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A Genetic Analysis Of Folate Transport In Chinese Hamster Ovary Cells

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A Genetic Analysis of Folate Transport in Chinese Hamster Ovary Cells

by

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ABSTRACT

Chinese Hamster Ovary cells that are about 50x more resistant to the cytotoxic action of methotrexate than wild-type cells were deficient in the ability to take up methotrexate. In the absence of any exogeneous folates these cells require 100-250x the level of folinic acid as do wild-type cells to support growth at a similar level. Two classes of mutants were distinguishable by their revertability for growth on folinic acid and ability to take-up folic acid. Revertants derived from one class were similar to wild-type cells in their ability to grow in medium containing low levels of folinic acid and in their sensitivity to methotrexate. In contrast, revertants from a second class were able to grow in medium containing low or no folinic acid, but retained their methotrexate resistance. Somatic cell hybrids formed between these two classes of mutants were non-complementing. These observations suggested that some but not all components may be shared between the transport systems mediating methotrexate and folic acid uptake.

The second class of methotrexate-resistant Chinese hamster ovary cells have been complemented to methotrexate sensitivity by transfection with DNA isolated from either wild-type Chinese hamster ovary or human G2 cells. Primary and secondary transfectants regained the ability to take up methotrexate in a manner similar to wild-type cells. To

assist in cloning the sequences responsible for this complementation, transfections have also been carried out with DNA from a wild-type cosmid library. Transfectants have been isolated which have regained methotrexate sensitivity, the ability to take-up methotrexate and were found to contain a limited number of transfected cosmid sequences. Three cosmid clones have been isolated from a primary methotrexate sensitive transfectant cosmid library which after being transfected into the mutant, rescued the methotrexate resistant phenotype of the mutant at a high frequency. Restriction endonuclease analysis of the cosmid clones determined that they overlapped extensively and shared, two regions of 6.6 kB and 20.9 kB DNA. These observations suggested that a gene involved in Mtx uptake is contained within these regions. This is the first report of the molecular cloning of a gene specific to Mtx uptake.

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No acknowledgement would be complete without acknowledging the support of Fred Williams. Fred was instrumental in helping me make the technical transition from the tissue culture hood to the bench and molecular biology and although he probably doesn't realize it, he will be sorely missed. The friendship provided by fellow graduate students in the microbiology department, Mary Anne, Janice, Lou, Keith and most recently Rick is acknowledged.

Lastly, I would like to thank Sue and Ed Alaszkiewicz for putting up with some of my extracurricular antics, their friendship will always be treasured.

I dedicate this thesis to the memory of my grandfather, Mr. Herman Hare.

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aminopterin	N-(p-((2,4-Diamino-6-pteridyl methyl) amino) benzoyl) glutamic
bp	base pairs
°C	degrees centrigrade
cDNA	complementary DNA
CHO	Chinese hamster ovary
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetracetic acid
EMS	ethyl methane sulphonate
FBS	fetal bovine serum
folic acid	N-(4-(((2-amino-,1,4-dihydro-4-oxo-6-pteridinyl) methyl) amino) benzoyl)- L-glutamic acid
folinic acid	N-(4(((2-amino-5,formyl-1,4-5,6,7,8-hexahydro-4-oxo-6-pteridinyl) methyl) amino) benzoyl) -L-glutamic acid
g	gravity
G418	geneticin
GPI	glycosylphosphatidyl inositol
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
K_T	affinity constant for transport
kD	kilodalton
LB	luria broth
mFBP	membrane bound folate binding protein

methotrexate	N-(p-((2,4-Diamino-6-pteridyl) methyl) methyl) amino) benzoyl glutamic acid
5-methyl tetrahydrofolate	N-(4-(((2-Amino-5-methyl-1,4-5,6,7,8-hexahydro-4-oxo-6-pteridinyl) methyl) amino) benzoyl) -L-glutamic acid
Mtx	methotrexate
neo	neomycin
Oua	ouabain
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PI PLC	phosphatidyl inositol specific phospholipase C
pyrimethamine	5-(4-Chlorophenyl)-6-ethyl-2,4-pyrimidine diamine
SDS	sodium dodecy sulfate
sFBP	soluble folate binding protein
SSC	sodium chloride sodium citrate buffer
TE	tris EDTA buffer
Tris	tris (hydroxymethyl)aminoethane

CHAPTER 1

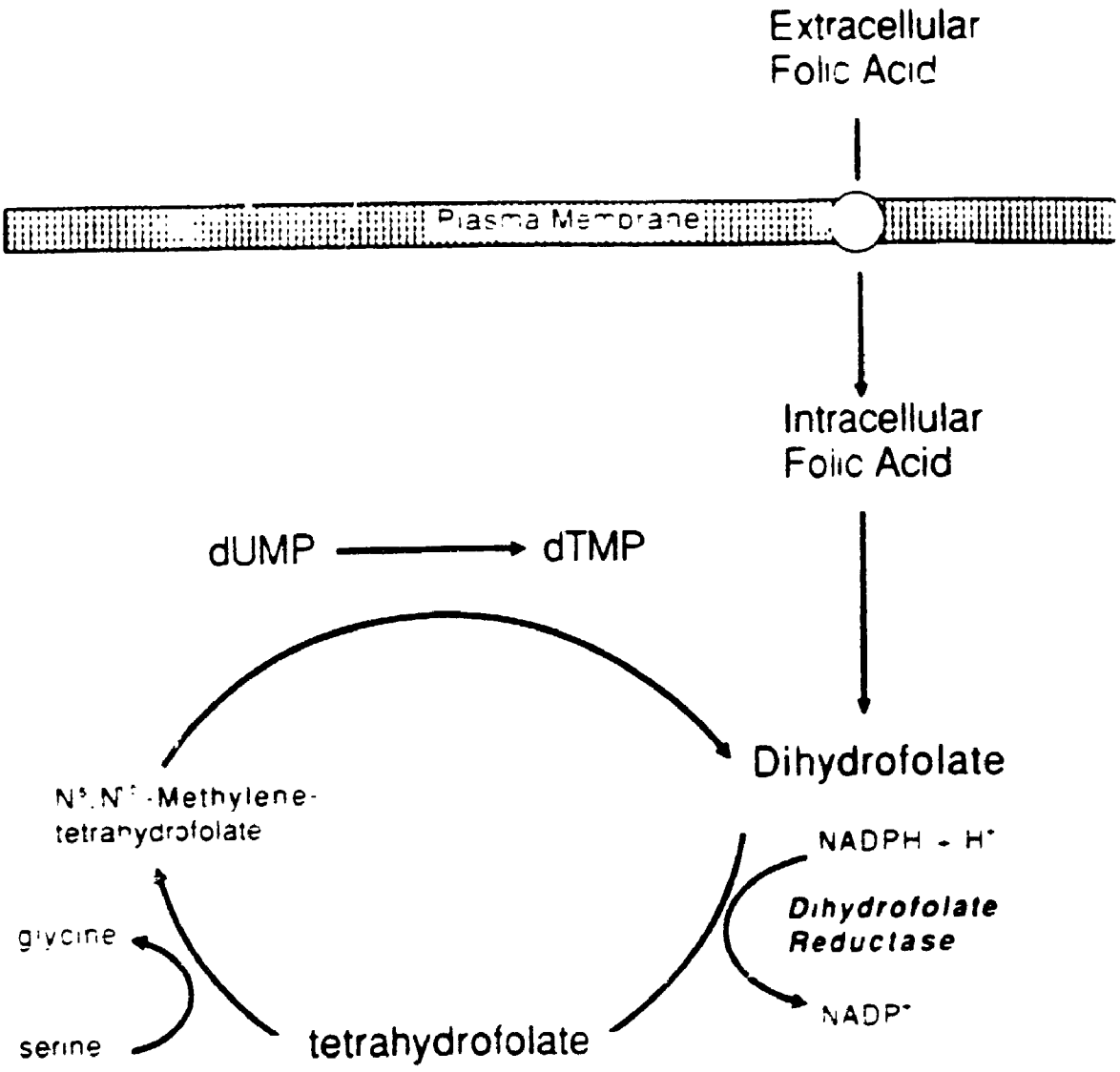
Introduction

1.1 Folate Metabolism

The importance of folates in mammalian metabolism was first demonstrated when a dietary factor, subsequently identified as folic acid, proved to be effective in curing megaloblastic anemia in pregnant women in India (Wills *et al.*, 1937). Folic acid was later found to have no direct role in cellular physiology, but rather it was the reduced forms of folic acid which were of metabolic importance. These folate derivatives are coenzymes for the transfer, oxidation, and reduction of single-carbon units used for the biosynthesis (Figure 1.1) of thymidylate, purine nucleotides, methionine, serine, glycine, and many other compounds (Blakely, 1969).

Rapidly dividing cells require an abundant supply of reduced folates to replenish the pool of precursors consumed in DNA and protein synthesis. Mammalian cells are unable to synthesize *de novo* the pteridine ring of folic acid, hence they obtain folic acid and/or reduced folates from their extracellular environment. Mammals obtain folates from their diet or bacteria within the intestinal lumen. The essential metabolic requirement of mammalian cells for folates has necessitated the development of a system or systems by which these cells can accumulate folates intracellularly against a physiological concentration gradient.

Figure 1.1 Outline of the metabolic pathway of folic acid and its derivatives. Folic acid is transported across the plasma membrane, where it subsequently becomes polyglutamated to reduce efflux. Folic acid is reduced to dihydrofolate by dihydrofolate reductase. This enzyme also catalyzes the reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate is the branch point of the pathway and can potentially feed into a number of pathways, one of which is shown here is important for providing precursors for DNA synthesis. A methyl group is transferred to tetrahydrofolate from serine thereby producing glycine, and N^5,N^{10}- Methylene-tetrahydrofolate. N^5,N^{10}-Methylene-tetrahydrofolate provides the methyl group for converting uridine to thymidine and in the process dihydrofolate is regenerated.


Extracellular
Folic Acid
Plasma Membrane
Intracellular
Folic Acid
dUMP
dTMP
Dihydrofolate
N⁵,N¹⁰-Methylene-
tetrahydrofolate
NADPH + H⁺
Dihydrofolate
Reductase
NADP⁺
glycine
serine
tetrahydrofolate

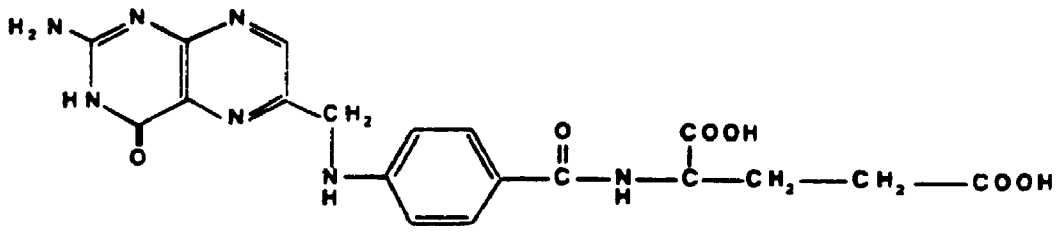
1.2 Methotrexate: An anti-folate

The fact that actively dividing mammalian cells have an absolute requirement for exogeneous folates has been exploited to develop compounds that may interfere with mammalian metabolism. Several folate analogs, termed anti-folates, have been developed for this purpose. One of the more successful folic acid structural analogs, is methotrexate (Mtx) or amethopterin (Cosulich and Smith, 1948), which is the N^{10}-methyl-4-aminopteroylglutamic derivative of folic acid (Figure 1.2).

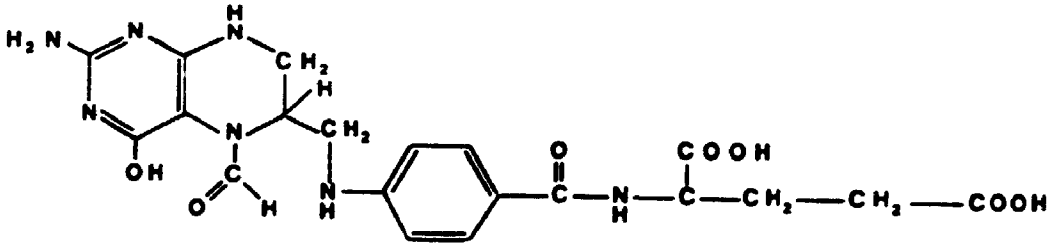
Mtx acts by inhibiting dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolate to tetrahydrofolate. Mtx is bound to the enzyme about 10,000 times more tightly than substrate folate (Werkheiser, 1961) and thus effectively prevents the regeneration of tetrahydrofolate, thereby blocking thymidylate synthesis, which leads to cessation of DNA synthesis and ultimately cell death (Jackson and Grindey, 1984).

The cytoxicity of Mtx and hence its effectiveness is directly dependent upon its free intracellular concentration. Several factors govern the intracellular accumulation of Mtx and likewise folates within cells, some of which include: extracellular concentration, influx across the membrane, efflux across the membrane, degree of polyglutamation which inversely affects efflux, level of binding to intracellular targets, and rate of

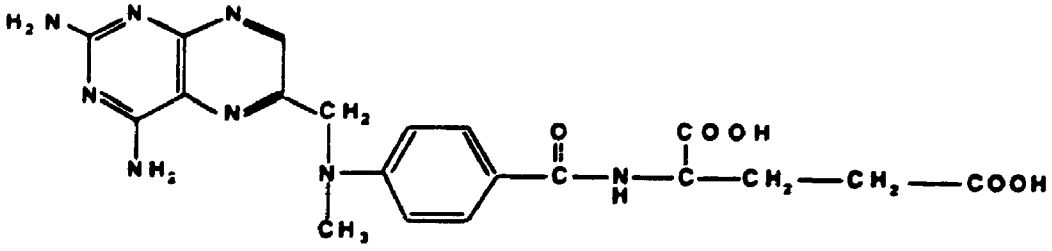
Figure 1.2 Chemical structures of folic acid, folinic acid and methotrexate. These structures are comprised of three basic units, a pteridine moiety which is linked to *p*-aminobenzoic acid, which in turn, is linked to the alpha-amino group of L-glutamate. Folinic acid is the tetrahydrofolate derivative of folic acid and contains a formyl group at the N^5 position. Methotrexate is structurally similar to folic acid with only two substitutions, replacement of the oxygen on C^4 with an amino group and the substitution of a methyl group for the H on N^{10}.



Folic Acid



Folinic Acid



Methotrexate

metabolism of folates.

Due to the cytotoxicity of this compound, Mtx has been successfully used for the treatment of malignancies (Bertino *et al.*, 1985), although cellular resistance does sometimes arise. Resistance develops clinically and in model systems, and at least four mechanisms have been identified: 1, kinetically altered intracellular target DHFR; 2, decreased intracellular accumulation most likely due to reduced uptake; 3, dramatic increase in the number of intracellular targets either due to overexpression or gene amplification; and 4, decreased folylpolyglutamate synthetase activity (McCloskey *et al.*, 1991) which results in increased efflux of the drug (Bertino *et al.*, 1979)(for reviews see Albrecht and Biedler, 1984; Schimke, 1984; Bertino and Rodenhuis, 1986).

