

Among the three polyoma oncogene expressing cell lines, MT12 was the most methotrexate-sensitive. The frequency of resistance to methotrexate was very low compared to CC1 cells. As in the case of ST1-5 cells, the methotrexate-resistant subclones derived from MT12 cells did not show amplification of the dhfr gene. Because
the frequency of methotrexate resistance was so low in MT12 cells, mutation of the \textit{dhfr} gene could be still a possible mechanism of resistance in these cells. Whatever the mechanism of resistance in MT12 cells, it appears that the frequency is not high enough to explain the lack of amplification as being due to preemption by this alternate mechanism. This raises the possibility that amplification of \textit{dhfr} gene is suppressed in this cell line and expression of the middle T oncogene may have a stabilizing influence on the cellular genome. This is consistent with the observation that cells expressing transforming genes are genetically stable with respect to their normal levels of sister chromatid exchange (Cemi et al, 1987).

When the growth kinetics of ST1-5, MT12, LT11 and CC1 cells were studied they were found to differ greatly in maximum cell density. This is likely to be due, at least in part, to varying degrees of contact inhibition. Since the cells were plated at high cell density in the methotrexate selection experiments, cell contact might play an important role in their early behavior in the selecting media. Although there is no correlation between growth kinetics and frequency of methotrexate resistant colonies for these cell lines, there is a correlation between maximum cell density and the fraction of methotrexate resistant colonies with \textit{dhfr} reductase gene amplification. The two cell lines with highest maximum cell densities, MT12 and ST1-5, did not show \textit{dhfr} gene amplification. On the other hand CC1 cells showed lowest maximum cell density and a high frequency of marked dihydrofolate reductase gene amplification. The LT11 cell line, which was intermediate with respect to maximum cell density, showed rare, low level gene amplification.
In the present study all three of the polyoma oncogene expressing lines tend to become resistant to methotrexate by mechanisms other than \textit{dhfr} gene amplification. Thus it is possible that all three polyoma T antigens suppress amplification of this gene. In a different study (Lucke-Huhle et al, 1987), when SV40 transformed human ataxia telangiectasia cells were made resistant to high concentrations of methotrexate by a series of step-wise selections, the resistance in these cells was acquired through amplification of \textit{dhfr} gene which was accompanied by loss of the SV40 sequences. This might be explained if the SV40 T antigens suppress \textit{dhfr} gene amplification. Reduced methotrexate transport and mutation of \textit{dhfr} gene alone can confer resistance to low concentrations of methotrexate, but resistance to higher methotrexate concentrations always involves \textit{dhfr} gene amplification (Hill et al, 1986). In order to achieve resistance to high levels of methotrexate, the cells would tend to lose any gene whose product was suppressing gene amplification. T antigens might alter methotrexate resistance by several mechanisms. Methotrexate transport has been reported to be regulated by cyclic AMP (Henderson et al, 1978). Involvement of polyoma T antigens in cyclic AMP metabolism is suggested by the observation that polyoma transformed rat 3T3 cells are uniquely sensitive to growth inhibition by cyclic AMP and evidence that all three of the polyoma T antigens are required for this sensitivity (Kamech et al, 1987). Poly ADP-ribosylation represents another intracellular signaling pathway that might mediate affects on methotrexate resistance. Relative to nontransformed 3T3 cells, SV40 transformed 3T3 cells show greatly increased poly ADP-ribosylation in response to methotrexate inhibition of the cell cycle (Sooki-Toth et al, 1987).
Accelerated Loss of Unstably Amplified Dihydrofolate Reductase Genes

Cytotoxic treatments can cause rapid loss of unstably amplified dihydrofolate reductase genes.

The mouse 3T3 derived R500 cell line is resistant to 250 uM methotrexate and carries approximately 200 copies of dhfr gene per cell (Brown et al, 1981). The karyotype of R500 cells has been described in detail (Hamkalo et al, 1985). The amplified genes, in these cells, are located on double minutes, and there is a great heterogeneity in both the numbers and sizes of the double minutes in the individual cells. Both acentric double minutes and marker chromosomes with centromeric sequences are present (Hamkalo et al, 1985). Double minutes are extrachromosomal and known to be unstable because they can be lost from the cells when grown in the absence of selecting drug. Loss of double minutes can occur because their lack of a functional centromere results in disproportionate segregation during mitosis. Inspite of their unstably drug resistance nature, R500 show numerous double minutes and retain significant methotrexate resistance after more than 100 population doublings in the absence of selecting drug, methotrexate. The complex kinetics of dhfr gene loss as detailed in the results of the present study may be a reflection of the heterogeneity of extrachromosomal elements carrying these genes.