As the major intracellular target for Mtx is DHFR, it is quite common to find changes in this enzyme in Mtx drug resistant cell lines. These changes can be either qualitative or quantitative in nature. Several Mtx resistant cell lines from various sources have been identified that have either functional differences in DHFR (Goldie *et al.*, 1981; Jackson *et al.*, 1975; Haber *et al.*, 1981; Blumenthal *et al.*, 1970; Flintoff *et al.*, 1976a; Albrecht *et al.*, 1972; Jackson and Niethammer, 1977) or increased levels of the enzyme (Kashket *et al.*, 1964; Perkins *et al.*, 1967; Rauino and Hakala, 1967; Nakamura and

Littlefield, 1972; Bostock *et al.*, 1979; Domin *et al.*, 1982; Bertino *et al.*, 1985; Albrecht *et al.*, 1972; Diddens *et al.*, 1983; Hakala *et al.*, 1961; Dedhar and Goldie, 1983; Tyler-Smith and Alderson, 1981; Littlefield, 1969; Lewis *et al.*, 1982; Melera *et al.*, 1980; Alt *et al.*, 1978). The functional changes in DHFR are the result of mutations within the coding sequences of DHFR, conferring the enzyme with new substrate binding parameters, V_{MAX}, and altered stability (Goldie *et al.*, 1981; Jackson *et al.*, 1975; Haber *et al.*, 1981; Blumenthal *et al.*, 1970; Flintoff *et al.*, 1976a; Albrecht *et al.*, 1972; Jackson and Niethammer, 1977). In most cases, DHFR has a lowered affinity for Mtx, such that analog induced cytotoxicity only occurs at much higher drug concentrations. In addition, kinetic alterations in DHFR are sometimes accompanied (Albrecht *et al.*, 1972) by increased rates of synthesis. DHFR has been found to be overexpressed in a number of cell lines and this is usually the result of gene amplification (Bostock and Tyler-Smith, 1981; Tyler-Smith and Alderson, 1981; Lewis *et al.*, 1982; Melera *et al.*, 1980; Alt *et al.*, 1978; Flintoff *et al.*, 1982). Gene amplification leads to the generation of multiple expressible copies of a gene, which thereby gives rise to high rates of gene product synthesis (Schimke, 1982). Cell lines that contain amplified copies of the DHFR gene survive in higher than normal concentrations of Mtx because Mtx transport cannot provide sufficient

intracellular levels of drug to effectively saturate all the DHFR. It should be mentioned that in one particular cell line, DHFR was overexpressed with no observable change in DHFR gene copy (Dedhar *et al.*, 1985). Presumably, this line overexpresses DHFR as the consequence of an alteration in the promoter of the gene. Gene amplication of DHFR in cells can lead to very high levels of Mtx resistance, as this mechanism can efficiently contend with high intracellular drug concentrations. The last two classes of Mtx resistant cell lines do not have alterations in DHFR, but are observed to have reduced intracellular accumulation of Mtx via decreased drug permeability or increased efflux. The cell lines with reduced Mtx permeability appear to have alterations in a cell surface component that mediates the uptake of Mtx and other folates. It is the properties of this last class of Mtx resistant cell lines that this thesis will address.

Mtx has been instrumental in determining the role that folate transport plays in anti-folate drug resistance and in delineating the systems that mediate the uptake of folates.

1.3 Folate Uptake: One or More Transport Systems?

Early studies indicated that there was a single component on the cell surface that mediated the transport of folates, including some anti-folates. Biochemical analysis of folate uptake in various mammalian

cell lines seemed to support this conclusion. Henderson *et al.* (1986a) reported that the uptake of folic acid in L1210 cells was competively inhibited by Mtx and that the K_i for inhibition was comparable to the K_T for Mtx influx and *vice versa*. Pretreatment of the cells with an activated ester of methotrexate, N-hydroxysuccinimide Mtx, which has been shown to effectively and specifically block Mtx uptake (Henderson, and Montague-Wilkie, 1983), inhibited folic acid uptake. In addition, the uptake of folic acid was inhibited by p-chloromercuriphenylsulfonate, an inhibitor of Mtx uptake. Finally, folic acid influx fluctuated with the anionic composition of the medium in the same fashion as Mtx uptake. Similar observations were made by Henderson *et al.* (1986b, 1987) using the human leukemia cell line CCRF-CEM.

Uptake of 5-methyltetrahydrofolate in monkey kidney cells appeared to be mediated by a specific cell surface receptor that had a high affinity for folic acid and reduced folates (Kamen and Capdevilla, 1986). In this system folic acid inhibited cellular binding and accumulation of 5-methyltetrahydrofolate. Binding of 5-methyltetrahydrofolate was inhibited by an antibody raised against a soluble plasma folate binding protein (FBP), later shown to be a product of the membra e bound FBP, which suggested that the cell surface receptor and the circulating FBP were immunologically related.

Further support for the one system hypothesis was

gathered by other investigators through analysis of folate uptake in human nasopharyngeal epidermoid carcinoma (KB) cell lines. It was demonstrated in KB cells that uptake of the reduced folate 5-methyltetrahydrofolate could be inhibited by the addition of antiserum raised against a human folic acid receptor in a concentration dependent manner (Antony, *et al.*, 1985). In addition, this antiserum was used to immunoprecipitate the component believed to be involved in reduced folate uptake. Similarly, a receptor from KB cells was purified by methotrexate affinity labelling which bound folic acid and was specifically immunoprecipitated by antiserum raised against the previously purified FBP from KB cells (Deutsch *et al.*, 1989).

Some reports have demonstrated the presence of multiple routes of entry for folates into cells. The evidence for mutliple influx routes stemmed from traditional biochemical analysis, complemented by the analysis of cell variants with aberrant folate uptake.

Yang *et al.* (1982, 1983) were the first to experimentally demonstrate the existence of multiple carrier mediated routes for folates into L1210 plasma membrane vesicles. They found a marked inconsistency in the K_T and K_i uptake parameters for various folates in reciprocal experiments. These observations formed the basis for their hypothesis that there were at least two systems involved in

the transport of folates in L1210 cells. These were: 1) a low capacity, high affinity route for the transport of Mtx and reduced folates, with little or no folic acid uptake; 2) a high capacity, low affinity pathway for all folates. Both of these systems were found to be differentially inhibited by several transport inhibitory compounds. But, the strongest evidence for the existence of multiple routes came from the observation that only influx through the high affinity/low capacity system was altered in vesicles prepared from a Mtx resistant uptake deficient L1210 cell line. In contrast, several L1210 variants have been isolated with increased uptake of Mtx (Sirotnak *et al.*, 1984, Yang *et al.*, 1988). Interestingly, these variants were found to have increased V_{MAX} for the uptake of Mtx with no apparent change in either Mtx influx K_T or folic acid uptake (Sirotnak *et al.*, 1984). Also, affinity labelling of the reduced folate carrier with aminopterin was reduced in the presence of Mtx but not folic acid (Yang *et al.*, 1988). These observations were later confirmed in an L1210 mutant with reduced Mtx uptake where there was no change in the kinetics for influx of folic acid (Sirotnak *et al.*, 1987). Furthermore, it was demonstrated that the system that mediated uptake of reduced folate exhibited low affinity for folic acid and was not expected to contribute significantly to the total influx of folic acid.

Direct biochemical evidence for the existence of

multiple influx routes for folates came from the design and use of folate affinity labels which could apparently selectively inhibit the uptake of either reduced folates or folic acid. A photoaffinity analog of Mtx was used to identify a plasma membrane protein from L1210 cells, whereas there was no labelling detected in L1210/R81, a Mtx resistant variant with reduced uptake (Price *et al.*, 1988). A protein of similar molecular weight was detected from both L1210 parental cells and the R81/L1210 mutant on Western blots developed with antisera from human FBP. Uptake of the analog was inhibited by Mtx but not folic acid. These observations suggested that the binding/transport proteins for folic acid and reduced folates were distinct but immunologically related.

Some of these results have been confirmed in cell lines that have been selected for aberrant transport of folates. The majority of Mtx deficient uptake variants are characterized by a reduced V_{MAX} for the uptake of Mtx and reduced folates (Sirotnak, 1985). In addition some of these variants require higher levels of reduced folates for growth than wild-type cells with little or no concomitant increase in folic acid for growth (Sirotnak *et al.*, 1985; Kano *et al.*, 1986; Underhill and Flintoff, 1989a). The fact that there is no corresponding increase in the requirement of folic acid for growth, provides further support for the presence of functionally distinct systems to mediate

transport of folates, evidence to support this follows.

A variant of CCRF-CEM was selected for overproduction of the reduced folate system through step-wise selection in low concentrations of folinic acid (Jansen *et al.*, 1990). These variants were found to have a 95 fold increase in V_{MAX} for Mtx, with no significant change in either the K_T or K_i for folic acid. Two mutants were isolated from CCRF-CEM, one, designated CEM-RF, expressed only the reduced folate/Mtx carrier. The other, CEM-FBP, lacked the reduced carrier but expressed FBP (Van Der Veer *et al.*, 1989). CEM-FBP was found to be highly resistant to Mtx compared to CEM-RF. At Mtx levels that were cytotoxic to CEM-FBP, reversal of cytotoxicity with addition of the reduced folate, folinic acid, was significantly impaired in this line compared to CEM-RF.

Recently, a variant of the K562 human erythroleukemia line was isolated which exhibited increased transport of methotrexate with only a slight increase in folic acid uptake (Matherly *et al.*, 1991) . This transport system was characterized as having a high affinity for Mtx and folinic acid, and a significantly reduced affinity for folic acid as evidenced by the poor ability of folic acid to competively inhibit Mtx uptake compared to reduced folates.

Previously our laboratory characterized two Mtx resistant mutants deficient in Mtx uptake (Flintoff and Saya, 1978; Flintoff and Nagainis, 1983; Underhill and

Flintoff, 1989a). One mutant, designated Mtx RII 5-3, was found to revert easily to Mtx sensitivity under appropriate back selection and all the revertants studied displayed wild-type like Mtx uptake kinetics. In contrast, the second mutant designated Mtx RII Oua^R 2-4 did not revert to Mtx sensitivity under any of the conditions studied. Both mutants were found to belong to the same complementation group. These data, taken together, suggested that some but not all components may be shared between the processes involved in folic acid and Mtx uptake. Genetic analysis of these two mutants will be described in greater detail in chapter 2.

Mutants that are altered in folate uptake have been instrumental in elucidating both the number and properties of the systems that mediate transport of folates. More importantly, it allowed investigators to study uptake of specific folates in the absence of contribution from additional folate uptake systems.

There are several conflicting reports as to the nature and number of folate influx pathways operating in mammalian cells and, in particular, cells of the same lineage. Analysis of folate uptake variants has helped resolve this problem. The apparent disparity in the observations of various groups working with seemingly identical cell lines may be explained by the demonstrated functional overlap and structural similarity of the components that mediate the

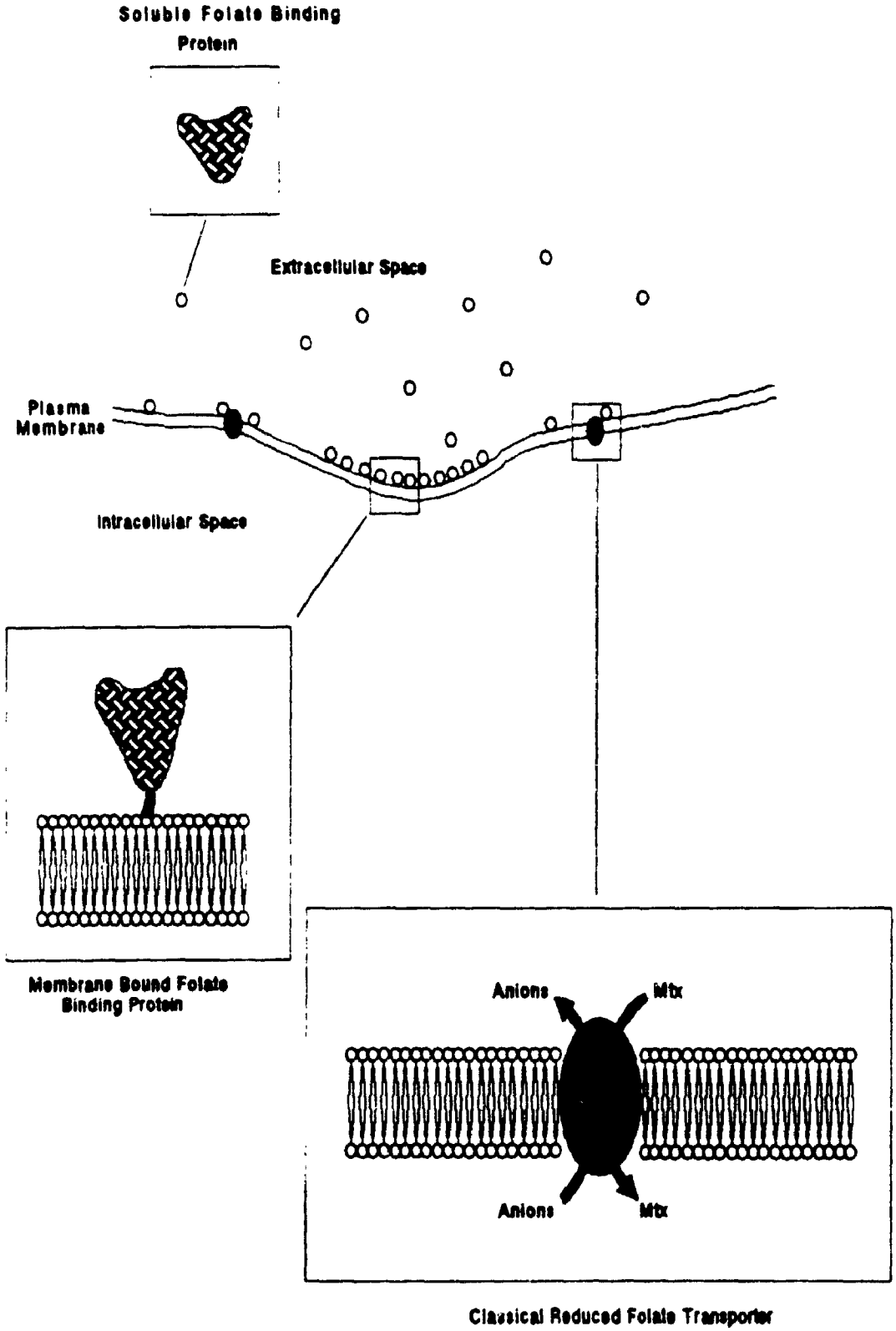
transport of folates, particularly the transport of folic acid. In addition, these observations may, in part, be the result of the conditions used to maintain the cells as well as the purity of the reagents used to perform the experiments.

Biochemical and genetic evidence presently suggests that in mammalian cells folate transport is mediated by at least two major classes of cell surface folate receptors, FBP and the reduced folate classical transporter (Figure 1.3). FBP is thought to transport, by receptor endocytosis (Kamen and Capedevilla, 1986; Rothberg *et al.*, 1990) or via transfer to the classical transporter (Kamen *et al.*, 1991), the bulk of folic acid and to a lesser extent reduced folates and their analogs. In contrast, the reduced folate "classical" transporter, is characterized by a high affinity for reduced folates and a low to medium affinity for oxidized folates (ie. folic acid). Furthermore, the expression of these components on the cell surface may be in response to the metabolic requirements of the cells (Kamen and Capedevila, 1986; Jansen *et al.*, 1990; Kamen *et al.*, 1991), cellular differentiation state (Sirotnak, 1985) and concentrations of folates in the surrounding environment (Kane *et al.*, 1986a).

1.4 Folate Binding Protein

1.4.1 Physical Properties of FBP

Figure 1.3 Schematic of folate transport in mammalian cells. Folic acid and to a lesser extent reduced folates can be transported by a membrane bound folate binding protein, which appears to mediate internalization by receptor endocytosis. The extracellular soluble component of folate binding protein is the product of the endoproteolytic cleavage of the membrane folate binding protein. Reduced folates appear to be taken-up through a transport system characterized by being temperature sensitive, energy dependent, requiring anion exchange and exhibiting Michaelis-Menten saturation kinetics.



Classical Reduced Folate Transporter

Many groups have proven the existence of a protein on the cell surface that can bind folic acid with a higher affinity than reduced folates and Mtx. This protein appeared in two forms, a soluble form and membrane associated, particulate form. Studies using pulse-chase experiments and cycloheximide treatment showed that the membrane bound folate binding protein (mFBP) was a precursor of the soluble folate binding protein (sFBP) (Kane *et al* , 1986a). Subsequently, protein sequencing of the amino terminus of both species and amino acid composition analysis confirmed that the sFBP was the product of mFBP (Luhrs *et al.*, 1987). Further studies showed that the membrane bound FBP was anchored to the membrane through a glycosyl-phosphatidylinositol tail (GPI) (Luhrs and Slomiany, 1989). Treatment of purified or native mFBP with phospholipase C specific for phosphatidylinositol (PI-PLC) removed the GPI tail, giving rise to functional soluble FBP (sFBP) (Luhrs and Slomiany, 1989). More recently it has been demonstrated that *in vivo*, sFBP actually resulted from the endoproteolytic cleavage of mFBP at a site approximately 28-31 amino acid residues from the carboxyl terminus (Elwood *et al.*, 1991), and that this conversion involved a metalloprotease. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) separation of this protein suggested that it had a molecular weight of approximately 36-50 kD depending on the origin, of which

approximately 12-25% was carbohydrate. It was also immunologically crossreactive with FBP's isolated from placenta, KB cells, rat liver and bovine milk. A number of folate binding proteins have been isolated from various sources and their high degree of homology at the amino acid sequence level suggested that they were evolutionarily highly conserved.

It appears that mammalian cells have a repertoire of FBPs, which could be expressed in accordance with their metabolic demands. Sadasivan *et al.* (1987) isolated and purified by folic acid affinity chromatography a membrane associated and soluble FBP from leukemia cells derived from human spleen. Similar to other FBP's, the membrane associated FBP had an apparent molecular weight of 35.5 kD on SDS-PAGE and both the purified sFBP and mFBP had similar amino acid compositions. Both FBP's exhibited a much higher affinity for oxidized folates rather than reduced folates and very low affinity for Mtx and folinic acid. Interestingly, these FBP's did not cross react with polyclonal antibodies raised against FBP's isolated from two other human leukemia cell lines, human placenta or KB cells.

1.4.2 Molecular Analysis of FBP

Recently, several groups have isolated cDNA's that code for the human FBP. Elwood (1989) and Sadasivan *et al.* (1989) isolated similar cDNA's for FBP from KB cells. Both

cDNA's detected an 1100-1200 base mRNA in KB cells. The Sadasivan clone had an open reading frame of 678 nucleotides which encoded a protein of 226 amino acids. whereas the clone isolated by Elwood (1989) had an open reading frame of 781 nucleotides which predicted a protein of 257 amino acids. The deduced amino acid sequence of both FBP cDNA's was found to be almost identical to the amino terminal sequence of the previously isolated KB FBP and human milk FBP. In addition, both FBP's shared over 90% homology with the bovine milk FBP at the amino acid level. The amino acid sequence of these two clones predicted the existence of a hydrophobic amino terminus consistent with a signal peptide and 3 potential sites for N-linked glycosylation. Neither predicted amino acid sequence contained a well-defined hydrophobic transmembrane motif, hence it is quite plausible that the protein is anchored to the membrane by the previously described GPI tail. In vitro translation of RNA transcripts from the Elwood cDNA inserts yielded a 30 kD and a 42 kD polypeptide in the absence and presence of microsomal membranes, respectively (Elwood, P.C., 1989). Furthermore, on Northern blot analysis, radiolabelled cDNA probes hybridized to a single mRNA species, that was expressed to a variable degree in human KB cells, placenta, brain, and epithelia mRNA but was not detectable in human liver mRNA.

In contrast, Ratham *et al.* (1989) have isolated two

distinct, but homologous cDNA's that coded for placenta FBPs, one that was similar to the previously characterized KB FBP and one that was only 68% homologous to the KB cell FBP. The deduced protein sequence of the dissimilar cDNA was characterized by a putative 16-residue amino-terminal signal peptide that would be cleaved into a mature polypeptide of 239 amino acids (Ratnam *et al.* , 1989). The mature protein contained two potential sites for N-linked glycosylation and a stretch of hydrophobic residues at the carboxy terminus that could form a transmembrane domain. Ratham *et al.* (1989) postulated that this novel FBP may represent a fetal folate transport protein. This indicated that the pattern of expression of folate uptake genes maybe governed by the differentiation state of the cell. FBP is believed to facilitate transport of folates by initially binding folate at the cell surface followed by either endocytosis of the ligand-receptor complex (Kamen and Capedevila, 1986; Kamen *et al.*, 1988; Deutsch *et al.*, 1989; Rothberg *et al.*, 1990) or transfer of folate to a "classical transporter" (Kamen *et al.*, 1991). Recent evidence suggests the GPI linked folate receptor is sequestered in numerous discrete clusters on the cell surface that are associated with caveolae (Rothberg *et al.*, 1990). Folate internalization appears to occur through a novel uncoated pit pathway that does not merge with clathrin-coated pits. Mutant analysis seems to support this conclusion, as Mtx resistant uptake defective mutants

are still able to transport folic acid, possibly, through the FBP, in the absence of a "functioning" classical reduced folate transporter (Jansen *et al.*, 1989; Van Der Veer *et al.*, 1989).

The classical reduced folate transporter is thought to be the preferred carrier-mediated route for uptake of reduced folates and Mtx, and to a lesser extent folic acid. The individual transport routes were initially classified based on folate affinities, although this distinction is becoming less clear in light of recent functional data.

1.5 Reduced Folate Classical Transporter

The influx of reduced folates, Mtx and to a lesser extent folic acid appeared to be facilitated by what was considered to be a classical carrier involving active transport. Transport of these compounds in various cell lines conformed to Michaelis-Menten saturation kinetics (Yang *et al.*, 1979; Sirotnak *et al.*, 1987), required energy (Yang *et al.*, 1979) and was both temperature and concentration dependent (Yang *et al.*, 1979; Antony *et al.*, 1985). Henderson *et al.* (1983, 1985a, 1985b, 1986, 1987) observed that Mtx uptake could be irreversibly inhibited by anions and that strong anionic buffers used in uptake analysis could significantly reduce Mtx influx. Anions appeared to be involved in Mtx uptake and it has been surmised that Mtx influx was coupled to anion exchange.

Mammalian cells were first shown to possess a specific receptor for Mtx on their cell surface by affinity crosslinking of Mtx and a Mtx binding protein with C^{14}-N-ethylmaleimide (M'Cormick *et al.*, 1979). The affinity labelled protein had a molecular weight of 56 kD on SDS-PAGE. Subsequent sepharose affinity chromatography with an Mtx ligand yielded three chromatographic peaks, all of which had Mtx binding potential with molecular weights of 67 kD, 63 kD, and 56 kD after SDS-PAGE separation. It was later found that these proteins may have represented nonspecifically labeled serum albumin from the culture medium, which tends to associate with Mtx and cell membranes (Price and Freishiem, 1987).

Shortly thereafter, a new affinity reagent was developed for the specific labelling of Mtx binding proteins on cell surfaces. Mtx and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide reacted to form a relatively unstable product that irreversibly inhibited Mtx transport in L1210 cells (Henderson, G.B., *et al.*, 1980). This Mtx affinity analog was found to be specific for the inhibition of Mtx uptake, as there was no apparent effect on uptake of two unrelated control compounds.

A more effective affinity labelling reagent was developed using the aforementioned reagent, Mtx, and N-hydroxysuccinimide. These reacted to form an activated ester of Mtx (NHS-Mtx) which was found to be a potent,

highly specific irreversible inihibitor of Mtx transport in L1210 cells (Henderson and Montague-Wilkie, 1983). This reagent subsequently was used to affinity label Mtx binding proteins in the plasma membranes of L1210 cells (Henderson and Zevely, 1984). An activated ester of [^{3}H]Mtx was used to covalently label a specific binding protein within L1210 plasma membranes. No labelling was observed in an L1210 subline deficient in Mtx uptake. The radiolabelled protein appeared as a single band with a molecular weight of 36 kD after separation of partially purified plasma membrane proteins by SDS-PAGE. In contrast, NHS-aminopterin, a structurally similar chemically reactive analog of NHS-Mtx was used to identify a protein of molecular weight 45-48 kD from L1210 cells by SDS-PAGE, which was thought to be the reduced folate transport system. It should be noted that labelling of cells with this reagent was specifically reduced in the presence of Mtx, but not with folic acid. In addition, the recovery of this labelled protein was found to be 40 fold greater from a L1210 variant that had been selected for increased transport of reduced folates as compared to a parental cell line. Conversely, no labelled protein was detected in an Mtx resistant uptake deficient L1210 mutant. Further support for the presence of a plasma membrane protein with a molecular weight of 46-48 kD, by SDS-PAGE, that specifically bound Mtx came from the use of photoaffinity analogues of Mtx (Price and Freisheim, 1987;

Price *et al.*, 1988).

These studies have been expanded to include the affinity labelling of Mtx binding proteins from human cell lines. Human leukemia cell line CCRF-CEM was found to contain a plasma membrane bound protein of molecular weight 44 kD, determined by SDS-PAGE, that was affinity labelled with NHS-Mtx (Jansen *et al.*, 1989). Matherly *et al.* (1991) have recently identified a protein of molecular weight 76-85 by SDS-PAGE of NHS-Mtx affinity labelled K562 human erythroleukemia cells. This protein was found to be present in an approximately 7-fold increased amount in a cell line that exhibited a 10 fold increase in Mtx influx V_{max} as compared to the parental cell line. The affinity labelling of Mtx binding proteins from both murine and human sources led to the identification of Mtx binding proteins with dramatically different molecular weights. Matherly *et al.* (1991) have suggested that this apparent disparity in molecular weights may be the result of varying degrees of glycosylation of the folate transport components.

1.6 Objectives

In many cases it appears that pretreatment of cells with a chemically reactive Mtx compound can inhibit Mtx, reduced folate and, somewhat surprisingly, folic acid uptake. Furthermore, several Mtx binding proteins that are identified by affinity labelling with Mtx affinity analogues

appear to cross-react with antibodies raised against FBP. As mentioned above, the functional overlap of the components mediating folate transport complicates the determination of the importance each system plays in total folate transport and their individual roles in anti-folate resistance.

Analysis of folate uptake in mutants has provided clear evidence for the existence of multiple, distinct systems mediating oxidized and reduced folate uptake, where traditional biochemical approaches have proved somewhat contradictory. A genetic analysis of anti-folate resistant mutants should further our understanding of the contribution these individual components make to cellular maintenance and drug resistance. These studies are described in chapter 2 and briefly, demonstrate that folic acid and Mtx uptake are functionally distinguishable.

The molecular properties of the reduced folate classical transporter have not been described. Therefore, the isolation and characterization of the gene for this transport system would facilitate an understanding of its role in total folate transport. To realize this goal, a strategy was developed for the isolation of a transporter gene by DNA-mediated gene transfer. These approaches will be described in greater depth in chapters 3 and 4.

CHAPTER 2

Mutant Chinese Hamster Ovary Cells with Defective Uptake Are Distinguishable by Reversion Analysis

2.1 INTRODUCTION

Previously Flintoff *et al.* (1976a) had isolated a series of Chinese hamster ovary (CHO) cells which are resistant to Mtx by virtue of an inability to take up the drug. These have been designated Class II Mtx resistant cells. All mutants characterized with this phenotype displayed collateral sensitivity to several lipophilic inhibitors of dihydrofolate reductase (Flintoff and Saya, 1978). In addition one mutant was shown not to bind Mtx at its cell surface nor to transport folic acid suggesting that Mtx and folic acid are transported by the same system in CHO cells (Flintoff and Nagainis, 1983). In this report we demonstrate that not all previously described RII cells have this property. One RII isolate was Mtx resistant but still able to transport folic acid in a similar manner as wild type cells. In addition, we show that these two independent isolates of the RII phenotype can be distinguished from each other by their revertability for growth on the reduced folate, folinic acid, as a source of folate.

Isolates selected for growth on low folinic acid levels from one class of RII cells were able to grow in medium containing low levels of folinic acid, to regain their sensitivity to Mtx and their ability to transport folic acid. In contrast, isolates obtained from the other RII Mtx resistant mutant were able to grow in medium containing low

or no added folinic acid, but retained their Mtx resistance and the ability to transport folic acid. Somatic cell hybrids formed between these two subclasses of RII cells were non-complementing. Taken together these results suggest that Mtx and folic acid share some but not all the components for uptake in CHO cells.

2.2 MATERIALS AND METHODS

2.2.1 Reagents and [^{3}H]Mtx Purification

Mtx, folic acid and ouabain (Oua) were purchased from Sigma Chemical Company. N^5-formyl tetrahydrofolate (folinic acid) was purchased from ICN Biochemicals. Geneticin (G418) was purchased from Gibco Canada Ltd. Ethyl methanesulfonate (EMS) and N-2-hydroxyethylpiperazine-n-2-ethanesulfonic acid (Hepes) were purchased from Kodak Chemicals and Boehringer Mannheim Co., respectively.

[3',5',7'-^{3}H]Mtx (56 Ci/mmol) and [^{3}H] Folic acid (26 Ci/mmol) were purchased from Dupont and Amersham, respectively. Immediately prior to use, ^{3}H Mtx and ^{3}H Folic acid were purified by thin layer chromatography as described previously (Flintoff and Nagainis, 1983). After purification both labelled compounds were greater than 98% pure.

2.2.2 Cells and Cell Culture

Wild-type Mtx-sensitive (Pro $^-$3), class II Mtx-resistant

Pro $^{-3}$ Mtx^{RII} 5-3, Pro $^{-4}$ Mtx^{RII} 2-4 and Pro $^{-4}$ Mtx^{RII} Oua^{R} 2-4 cell lines have been previously described (Flintoff *et al.*, 1976a; Flintoff *et al.*, 1976b) and are designated in this report as wild-type, Mtx RII 5-3, Mtx RII 2-4 and Mtx RII Oua^{R} 2-4 respectively. Cells were maintained in suspension culture in alpha MEM medium (normal medium) supplemented with 8% Nu-Serum (Collaborative Research) and 2% fetal bovine serum. The final concentration of folic acid in this medium is 2 X 10^{-6} M. Isolates obtained by selection for growth in low levels of folinic acid were maintained in folic acid free alpha medium (selective medium), which was supplemented with 10% dialyzed fetal bovine serum and an appropriate amount of folinic acid as indicated in the text.

2.2.3 Selection of Cells Growing On Low Folinic Acid

Isolates from Class II Mtx resistant mutants were selected for their ability to grow in media containing low folinic acid either spontaneously or after treatment with EMS. The cells were treated with 300 ug/ml EMS for 18 hours in normal medium. This treatment resulted in a 40% cell survival. After 3 days of recovery in normal medium the cells were assayed for colony formation in selection medium containing various levels of folinic acid. Colonies were picked, cloned by limiting dilution, tested for Mtx resistance and for growth in media containing low levels of

folinic acid, and used for further analysis. Independently selected lines in which resistance to Mtx was reverted were given the designation Rev Mtx RII 1,2... etc. Isolates which could grow in medium containing low levels of folinic acid (2 X 10^{-5} M) but retained their Mtx resistance were designated as Mtx RII Fl,2... etc and Mtx RII Oua^R Fl,2... etc.