Hydroxyurea is a known cytotoxic agent. It is an inhibitor of ribonucleotide reductase (Engston et al, 1979; Krakoff et al, 1968), an enzyme essential for DNA synthesis. It is used for treatment of certain cancers, such as chronic granulocyte leukemia or polycythemia vera (Waaserman and Brown, 1882). Hydroxyurea is also known as a potent teratogen. At subcytotoxic concentrations, hydroxyurea can cause
greatly accelerated loss of amplified $dhfr$ genes both in 3T3-derived R500 cell line and 3T6-derived R.3 cell (Snapka and Varshavsky, 1983). Both of these cell lines are unstably methotrexate-resistant and contain amplified $dhfr$ genes on double minutes. In the same study concentrations of hydroxyurea which could enhance loss of unstably amplified genes did not have any effect on the loss of stably amplified $dhfr$ genes, located in HSRs (Snapka and Varshavsky, 1983). Since transient amplification of specific genes is known to occur during normal development of certain eukaryotes (Spradling and Mahowald, 1980) and because some cancers are known to arise due amplification of specific oncogenes (Schimke et al, 1980; Varshavsky, 1981; Barker, 1982; Cowell, 1982; Hamlin and Biedler, 1981; Schwab et al, 1983; Collins and Groudine, 1982), the induced loss of amplified genes by hydroxyurea might be the basis of its antineoplastic or teratogenic effects.

The loss of amplified dihydrofolate reductase genes was linear throughout the first passage in hydroxyurea in which most of the loss occurs, and no shift of gene dosage from the nucleus to the cytoplasm could be detected (Snapka and Varshavsky, 1983). In the present study it was found that low level inhibition of ribonucleotide reductase is sufficient but not necessary for rapid loss of unstably amplified $dhfr$ genes in the R500 cell line. Subcytotoxic concentrations of several unrelated drugs were able to cause rapid loss of $dhfr$ gene dosage. All of the drugs studied reduced the growth rate of R500 cells in a concentration dependent manner. This was expected since the cell cycle is lengthened by cytotoxic and cytostatic agents of all classes (Mauro et al, 1986). G-2 delay and a lengthened S-phase are seen in cells exposed to antimetabolites and DNA damaging agents such as topoisomerase inhibitors. Low concentrations of these drugs were found to
efficiently induce rapid drops in amplified \textit{dhfr} gene dosage. A lengthened G1 phase is
typical of cytostatic treatments such as growth in low serum (Todaro et al, 1965; Burk,
1970; Temin, 1970). A pronounced reduction in growth rate was observed when cells
were grown in media containing 2.5\% serum, yet the rate of loss of amplified \textit{dhfr} genes
was similar to or slightly lower than that of the control cells. DFMO showed complex
effects on rate of loss of amplified genes, from R500 cells, with an apparent concentration
optimum and a return to a slower rate of gene loss at the highest concentration. This drug
causes a prolonged G1 phase and is considered cytostatic, however it can also interfere
with S-phase of some cell types, notably transformed cells (Porter and Janne, 1987).
There was no correlation between the rate or extent of \textit{dhfr} gene loss and other indicators
of low level stress such as reduction in growth rate and increased cell volume. This
suggests that drug induced loss of amplified genes is not a simple function of the level of
stress experienced by the cells.

Amplified genes carried on double minute chromosomes are thought to be lost by
becoming stranded in the cytoplasm where they undergo micronucleation and subsequent
loss (Cowell, 1982; Schimke et al, 1980). Large numbers of micronuclei are a feature of
the R500 cells both when cells are maintained in methotrexate and after extensive growth
in the absence of methotrexate (Hamkalo et al, 1985). The lack of correlation between
increased cell volume and amplified gene loss taken together with the earlier observation
that there is no detectable shift of \textit{dhfr} dosage from nucleus to the cytoplasm by
hydroxyurea (Snapka and Varshavsky, 1983) argues against the idea that cytotoxic drugs
increase the probability of amplified gene loss simply by increasing cell volume.
In this study, plating efficiency and trypan exclusion data, did not indicate any significantly increased cell death under the treatment conditions used in gene loss experiments. Thus the loss of \textit{dhfr} gene dosage was plotted as a function of time rather than as a function of estimated population doublings. This is more conservative since cells in cytotoxic or cytostatic drugs are unlikely to go through more cell divisions in a given period of time than the control cells which are grown in the absence of any drug. The absence of a detectable increase in the cell death does not rule out a role for cell killing in the gene loss effect. It is possible that specific cell subpopulations are killed or slowed to different extents. Selective action against a small subpopulation might not be detected by these methods. As discussed below, the results suggest that low level cytotoxic stress selectively targets those cells with the highest levels of \textit{dhfr} gene amplification, causing the rapid emergence of a cell population with greatly reduced gene dosage.
Hypersensitivity to Low Level Cytotoxic Stress in R500 Cells with High Levels of Dihydrofolate Reductase Gene Amplification