2.2.4 Resistance Level

The resistance of the various cell lines to drugs was expressed by the D_{10} value, the drug concentration that reduces cell survival to 10%. These values were determined either by complete dose response curves or by plating 100 cells at various drug concentrations in 24-well Costar plates as previously described (Flintoff *et al.*, 1976a). All these plating experiments were carried out in alpha medium supplemented with 10% dialyzed fetal bovine serum. After 8 days incubation at 37° C in a 5% CO_2 atmosphere, colonies were stained with 1% methylene blue in 50% methanol. Colonies of >50 cells were counted and used to determine relative plating efficiencies. The D_{10} values for growth in folinic acid or folic acid containing media were obtained in a similar manner. In this case, the D_{10} represents the concentration of folinic acid or folic acid allowing 10% cell survival.

2.2.5 Determination of Drug Uptake and Kinetic Analysis

Cells growing in the appropriate medium were harvested in mid-log phase (2-6 X 10^5 cells/ml), washed 2 times with cold uptake buffer (0.15M Hepes, 1mM $MgCl_2$ pH 7.3) and resuspended to a density of 0.8 - 1.0 X 10^7 cells/ml. Cells were incubated with shaking at 37° C for 10 minutes, uptake was initiated by the addition of ^{3}H-Mtx or ^{3}H-Folic acid to a final concentration of 0.25 uM and 0.40 uM, respectively. Uptake was terminated at various times by diluting a 0.5 ml aliquot into a 20-fold excess of cold uptake buffer. Samples were centrifuged at 780g for 5 minutes and washed once with cold uptake buffer. Cells were suspended in water and quantitatively transferred to scintillation vials containing Protosol (DuPont) and Omnifluor and counted in a Beckman Scintillation Counter.

Kinetic analyses employed the same method as described above, except assays were designed to contain labelled drug with differing specific activities (100-800 cpm/pmol) but the same number of counts. Samples were taken in the linear range of uptake at four time points. Kinetic parameters were determined by double reciprocal plots of 1/(the initial rate of uptake) as a function of 1/(substrate concentration). Linear regression was performed using Lotus 123 to give the best straight line of fit. The reciprocal of the X and Y intercepts of this line yield the K_T (uM) and V_{MAX} (pmol/min/mg protein) kinetic parameters for uptake,

respectively. Protein was determined by the method of Bradford (Bradford, 1976) using the BioRad Protein Microassay.

2.2.6 Dihydrofolate Reductase Assay

Cell extracts were prepared and dihydrofolate reductase (DHFR) assayed as previously described (Flintoff *et al.*, 1976b) using ^{3}H folic acid as substrate. In short, approximately 1 X 10^{8} cells were washed 2X with PBS and resuspended in water to a concentration of 1 X 10^{8} cells/ml. Cells were sonicated and cellular debris was removed by centrifugation at 35,000 rpm for 45 min. at 4° C in a Ti70 rotor. Appropriately diluted extracts were assayed for DHFR activity with 0.25 uCi/ml [^{3}H]folic acid and an excess of nicotinamide adenine dinucleotide hydrogen phosphate (400 nM) in a 0.11 M potassium acetate buffer (pH 5.0). Assay samples were incubated at 37° C for 30' and the reactions were terminated by the addition of an excess of cold folic acid. Unreacted substrate was precipitated with 1/5 volume of a mixture of 1:4 glacial acetic acid/1M zinc sulphate. The precipitate was removed by centrifugation at 780g for 10' and an aliquot of the supernatant was added to protosol (NEN) and Aquasol Scintillation fluid (NEN) and counted in a Beckman scintillation counter. Enzyme activity was expressed as nmol folic acid reduced per mg protein per min at 37° C. The IC_{50}, the drug concentration inhibiting

enzyme activity 50% was determined for each extract by adding various concentrations of Mtx to the reaction mixtures.

2.2.7 Transfection

G418 resistant Mtx RII 5-3 cells were generated by transfection with pSV2NEO using the procedure described by Chaney et al. (Chaney *et al.*, 1986). Briefly, 4 X 10^5 cells were incubated for 6h at 37° C with 2 ug pSV2NEO in the presence of 1ug/ml polybrene, at the end of this period cells were treated for 4' with 30% dimethyl sulfoxide (DMSO) in normal medium. Cells were allowed to recover in normal media for 24 hours and were then placed under selection in normal media containing 1.5 mg/ml G418. Colonies arose at a frequency of 6 X 10^{-4}, were picked after 10 days and designated as Mtx RII 5-3 Neo^R.

2.2.8 Cell-Cell Hybridization

Hybrid cells were formed by polyethylene glycol induced fusion between the two lines Mtx RII 5-3 Neo^R and Mtx RII Oua^R 2-4 using a previously described procedure (Flintoff, 1984). Cell lines were co-cultivated and allowed to reach subconfluency in six well trays before they were treated for 1 min with 1.5 ml of 80% polyethylene glycol 8000 (PEG), 10% DMSO in alpha medium. Cells were subsequently washed 3 times with 10% DMSO in alpha medium and once with normal

medium. Normal medium was added to the trays and cells were incubated overnight, following this the cells were trypsinized and replated at low density in selective medium. Hybrids were selected for growth in normal medium containing 2 mM ouabain and 1.5 mg/ml G418, conditions under which neither parental cell line can grow. The frequency of hybridization formation was 5×10^{-4}. Parental cell survival under these conditions was $<5 \times 10^{-6}$.

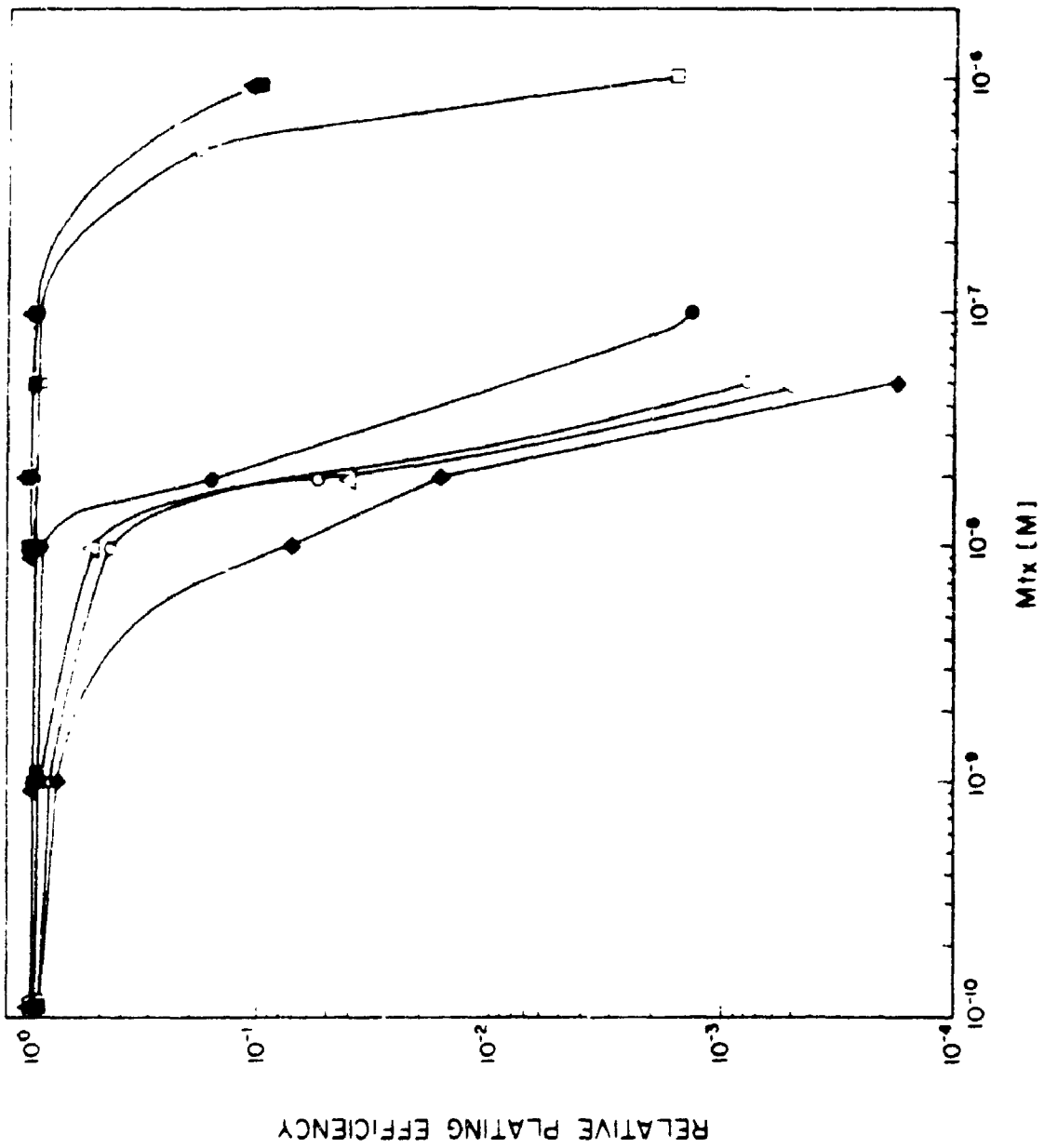
The hybrid nature of the cells was determined by karyotype analyses. Cells growing exponentially in T-25 tissue culture flasks were incubated with 0.25 ug/ml colcemid (Gibco) for 3.5 hours at 37° C. Cells were gently trypsinized from the flask by treatment with a 0.75 M potassium chloride hypotonic solution containing 0.025% trypsin and 0.25 ug/ml colcemid for 4 hours at 37° C and then washed with a fixing solution (3:1 methanol:glacial acetic acid). Slides were prepared, stained with Giemsa stain and 20 representative chromosome spreads were counted for each line.

2.3 RESULTS

2.3.1 Phenotype of RII Mutants

CHO Mtx deficient transport mutants (RII) are approximately 50-fold more resistant to the cytotoxic action of Mtx than wild-type cells (Figure 2.1) and have a

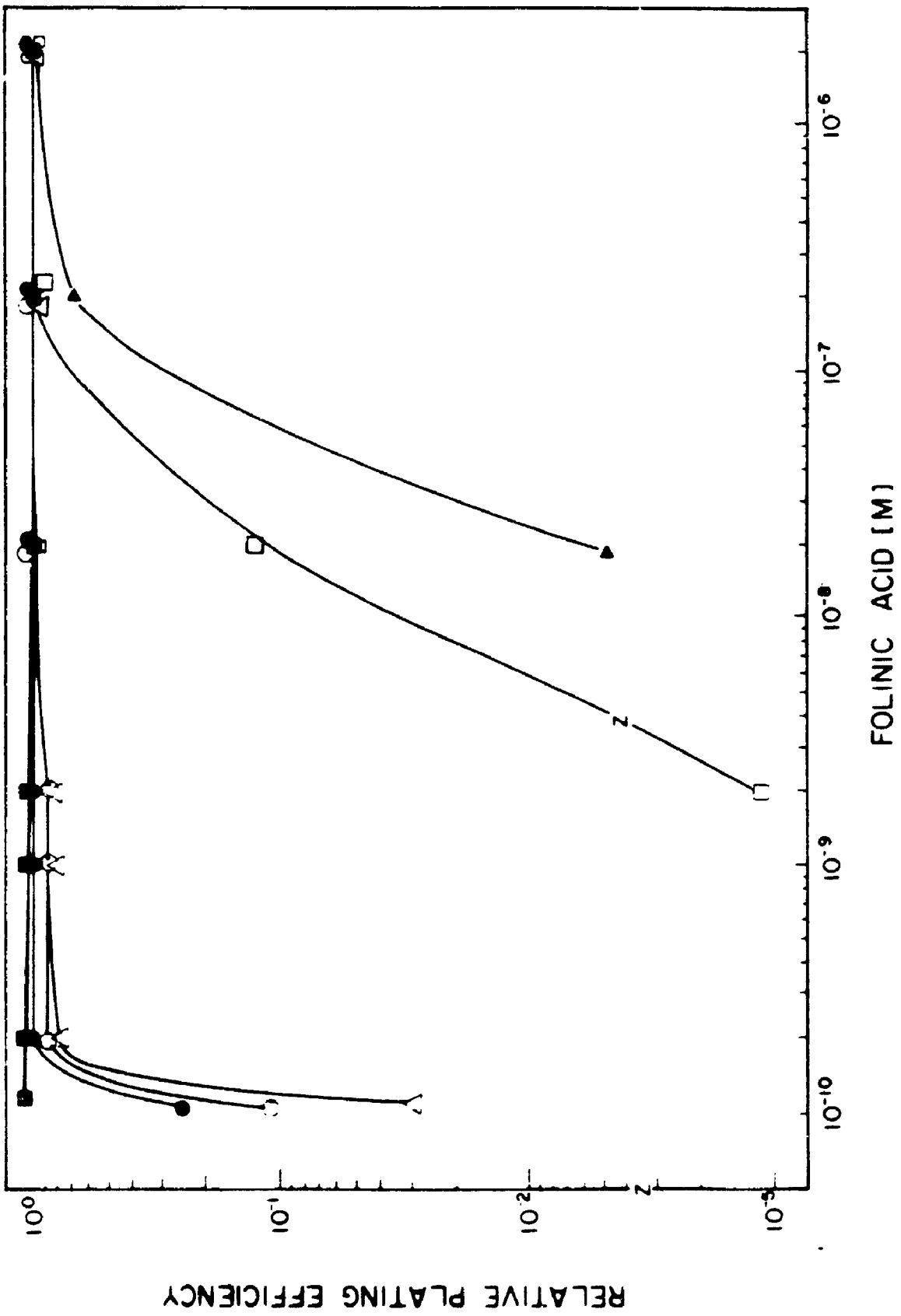
Figure 2.1 Dose reponse curves for cells growing in Mtx. Exponentially growing cells were plated in selective medium with various concentrations of Mtx. ● , wild-type; ◆ , wild-type maintained in selective medium supplemented with folinic acid at 2 X 10^{-5} M; □ , Mtx RII 5-3 mutant; ○ , spontaneous Rev Mtx RII 1; △ , EMS generated Rev Mtx RII 2; ▲ , Mtx RII Oua^{R} 2-4 mutant; ■ , spontaneously generated Mtx RII Oua^{R} F1.


RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-7}
10^{-9}
10^{-9}
10^{-10}
Mix [M]

markedly reduced uptake of the drug.

In several Mtx deficient uptake mutants that have been examined (mouse L1210 and human lymphoblastic leukemia cells, MOLT-3) there is a requisite increase for reduced folate to maintain growth as compared to wild-type cells (Kano *et al.*, 1986; Sirotnak *et al.*, 1987). Mtx and reduced folates are believed to be transported by the same influx system. Hence a decreased uptake of Mtx would also be associated with decreased uptake of reduced folates. In order to offset the inefficiences of reduced folate uptake, these mutants require higher levels of these compounds. Increased requirement for folic acid in these lines is not as well documented. To test whether this requirement for folates was occurring in CHO cells, wild-type and mutant RII cells were grown in medium deficient in folic acid and supplemented with various levels of folinic acid as a source of folate. As shown in Figure 2.2 the RII cell line Mtx RII 5-3 required approximately 100 times the amount of folinic acid as compared to wild-type cells to attain the same level of growth. Another independently selected Class II isolate Mtx RII Oua^R 2-4, also required higher levels of folinic acid to maintain similar growth as wild-type cells. In this case the line required 250 times the level of folinic acid as the wild-type cells (Figure 2.2). The requirement for higher levels of folates of the RII mutant was also demonstrated for folic acid. This requirement, however was

Figure 2.2 Dose resonse curves for cells growing in folinic acid. Exponentially growing cells were plated in selective medium with various concentrations of folinic acid.
● , wild-type; □ , Mtx RII 5-3; △ , Rev Mtx RII 1; ○ , Rev Mtx RII 2; ▲ , Mtx RII Oua^R 2-4; ■ , Mtx RII Oua^R F1.


RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
FOLINIC ACID (M)

less dramatic than for folinic acid. The Mtx RII 5-3 mutant required about 2-3 times the wild-type level of folic acid for growth, whereas Mtx RII Oua^R 2-4 mutant needed 10 times the wild-type level of folic acid to support growth. The folic acid $D_{1/2}$s for these various cell lines were 28 nM, 83 nM and 9nM, respectively.

2.3.2 Selection of RII Isolates For Growth in Low Folinic Acid

The dependence of RII cells on folinic acid for growth suggested the possibility of a selection scheme in which to isolate RII cells which may have reverted back to a wild-type phenotype for folinic acid requirement and perhaps Mtx sensitivity. Putative RII revertants were isolated on the basis of their ability to grow in folic acid deficient media supplemented with 2 X 10^{-9} M folinic acid. This level of folinic acid supported growth of Mtx RII 5-3 and Mtx RII Oua^R 2-4 at a frequency of 10^{-4} and $<10^{-5}$, respectively. To increase these frequencies these Mtx RII lines were mutagenized with EMS. EMS mutagenesis increased the ability of these lines to grow on low levels of folinic acid at least a 100 fold. For the Mtx RII 5-3 line this frequency was frequency 1.9 X 10^{-3} and for the Mtx RII Oua^R 2-4 the frequency was 5.2 X 10^{-3}. Several independently selected spontaneous and EMS-generated isolates were tested for their resistance to Mtx and their ability to grow in various

levels of folinic acid.

Both spontaneously generated and mutagen induced isolates from the Mtx RII 5-3 line regained a wild-type sensitivity to Mtx. The dose response curves for two representatives are shown in Figure 2.1. In addition, these isolates were able to grow in levels of folinic acid of 10^{-9}-10^{-10}M, which was similar to the level required for growth by wild-type cells (Figure 2.2). A total of 12 independently selected isolates were examined; 5 representatives are shown in Table 2.1. All isolates obtained from Mtx RII 5-3 displayed similar sensitivities to Mtx as wild-type with their D_{10} values ranging from 2-4 X 10^{-8} M. Their response to folinic acid was not nearly as consistent, folinic acid D_{10} values ranged from 6 X 10^{-10} M to below 1 X 10^{-10} M (Table 2.1). Thus all isolates obtained from this RII line regained sensitivity to Mtx and required much lower levels of folinic acid than the parental RII line for growth. This implied that the alterations in this RII isolate that conferred Mtx resistance and a high folinic acid growth requirement were similar.

Isolates obtained from the Mtx RII Oua^R 2-4 line, however, behaved somewhat differently. Of the 4 isolates examined all were able to survive on low levels of folinic acid, but retained their resistance to Mtx (Table 2.1). Similar results were also obtained on isolates derived from the immediate parent of the RII Oua^R 2-4 line (Table 2.1).

Table 2.1

Summary of Mtx resistance and folinic acid dependence of wild-type, various RII mutants and folinic acid selected RII isolates.

Cell Line	Mutagenesis	Mtx D_{10} (M)[a]	Folinic D_{10} (M)[a]
Wild-type	-	$2X10^{-8}$	$1X10^{-9}$
Wild-type[b]	-	$8X10^{-9}$	$<1X10^{-10}$
Mtx RII 5-3	-	$8X10^{-7}$	$4X10^{-8}$
Rev Mtx RII 1	-	$2X10^{-8}$	$2X10^{-10}$
Rev Mtx RII 2	+	$2X10^{-8}$	$1X10^{-10}$
Rev Mtx RII 3	-	$4X10^{-8}$	$<1X10^{-10}$
Rev Mtx RII 4	+	$2X10^{-8}$	n.d.[c]
Rev Mtx RII 5	-	$2X10^{-8}$	$6X10^{-10}$
Mtx RII Oua^R 2-4	-	$6X10^{-7}$	$1X10^{-7}$
Mtx RII Oua^R F1	-	$5X10^{-7}$	$<<1X10^{-10}$
Mtx RII Oua^R P2	+	$6X10^{-7}$	$1X10^{-10}$
Mtx RII Oua^R P3	+	$6X10^{-7}$	$5X10^{-10}$
Mtx RII Oua^R P4	+	$6X10^{-7}$	$1X10^{-9}$
Mtx RII 2-4	-	$6X10^{-7}$	$2X10^{-8}$
Mtx RII F1	-	$6X10^{-7}$	$8X10^{-8}$

[a] Concentration of drug at which 10% of the plated cells survive.

[b] Wild-type cells were maintained in selective medium supplemented with folinic acid to a concentration of $2 X 10^{-9}$ M.

[c] not done

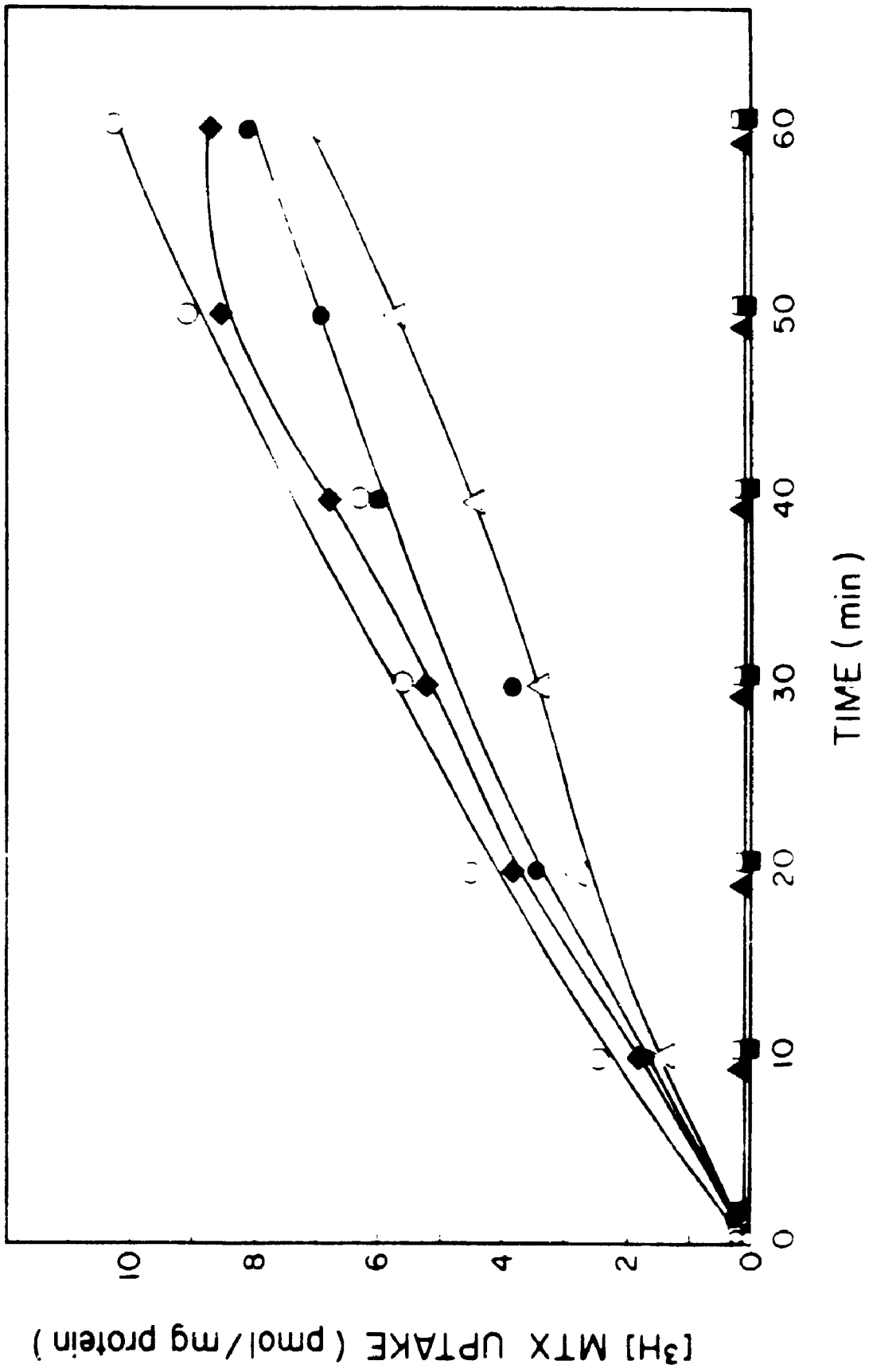
This RII line was Mtx resistant but not ouabain resistant. One spontaneously generated isolate Mtx RII Oua^R F1 survived in media lacking exogeneously added folic acid or folinic acid. The ability to select for isolates that could grow on low levels of folinic acid without altering the Mtx resistant phenotype suggested that in this class of RII cells the 2 phenotypic properties of Mtx resistance and folinic acid requirement were not identical.

2.3.3 Methotrexate Uptake Properties

RII mutants were originally characterized by their inability to accumulate Mtx intracellularly (Flintoff *et al.*, 1976a). If the isolates obtained from the Mtx RII 5-3 were true revertants as their phenotype suggested, then they should have regained the ability to take-up Mtx. To test this, several of these isolates were assayed for their ability to accumulate Mtx. As shown in Figure 2.3 two representative isolates accumulated ^3H Mtx intracellularly in a similar manner as the wild-type cells, although the level of drug accumulated was approximately 15% less than that of wild-type cells grown in either normal or folinic acid supplemented media. In contrast, the isolates from the Mtx RII Oua^R 2-4 line were unable to take-up the drug (Figure 2.3), confirming the previously demonstrated Mtx resistant phenotype.

Since isolates from the Mtx RII 5-3 had reverted so

Figure 2.3 Cellular uptake of 0.25 uM ^{3}H-mtx. Cells were incubated at 37° C, and ^{3}H-Mtx uptake measured as described in Materials and Methods. ◆ , wild-type maintained in normal medium; ○ , wild-type maintained in selective medium supplemented with folinic acid at 2 X 10^{-9}M; ▲ , Mtx RII 5-3; ● , Rev Mtx RII 1; △ , Rev Mtx RII 2; □ , Mtx RII Oua^{R} 2-4; ■ , Mtx RII Oua^{R} F1.


[3H] MTX UPTAKE (pmol / mg protein)
0
2
4
6
8
10
0
10
20
30
40
50
60
TIME (min)

that now they were able to transport Mtx, it was of interest to determine the kinetic parameters associated with this uptake. Two Mtx sensitive revertants were examined from each of the spontaneous and EMS treatments. In each case, the Mtx RII 5-3 revertants exhibited a K_T for Mtx uptake ranging from 0.33-0.62 uM which was similar to that of the wild-type cells (Table 2.2). The V_{max} for uptake ranged from 0.6-1.0 pmol/min/mg protein, which was also similar to that of wild-type cells (Table 2.2).

2.3.4 Folic Acid Uptake

Previously it has been shown that the Mtx RII 5-3 cell line was incapable of accumulating folic acid internally at extracellular concentrations in the 0.8 uM range (Flintoff and Nagainis, 1983). This was confirmed for a concentration of 0.4 uM (Figure 2.4). If Mtx and folic acid uptake occur via the same transport system, then the Mtx transporting revertants should now also transport folic acid. To investigate this one representative was examined for its ability to take up radiolabelled folic acid. As shown in Figure 2.4 this isolate could accumulate folic acid. However, the kinetics of uptake differed somewhat from that of wild-type cells. Over the time course examined this line accumulated more folic acid than wild-type cells maintained in normal media but about a half of that accumulated by wild-type cells maintained in low levels of folinic acid.

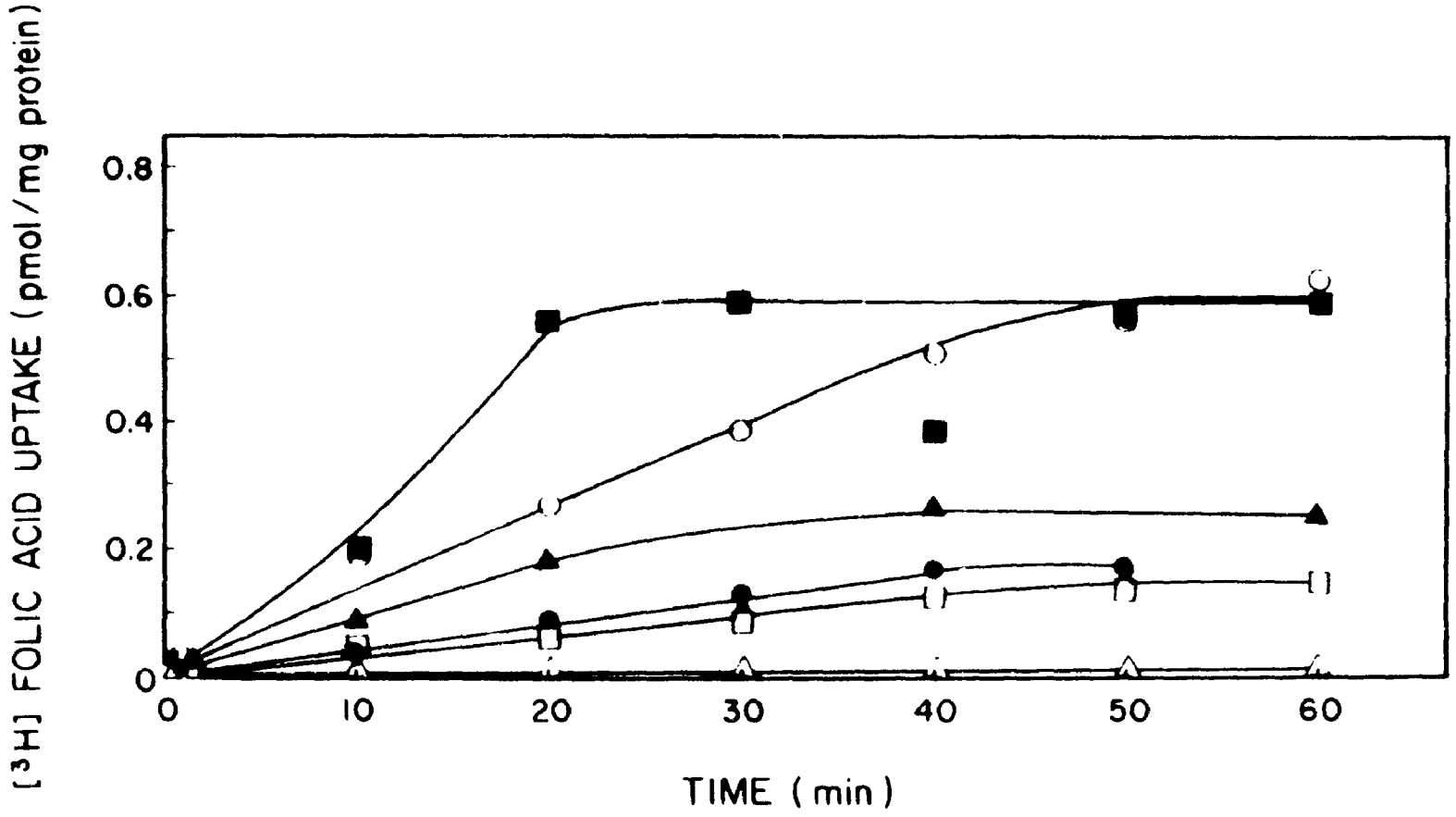
Table 2.2

Kinetic parameters for uptake of [^{3}H] Mtx.