Exposure of R500 cells to low levels of cytotoxic stress greatly accelerates the loss of unstably amplified \(dhfr\) genes. As discussed earlier, either amplified genes or the cells with higher numbers of amplified genes could be the targets of low level cytotoxic stress. To understand the drug-induced loss of amplified genes, the highly methotrexate resistant mouse R500 cell line was flow sorted into two subpopulations with higher and lower average \(dhfr\) gene dosage respectively. These two subpopulations were found to differ significantly in their sensitivity to low level cytotoxic stress. When the R500\(^+\) (subpopulation with higher copy number of dihydrofolate reductase genes) and the R500\(^-\) (subpopulation with lower copy number of dihydrofolate reductase genes) were compared for their growth and cloning efficiency in the presence of subcytotoxic concentration of hydroxyurea and camptothecin, the cells with higher gene copy number were always much more sensitive compared to the cells with a lower levels of amplified \(dhfr\) genes. The differential sensitivity was also observed in the presence of methotrexate. Although both subpopulations were derived from the same parent cell line, the differences in their growth behavior and cloning efficiency were dramatic. These findings suggest that within a population of cells with unstably amplified genes, there may be differences in sensitivity to cytotoxic stress which are associated with the level of gene amplification. This difference in sensitivity to stressors may be likely a key factor in drug induced loss of amplified genes. That low level cytotoxic stress does not increase rate of loss of stably amplified genes (Snapka and Varshavsky, 1983), can be explained by the fact that the induced gene loss is based on differences in the sensitivity to stressors that are correlated
with the amplified gene dosages within a population. Since stably amplified genes are found as HSRs, integrated into cellular chromosomes, they are evenly partitioned between daughter cells at mitosis. In such a population, the distribution of amplified gene copy number is very narrow relative to that seen in a cell population with unstably amplified genes (Schimke et al, 1980).

In this study the cell sorting procedure was itself stressful and thus the subpopulations obtained, although differing in \( dhfr \) gene copy number, may not completely represent the corresponding subpopulations of the parental R500 population. That this may be so is suggested by the observation that both sorted populations showed maximum cell densities significantly lower than that of the unsorted population. The slow growth of R500 cells (Snapka and Varshavsky, 1983; and this study) and R500+ (this study) in low concentrations of hydroxyurea appears to be mainly due to lowered maximum cell density and early contact inhibition. Also the maximum R500 cell density was much lower than that of the R500+ population. These observations suggest that increased cell size may be related to \( dhfr \) gene amplification and the ability of stressors to accelerate loss of amplified genes in populations of unstably methotrexate resistant cells.

Flow cytometry and in situ hybridization techniques have been used to study the normal loss of unstably amplified genes from drug resistant cell populations (Kaufman et al, 1981; Kaufman and Schimke, 1981; Shen et al, 1988). All of these studies show that loss of unstably amplified genes is very heterogenous as expected for a process that is based on the random distribution of double minutes at each mitosis. Since the cells with fewer double minutes have a replication advantage, they quickly take over the population (Kaufman et al, 1981). However, cells losing unstably amplified genes in the absence of
the selecting drug tend to generate a subpopulation with an amplification level significantly higher than any seen in the original population (Kaufman et al, 1981; Kaufman and Schimke, 1981). Although the population as a whole is losing gene copy number, these cells undergo a transient gain in gene copy number. If these highly amplified cells were selectively killed or inhibited, the rate of gene loss for the population would be sharply accelerated. The experimental results from this study suggest that within a heterogenous population, cells with the highest gene dosage may be selectively targeted by low level cytotoxic stress. This selective sensitivity may be due to total stress level experienced by the cells. Stressors typically slow cell growth and increased cell size (Ross, 1981; Sinclair, 1967). It may be that maintenance of amplified genes is stressful to the cells. The observation that gene amplification is inversely related to cell growth rate (Kaufman et al, 1981) supports this idea, as does the observation that R500⁺ cells appear to be larger than R500⁻ cells. The additional stress from low concentrations of cytotoxic drugs might raise those cells with the highest levels of gene amplification above a threshold beyond which survival or cell division would be compromised.

Selective action by cytotoxic drugs against cells with high levels of dhfr gene amplification is the probable basis for the phenomenon of drug induced loss of unstably amplified genes. Differentiation may also play a role in drug induced loss of unstably amplified genes. Subcytotoxic concentrations of cytotoxic drugs have a strong differentiating effect on transformed cells both in vivo and in vitro (Waxman, 1988). Gene amplification is typically seen in either cancer cells or established cell lines which have undergone immortalization (Wright et al, 1990; Tlsty, 1990). If normal differentiated cells and primary cultured cells do not have the facility for gene amplification, the rapid
loss of amplified genes during subcytotoxic drug treatment may be due to alterations in the state of cellular differentiation. The recent observation that human promyelocytic leukemia cells lose amplified c-myc genes in parallel with differentiation into granulocytes supports this model (Shima et al., 1989).
BIBLIOGRAPHY