Cell Line	K_T (uM)	V_{MAX} (pmol/min/mg protein)
Wild-type	0.55	1.10
Rev Mtx RII 1	0.53	1.00
Rev Mtx RII 2	0.62	0.90
Rev Mtx RII 3[a]	0.33	0.60
Rev Mtx RII 4[a]	0.42	0.90

[a] Cells were maintained in normal medium, all other lines were maintained in folinic acid at a concentration of 2 X 10^{-9} M.

Figure 2.4 Cellular uptake of 0.4 uM ^{3}H-folic acid. Cells were incubated at 37° C and uptake was determined as described in Materials and Methods. ● , wild-type maintained in normal medium; ○ , wild-type maintained in selective medium supplemented with folinic acid at 2X10^{-9}M; △ , Mtx RII 5-3; ▲ , Rev Mtx RII 1; □ , Mtx RII Oua^{R} 2-4; ■ , Mtx RII Oua^{R} F1.


[3H] FOLIC ACID UPTAKE (pmol/mg protein)
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
TIME (min)

The uptake of folic acid for the Mtx RII Oua^R 2-4 isolate was somewhat different than the RII 5-3 line. This line accumulated folic acid in a manner similar to wild-type cells. Isolates selected from this line for growth in low levels of folinic acid, not surprisingly could also accumulate folic acid. The representative shown in Figure 2.4 accumulated similar levels of folic acid as wild-type cells maintained in 2 X 10 $^{-9}$ M folinic acid. The initial rate, however, was much faster enabling satu. .tion to be reached in half the time required for the wild-type cells at this level of exogeneous folic acid. The difference in uptake between wild-type cells maintained in normal and selective media may result from binding sites being unoccupied in the latter conditions (Kamen and Capdevila, 1986).

Growth of cells in low levels of folinic acid resulted in an apparent 2-3 fold increase in the amount of folic acid accumulated as compare to those maintained on high levels (Figure 2.4). Conversely, there appears to be no significant increase in Mtx uptake under the same conditions (Figure 2.3).

2.3.5 DHFR

To determine whether changes had occurred in DHFR in these isolates, extracts were prepared and enzyme activity assayed as described in Materials and Methods. In all cases

examined there appeared to be no significant qualitative or quantitative differences in the DHFR activity among these isolates and that of wild-type cells (Table 2.3).

2.3.6 Cell-Cell Hybridization

The results above suggested that at least two different alterations were present in the Mtx Class II phenotype of CHO cells. One could be easily reverted for both folinic acid dependence and Mtx resistance and a second in which reversion for folinic acid dependence occurred but Mtx resistance remained. Previous work has shown that all Class II resistant phenotypes were genetically recessive when combined with wild-type cells in somatic cell hybrids (Flintoff *et al.*, 1976b). Thus, it was of interest to determine whether different genetic loci where involved in these two Class II phenotypes. Somatic cell hybrids were formed between Mtx RII 5-3 Neo^S and Mtx RII Oua^r 2-4 cell lines and resultant hybrids were tested for Mtx sensitivity. In all cases examined, the hybrids were as resistant to Mtx as the parental lines (Table 2.4). This indicated that non-complementating loci in the two lines were involved in generating the drug resistant phenotype.

2.4 DISCUSSION

The data presented in this chapter show that the

Table 2.3

Dihydrofolate reductase activity of cell lines.

Cell Line	Relative Enzyme Activity[a]	Affinities of DHFR for Mtx I_{50}[b] (M)
Wild-type	1.0	7 X 10^{-9}
Mtx RII 5-3	0.6	2 X 10^{-8}
Mtx RII Oua^{R} 2-4	1.0	2 X 10^{-8}
Rev Mtx RII 1	0.5	2 X 10^{-8}
Mtx RII Oua^{R} F1	1.0	1 X 10^{-6}

[a] Crude cell extracts were prepared and assayed as described in Materials and Methods. Relative activity is expressed as the ratio of activities in the various cell line extracts to wild-type extract. The wild-type line had an average activity of units/mg.

[b] I_{50} is the concentration of Mtx required to inhibit 50% of the DHFR activity.

Table 2.4

Properties of somatic cell hybrids.

Cell Line	D_{10}(nM) Mtx[a]	Modal Chromosome[b] Number
Wild-type	20	21
Mtx RII Neo[R] 5-3	800	20
Mtx RII Oua[R] 2-4	600	20
Hybrid 1	800	40
Hybrid 2	800	41
Hybrid 3	800	41
Hybrid 4	800	41
Hybrid 5	800	41

[a]Concentration of drug at which 10% of the plated cells survive.

[b]Twenty spreads from each line were counted.

resistant Mtx RII phenotype in CHO cells was comprised of at least two classes that could be distinguished by several criteria. These include the nature of the revertants obtained by selection for growth on low folinic acid and the uptake of folic acid.

The first class demonstrated by the Mtx RII 5-3 mutant was characterized as being Mtx resistant, folinic acid dependent and incapable of accumulating both folic acid and Mtx intracellularly at the concentrations tested. Mtx RII 5-3 mutants reverted easily. These revertants selected for growth on low folinic acid were phenotypically similar to wild-type with respect to both Mtx sensitivity and folinic acid dependence. Revertants of Mtx RII 5-3 that had been previously selected for pyrimethamine resistance (Flintoff and Saya, 1978) also showed the concomitant decrease in folinic acid dependence with increasing sensitivity to Mtx (unpublished data). In addition, independently isolated revertants consistently had K_T's for Mtx influx which were similar to those of wild-type cells and were capable of transporting folic acid. The exact nature of the original mutation in this line is unclear at the present time. The simultaneous reversion of both Mtx and folic acid uptake indicates that these two compounds share a common component in the uptake process. The observation that all revertants recover a similar K_T for Mtx uptake to that of wild-type would suggest but does not prove that the original

alteration may not be in a carrier component *per se* since it would seem unlikely that reversion in every case would result in a carrier with similar kinetic parameters. Perhaps the original mutation is in a regulatory component which is involved in both Mtx and folic acid uptake.

A second class demonstrated by the Mtx RII Oua^R 2-4 mutant was characterized as being folinic acid dependent, Mtx resistant, capable of transporting folic acid, but incapable of accumulating Mtx intracellularly. Isolates selected for growth on low folinic acid medium were phenotypically unaltered for Mtx resistance and exhibited variability in their growth requirement for folinic acid. Folic acid transport in the absence of Mtx transport in this isolate suggests that folic acid and Mtx transport are mediated by either physically distinct domains on a single component or by separate components.

Somatic cell hybrids of Mtx RII 5-3 Neo^R and Mtx RII Oua^R 2-4 were Mtx resistant, indicating that the loci involved were non-complementing. By definition these lines must have lesions residing within similar loci. Thus one possible explanation for the phenotypes of Mtx RII 5-3 and Mtx RII Oua^R 2-4 would be that Mtx RII 5-3 is down regulated for a protein common to both Mtx and folic acid transport, and Mtx RII Oua^R 2-4 expresses an altered Mtx binding or transport protein that no longer binds or transports Mtx. Alternatively, it is possible that these

mutations affect different genes which are noncomplementing. In this situation, because of subunit interactions, the complex becomes inactive if either subunit is defective. This has been demonstrated in genes controlling microtubule function in *Drosophila* (Regan and Fuller, 1988). These mutants appear to be different from a mutant of L1210 cells in which the transport system for Mtx appears to be upregulated (Sirotnak *et al.*, 1984).

At present it is difficult to distinguish amongst these various possibilities to explain the Mtx RII phenotypes. It is also equally difficult to ascertain whether folic acid and Mtx and reduced folates are transported by separate systems linked by a common component(s) or one system with several distinct domains.

The studies described in this chapter show that folic acid and Mtx uptake can be functionally distinguishable but that some common component or components are shared. Clearly additional insight will be obtained into these processes when it is possible to identify the specific components involved. One approach that is currently underway in our laboratory is the use of molecular biological techniques to identify genes that mediate folate uptake.

CHAPTER 3

Complementation of a Methotrexate Uptake Defect in Chinese Hamster Ovary Cells by DNA-Mediated Gene Transfer

3.1 INTRODUCTION

In a recent report, we have described a Mtx resistant Chinese hamster ovary cell designated Mtx RII Ouas 2-4 that was unable to transport Mtx, but was able to take up folic acid in a manner similar to wild-type cells (Underhill and Flintoff, 1989a). Under conditions of folic acid free medium, this line required 250X the level of folinic acid as did wild-type cells to maintain optimum growth. This requirement for high levels of folinic acid in otherwise folic acid free medium is a common property demonstrated by several mutants defective in Mtx uptake (Kano *et al.*, 1986; Sirotnak *et al.*, 1985). Pseudorevertants selected from this cell line for growth on low levels of folinic acid retained their resistance to Mtx. Because the Mtx resistant phenotype was a genetically recessive trait (Flintoff *et al.*, 1976b) it should be possible to complement this phenotype by transfection with DNA from a Mtx sensitive or wild-type cell. Such an approach of DNA mediated gene transfer has been previously used to complement several recessive mutations and to identify genes that encode proteins that either represent a small fraction of total cellular protein or are difficult to isolate (Diatloff-Zito, *et al.*, 1990; Sekiguchi *et al.*, 1987; Shaham *et al.*, 1987; Weber *et al.*, 1988; Westerveld *et al.*, 1984; Lowy *et al.*, 1980). In this report we demonstrate that it was possible

to isolate cells that had regained their sensitivity to Mtx after transfection with genomic DNA from either wild-type CHO or human G2 cells.

3.2 MATERIALS AND METHODS

3.2.1 Cells

The source and routine propagation of the wild-type and mutant CHO Mtx RII Ouab^R 2-4 cells have been previously described (Flintoff *et al.*, 1976a; Flintoff *et al.*, 1976b; Underhill and Flintoff, 1989a). The human G2 (G2 hepatoma) cells (Podskanlny *et al.*, 1985) were obtained from Dr. G. Mackie (University of Western Ontario) and were maintained as monolayer cultures under similar conditions as t' e CHO cells.

3.2.2 DNA Isolation

High molecular weight DNA was isolated from exponentially growing cells by the procedure of Gross-Bellard *et al.* (1978) as follows. Monolayer cultures in T-150 flasks that had reached 75 % confluency were washed with cold phosphate buffered saline (PBS) and incubated for 6 hours at 37° C in 3 ml of STE (100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA) which contained 150ng Proteinase K (Boehringer Mannheim) and SDS at a final concentration of 0.5%. At the end of this period, an

additional aliquot of Proteinase K was added and incubations were continued overnight. High molecular weight DNA was gently purified from protease digested cellular debris by serial extraction with phenol-saturated STE, followed by extraction with chloroform and finally dialyzed against TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) for 3 changes.

3.2.3 Transfection

Transfection of DNA into recipient Mtx RII Oua^R 2-4 cells was carried out by the polybrene procedure described by Chaney *et al.* (1986). Briefly, 5 X 10^5 Mtx RII Oua^R cells per T-75 flask were treated for 6 hours with 3 ml of alpha medium supplemented with 10 % fetal bovine serum (FBS), 10-60 ug DNA sheared by passage through a 21 gauge needle 3 times to an approximate size of 40-50 kB, and 30 ug of polybrene (Aldrich Chemical Company). Subsequently, the DNA mixture was removed and replaced with 5 mls of alpha MEM medium containing 10% FBS and 30% DMSO sulfoxide for 4'. The solution was removed, the cells washed in alpha medium containing FBS, and allowed to recover for 24 hours. The cells were washed with phosphate buffered saline and fed alpha medium deficient in folic acid and supplemented with 10% dialyzed FBS and 2 X 10^{-9} M folinic acid. Individual colonies were picked 2 to 3 weeks later and subjected to further analysis. Transfectants generated with wild-type CHO DNA were given the designation

T1,T2, etc. and those generated with human DNA were designated HT1, HT2, etc. Secondary transfectants generated with DNA from the Mtx sensitive HT2 cell line were given the designation HT2-1, HT2-2, etc.

3.2.4 Southern Blot

High molecular weight DNA was digested with a 6 fold excess of EcoR I (Pharmacia) according to the supplier's recommended conditions, separated on an 0.8% agarose gel and transferred onto nitrocellulose paper by the capillary transfer method of Southern et. al. (Southern, 1975). Blots were prehybridized for 24 hours at 42° C in 50% formamide, 5X SSC (1X SSC is 0.15M NaCl, 0.015M Na Citrate), 1X Denhardt's solution, 50 ug/ml salmon sperm DNA, 300 ug/ml yeast tRNA, 0.1% SDS, 50mM sodium phosphate pH 6.5 and 10% dextran sulphate. Sheared high molecular weight human DNA was nick translated with ^{32}P dCTP (ICN, 3200 Ci/mmmole) and ^{32}P dGTP (ICN, 3200 Ci/mmole) using the Bethesda Research Laboratory nick translation kit to a specific activity of 5 X 10^{8} cpm/ug. DNA was added to the prehybridization cocktail to a final concentration of 3.8 ng/ml. After 48 hours at 42° C blots were washed twice with 2X SSC-0.1% SDS at 23° C, 3 times with 0.1X SSC-0.1% SDS at 58° C and rinsed with 0.1X SSC-0.1% SDS and exposed at -70° C to an X-ray film with Dupont Cronex intensifying screens.

3.2.5 Phenotype Testing

The resistance of various cell lines to Mtx was determined by dose response curves as previously described (Flintoff *et al.*, 1976a) and expressed as D_{10} values, the drug concentrations that reduced cell survival to 10%.

3.2.6 Drug Uptake and Kinetic Analyses

The uptake of 0.25 uM of ^{3}H-labelled Mtx into various cell lines was determined as previously described (Underhill and Flintoff, 1989a), except uptake was performed on monolayer cultures rather than in suspension. The uptake was normalized to pmoles/mg of protein. Protein was determined by the method of Bradford (Bradford, 1976) using the BioRad Protein Microassay.

Kinetic analyses of uptake employed the same method as above except assays were designed to contain various levels of labelled drug with varying specific activities. Samples were taken in the linear range of uptake at 3 time points, graphed by double reciprocal plots and the kinetic parameters determined.

3.3 RESULTS

3.3.1 Phenotypic Properties of Mutant

The observation that Mtx-resistant transport deficient cells required 100-250x the wild-type level of folinic acid

in otherwise folic acid deficient medium to maintain optimum growth provided an opportunity to use this as a potential reversion scheme for the Mtx-resistant phenotype (Underhill and Flintoff, 1989a). Lines selected from some Mtx RII mutants that grew on low levels of folinic acid were also reverted to Mtx sensitivity. The frequency of reversion for growth on low levels of folinic acid and to Mtx sensitivity was $< 10^{-4}$ (Underhill and Flintoff, 1989a). In another case, pseudorevertants from from Mtx RII Oua^R 2-4 were obtained that were able to grow on low levels of folinic acid but retained their resistance to Mtx. In this case the frequency for reversion for growth on low levels of folinic acid was $\sim 1 \times 10^{-6}$, but reversion back to Mtx sensitivity was $< 1 \times 10^{-7}$. This low reversion of the Mtx resistant phenotype in the Mtx RII Oua^R 2-4 cells under these selective conditions raised the possibility of using this line and these conditions to complement the Mtx resistant phenotype by transfer of DNA from wild-type Mtx sensitive cells. Surviving colonies could be easily tested to determine whether a transfection event or a reversion event had occurred since the former should yield isolates with a Mtx sensitive phenotype and the latter, a resistant phenotype.

3.3.2 Generation of Primary Transfectants

Mtx RII Oua^R 2-4 cells were transfected with sheared

DNA from either wild-type CHO or human G2 cells. As shown in Table 3.1 and Figure 3.1, about 65% of all the tested isolates surviving these selective conditions had regained their sensitivity to Mtx. The remaining 35% retained the parental phenotype of Mtx resistance and most likely represented those that have reverted for folinic acid requirement, as this frequency was comparable to the spontaneous reversion frequency of 1 x 10^{-6}. In a similar experiment, when these cells were transfected with DNA isolated from the Mtx RII Oua^R 2-4 cells, no Mtx sensitive isolates were obtained; all isolates examined that survived under low folinic acid growth were Mtx resistant.

To confirm that the Mtx sensitive isolates generated by this primary transfection with human DNA represented true transfectants, DNA was isolated from several of these lines and tested for the presence of human specific DNA sequences by hybridization with a radiolabelled total human DNA as probe. Figure 3.2 shows an autoradiogram of 3 Mtx sensitive isolates obtained with human DNA as donor. All these lines contained a human specific DNA fragment of about 9.5 kB in size with some signal in the lower molecular weight region. DNA from the recipient CHO cells, under these conditions, showed no hybridization with the human DNA as probe. One isolate, HT10 that was Mtx sensitive and obtained after transfection with human DNA showed no significant hybridization with the human probe under conditions of high

Table 3.1

Frequency of Mtx^{s} colonies after transfection[a] with either wild-type CHO DNA, human DNA or secondary transfectant DNA.

DNA Source	Colonies	No. of colonies tested	No. of Mtx^{s} colonies[b]	Frequency of Mtx^{s} (colonies/30 ug DNA per 10^{6} cells plated)
No DNA	31	10	0	
Primary transfection				
Wild-type CHO	4	4	2	0.8
Wild-type CHO	5	5	4	1.6
Human G2	16	13	8	0.8
Mtx RII Oua^{R} 2-4	9	9	0	<0.03
Secondary Transfection				
HT2	8	8	6	0.8
HT7	5	5	4	0.5

[a] Mtx RII Oua^{R} 2-4 cells were transfected as described in Materials and Methods.

[b] Mtx^{s} isolates are defined as having Mtx sensitivity similar to that of wild-type CHO cells with a D_{10} of 2×10^{-5} M.

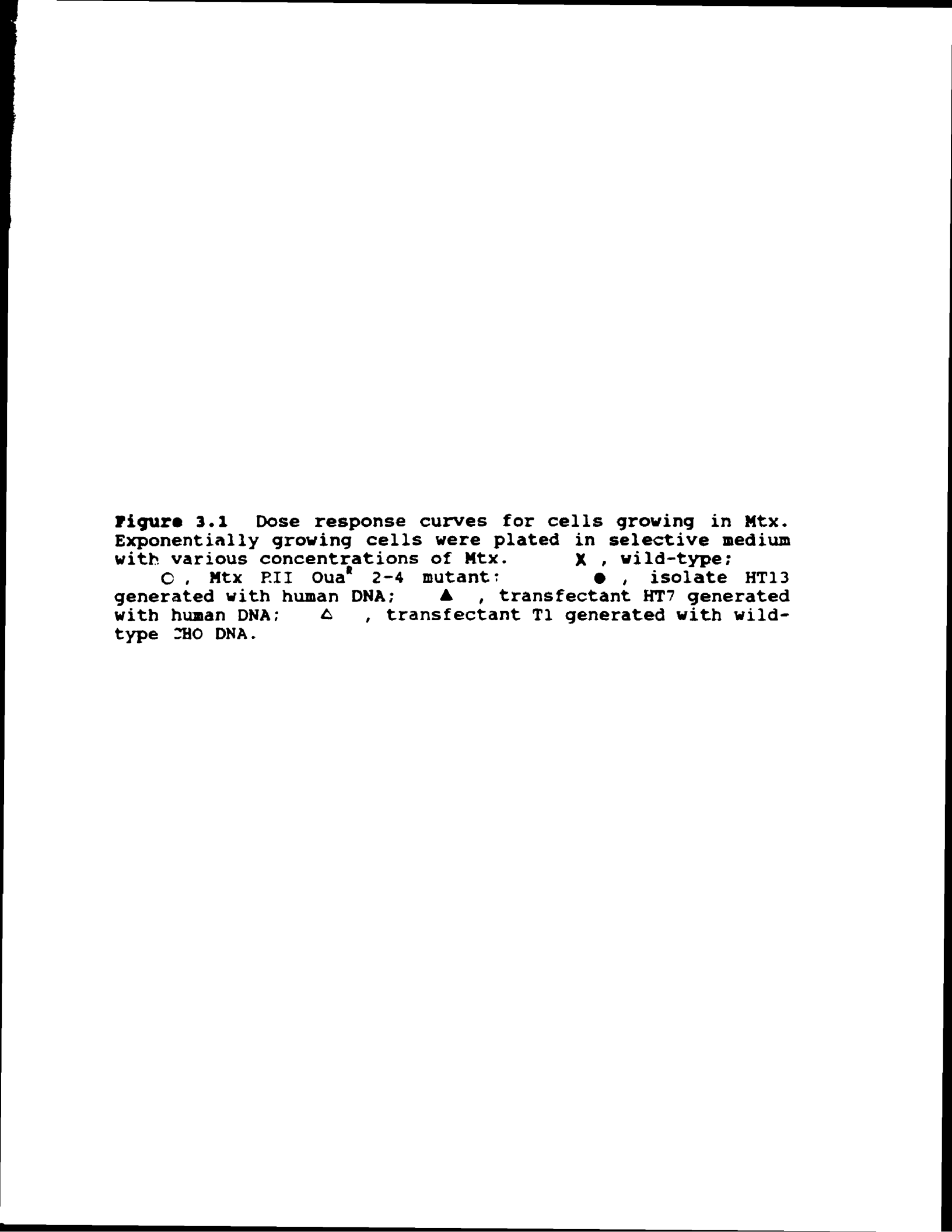
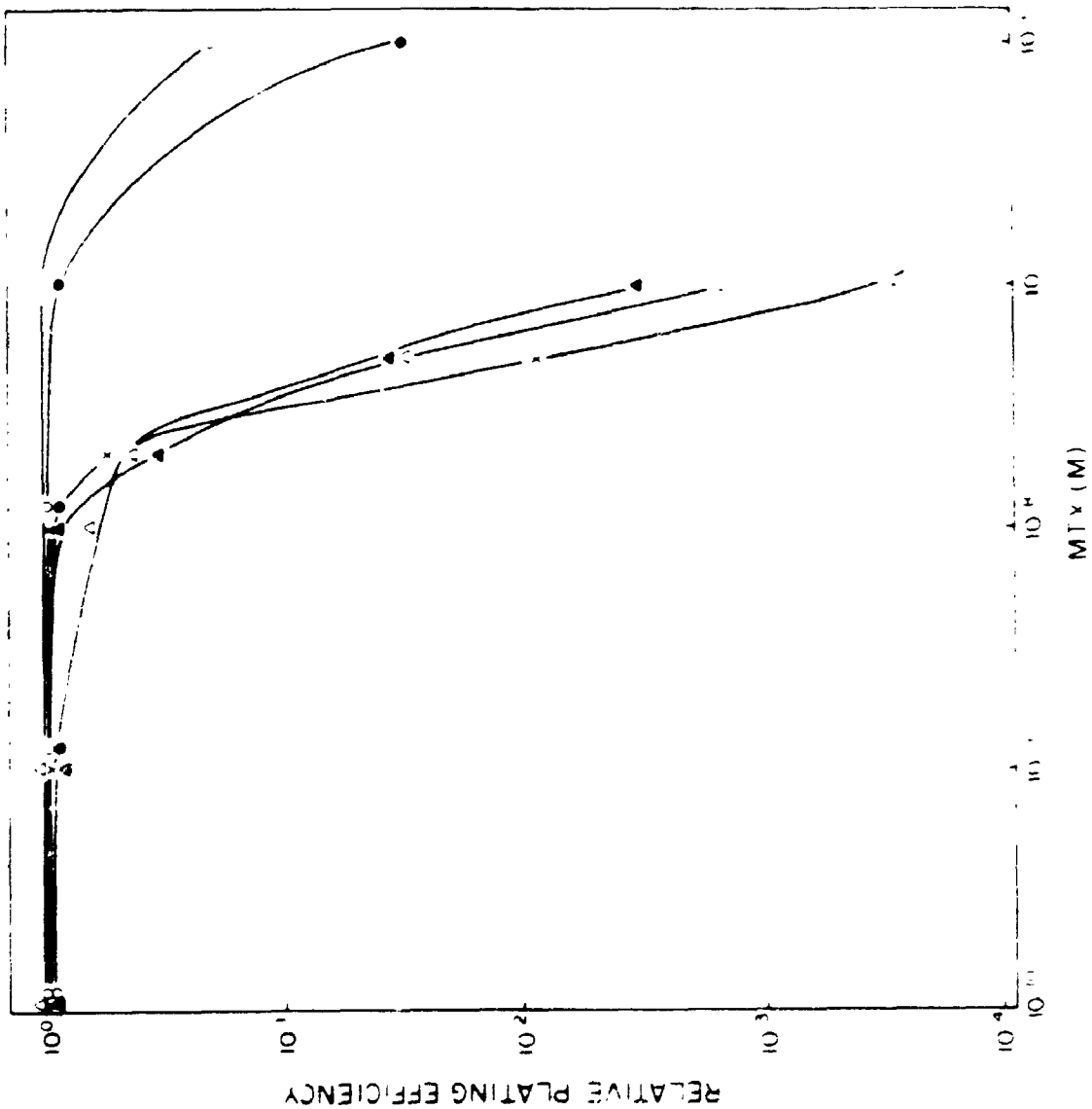


Figure 3.1 Dose response curves for cells growing in Mtx. Exponentially growing cells were plated in selective medium with various concentrations of Mtx. X , wild-type; ○ , Mtx RII Oua^{R} 2-4 mutant; ● , isolate HT13 generated with human DNA; ▲ , transfectant HT7 generated with human DNA; △ , transfectant T1 generated with wild-type CHO DNA.

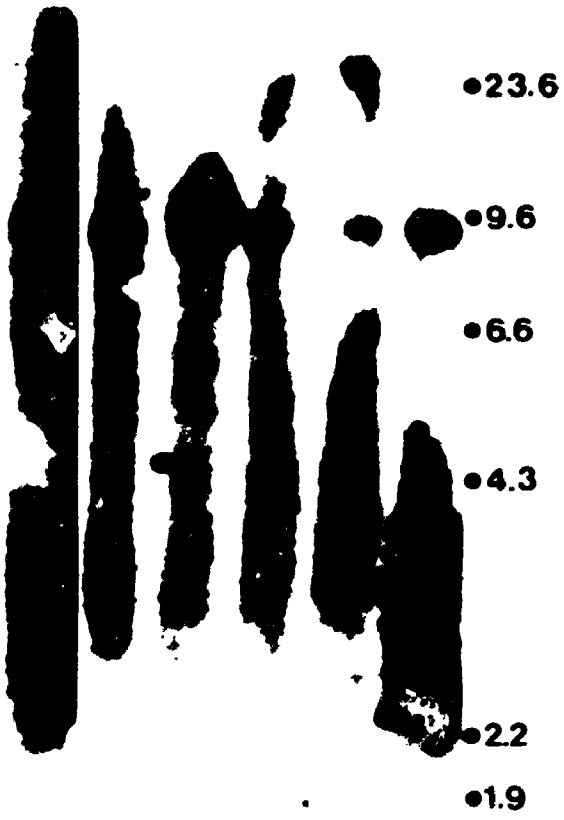

RELATIVE PLATING EFFICIENCY
MTX (M)

stringency (Figure 3.2). The ability to detect human sequences within these transfectants is a function of the amount of human DNA taken up during transfection and its subsequent stabilization and incorporation into the recipient cellular genome. That not all Mtx sensitive isolates examined appeared to contain human specific sequences, does not discount the possibility that these sequences could be present in low abundance and hence not be detectable. A similar variability in the amount and detection of human DNA sequences in transfected systems using human DNA has been reported by others (Sekiguchi *et al.*, 1987; Shaham *et al.*, 1987; Weber *et al.*, 1988). Furthermore, Dulhanty *et al.* (1988) showed that the transfected human sequences responsible for rescue of DNA-repair in a CHO mutant lacked highly abundant human repetitive sequences altogether. Thus, there was a strong correlation with the presence of human DNA sequences and the Mtx sensitive phenotype in these primary transfectants generated using human DNA as donor. Based upon the phenotypic properties of the recipient cell line, it seems reasonable that the isolates obtained using DNA from the wild-type CHO cells as donor also represented true transfectants.

3.3.3 Generation of Secondary Transfectants

Secondary transfectants were generated to determine

Figure 3.2 Autoradiograms of Southern hybridizations of wild-type CHO and human DNA generated transfectant DNAs and total nick translated ^{32}P labelled human DNA. 15 ug of DNA from each transfectant line and the CHO line Mtx RII Oua^{R} 2-4 was digested with EcoR I and subjected to electrophoresis in a 0.8% agarose gel. Lane representation: A, wild-type CHO; B,C,D,E mtx sensitive transfectants HT10, HT4, HT2, HT1, generated with human DNA; F,G,H Mtx sensitive transfectants HT2-1, HT2-2, HT2-3 generated with DNA from HT2. Circles indicate the position of molecular weight markers generated by digestion of phage λ DNA with Hind III.



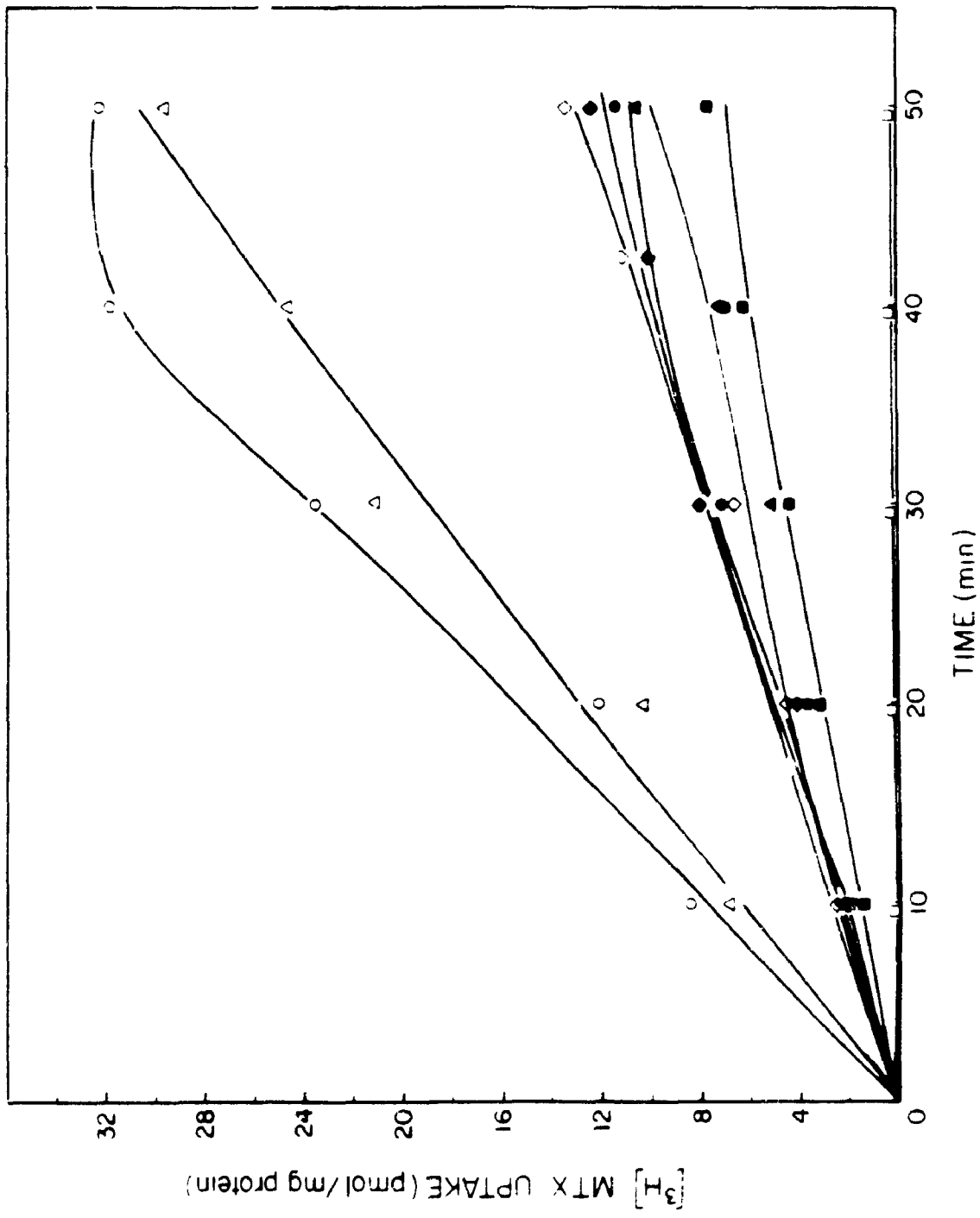
A B C D E F G H

whether a similar pattern of human DNA sequences were retained. DNA was isolated from the Mtx sensitive HT2 transfectant and used to transfect the Mtx RII Oua^{R} 2-4 cells using the method described above. Several Mtx sensitive isolates were obtained and DNA from three of these HT2-1, HT2-2 and HT2-3 were analyzed for the presence of human specific DNA sequences. As shown in Figure 3.2 the hybridization pattern in the secondary transfectants was similar to that of the primary ones. The presence of a similar human specific DNA fragment in several independently selected primary and secondary transfectants suggests that the function conferring Mtx sensitivity on these transfectants may be linked to this fragment of DNA.

3.3.4 Analysis of Methotrexate Uptake in the Transfectants

If these transfectants did in fact represent complementation of the Mtx resistant phenotype then they should have regained the ability to take up Mtx in a manner similar to wild-type cells. As shown in Figure 3.3, representative primary and secondary transfectants accumulated labelled Mtx, albeit not to the same level as in wild-type cells. This is consistent with their Mtx sensitive phenotype. The K_T associated with the newly acquired Mtx uptake in Mtx sensitive transfectants was, 0.4 uM for both T1 and HT7, not unlike that of wild-type CHO or human G2 cells which had a K_T of approximately 0.6 uM, 1.1

Figure 3.3 Cellular uptake of 0.25 uM ^{3}H-Mtx. Cells were incubated at 37°C, and ^{3}H-Mtx uptake measured as described in Materials and Methods. △ , wild-type; □ , Mtx RII Oua^{2} 2-4; ● , transfectant T1 generated with wild-type CHO DNA; ▲ , transfectant T11 generated with wild-type CHO DNA; ■ , Mtx sensitive HT2 generated with human DNA; ○ , Mtx sensitive HT7 generated with human DNA; ◇ , Mtx sensitive secondary transfectant HT2-1; ◆ , Mtx sensitive secondary transfectant HT2-^.


[3H] MTX UPTAKE (pmol/mg protein)
0
4
8
12
16
20
24
28
32
0
10
20
30
40
50
TIME (min)

uM respectively. Since the K_T values for Mtx uptake in both the parental human G2 and wild-type CHO cells were similar it is not possible to functionally distinguish between wild-type or human DNA generated transfectants. In some cases, the transfectants had a reduced V_{MAX} for Mtx uptake compared to wild-type CHO cells, a representative transfectant T1 was found to have a V_{MAX} for Mtx uptake of 0.4 pmol/min/mg protein, which is approximately 40% of that of wild-type cells. Because transfected DNA may integrate at random sites, perhaps this was a consequence of association in the genome of the recipient cell where expression was reduced.

3.4 DISCUSSION

The availability of a Mtx resistant line with a defect in drug uptake that showed no detectable reversion to Mtx sensitivity under low folate growth conditions has provided a unique opportunity to use DNA mediated gene transfer to complement this Mtx resistant phenotype. Isolates obtained using either wild-type CHO or human cells as a source of donor DNA regained the sensitivity to Mtx, the ability to take-up Mtx, and in the case of human donor DNA the presence of human specific DNA sequences.

This ability to complement by DNA mediated gene transfer the Mtx resistant phenotype in the Mtx RII Oua^R 2-4 cell line and to regain the ability to transport Mtx is to

our knowledge the first example of such an observation for this phenotype. At present it is not known what the nature of the alteration is in this cell line that confers resistance to the drug. The ability to revert these cells to Mtx sensitivity by DNA mediated gene transfer coupled with the outlined selection scheme provides an opportunity to design strategies to clone the gene responsible. The availability of such a gene should allow identification of the mutational basis for the resistant phenotype and be useful in the molecular analysis of the Mtx uptake system.

CHAPTER 4

Complementation of a Methotrexate Uptake Deficiency by DNA-Mediated Gene Transfer: Transfection with DNA from Cosmid Libraries

4.1 INTRODUCTION

An alternative approach to traditional biochemical analysis in examining folate transport involves the isolation of genes that encode folate uptake or binding proteins. Recently three groups have successfully cloned a gene encoding a human folic acid binding protein (Elwood, 1989; Sadasivan and Rothenberg, 1989; Ratham *et al.*, 1989). This gene was isolated by screening cDNA libraries with an oligonucleotide synthesized from a partial amino acid sequence of a folic acid affinity purified protein. At this time it is not completely clear what role if any this gene product may have in Mtx uptake. In order to isolate a gene specific to Mtx uptake we have employed an alternative method that involves transfer of genetic material from a Mtx uptake competent cell line into that of a cell line solely deficient in Mtx uptake.

Previously we have described the complementation of the Mtx uptake defect by genomic DNA from wild-type Chinese hamster ovary (CHO) cells and human cells (Underhill and Flintoff, 1989b.). In this chapter we extend these studies by demonstrating the feasbility of rescue of this mutant phenotype by cosmid libraries and describe a novel strategy for the quick retrieval of complementing sequences. Such approaches have facilitated the isolation of cosmid clones which rescue at high frequency, the Mtx resistant phenotype

of the mutant.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

Mtx was purchased from the Sigma Chemical Company. N^5-formyl-tetrahydrofolate (folinic acid) was obtained from ICN Biochemicals. Geneticin (G418) and N-2-hydroxyethylpiperazine (HEPES) were purchased from Gibco Canada Ltd. and Boehringer Mannheim Co., respectively. Polybrene was obtained from Aldrich Chemical Co.

[3',5',7'-^{3}H] Mtx (20 Ci/mmol) was purchased from Amersham. Immediately prior to use, [^{3}H]-Mtx was purified by thin layer chromatography as described previously (Flintoff and Nagainis, 1983.).

4.2.2 Cells

Wild-type Mtx sensitive (Pro ^{3}), class II Mtx-resistant Pro ^{4} Mtx RII Oua^R 2-4 (designated as Mtx RII Oua^R 2-4) have been previously described (Flintoff *et al.*, 1976a; Flintoff *et al.*, 1976b). Cells were maintained in suspension culture in alpha medium supplemented with 8% Nu-Serum (Collaborative Research) and 2% fetal bovine serum. Isolates sel cted for their ability to grow in medium supplemented with low levels of folinic acid were maintained in selective medium, a folic acid free alpha medium with 10% dialyzed fetal bovine serum

and 2 nM folinic acid.

4.2.3 Phenotype Testing

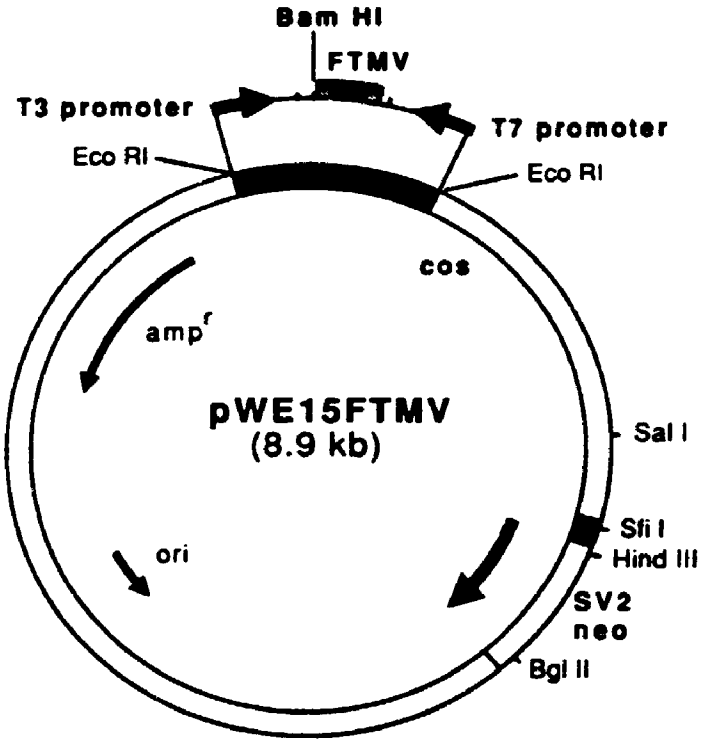
The resistance of various cell lines to Mtx was determined by dose-response curves as previously described (Flintoff *et al.*, 1976a). Resistance is expressed as a D_{10} value, the drug concentration that reduced cell survival to 10%. Isolates were considered to be Mtx-sensitive if they had a Mtx D_{10} value similar to wild type CHO cells of 2×10^{-5}M. The D_{10} values for growth in folinic acid and folic acid-containing media were performed in a similar manner. In this case, the D_{10} represents the concentration of folinic acid or folic acid allowing 10% cell survival.

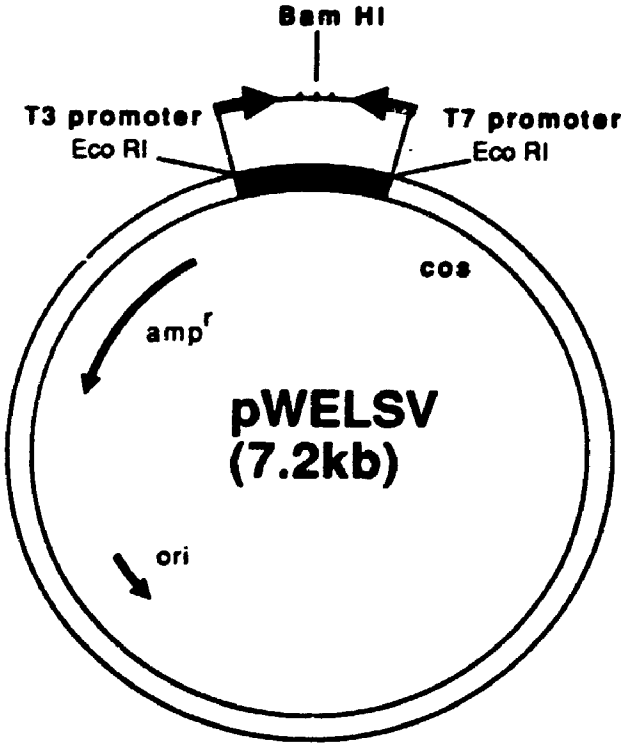
4.2.4 Construction of Cosmid Vectors pWE15FTMV and pWELSV

4.2.4.1 pWE15FTMV

Cosmid libraries for primary transfections were constructed in a modified pWE15 cosmid vector (Appendix 1). As shown in Figure 4.1, a 300 bp fragment from the fox tail mosaic virus (FTMV) was inserted into pWE15 (Wahl *et al.*, 1987) to produce the vector pWE15FTMV. pWE15FTMV was constructed from pWE15 and p137e (kindly provided by Dr. J. Bancroft, University of Western Ontario), a pSP65 derivative which contains part of the FTMV genome. A Sma I fragment containing FTMV genomic sequences was isolated from p137e. This fragment was ligated to Bam Hl linkers and digested with Bam HI and Bgl II, thereby generating a 300 bp Bam HI-

Figure 4.1 Maps of the cosmid vectors pWE15FTMV and pWELSV. A. pWE15FTMV cosmid vector was assembled by inserting a 300 bp Bam Hl -Bgl II fragment from the fox tail mosaic virus (FTMV)into the Bam Hl cloning site of pWE15, such that a single Bam Hl cloning site is retained. B. pWELSV was constructed from pWE15 by removal of the SV 40 sequences between the Sal 1-Bgl II restriction enzyme sites in pWE15 as described in Materials and Methods.


Bam HI
FTMV
T3 promoter
T7 promoter
Eco RI
Eco RI
cos
amp^r
pWE15FTMV
(8.9 kb)
Sal I
Sfi I
Hind III
ori
SV2
neo
Bgl II


Bam HI
T3 promoter
T7 promoter
Eco RI
Eco RI
cos
amp^r
pWELSV
(7.2kb)
ori

Bgl II fragment. pWE15 was digested with Bam Hl and ligated to the purified FTMV fragment, such that one Bam Hl cloning site is retained adjacent to the FTMV fragment. Transformation of JM109 *Escherichia coli* cells with the ligation mixture was accomplished using electroporation with the BioRad Gene Pulser unit according to the manufacturers directions. Transformants were selected on Luria broth (LB) agar plates supplemented with 100 ug/ml ampicillin and those transformants harboring FTMV sequences were identified by probing colony lifts with a radiolabelled Sma I fragment from pl37e. DNA was isolated from several positive clones and the presence of an FTMV sequence was confirmed by restriction digestion with Eco RI. Futhermore, double restriction enzyme digests of DNA from one clone with Hind III and Bam Hl showed that the FTMV sequence mapped between the Bam Hl site and the T7 promoter (Figure 4.1). DNA from this clone was isolated, termed pWE15FTMV and used for library construction.

4.2.4.2 pWELSV

pWELSV was constructed by removing the Sal I-Bgl II fragment that supposedly contained most of the pSV2neo derived neo gene, from pWE15 (Figure 4.1). pWELSV was digested with a 4-fold excess of Sal I and Bgl II, single stranded DNA overhangs were repaired with T4 DNA polymerase

and ligations were set up at low DNA concentrations (25 ug/ml) to favour re-circularization. Ligation mixtures were used to transform JM 109 cells as outlined above. Transformants were selected on LB agar with 50 ug/ml ampicillin and individual colonies were subsequently tested for growth on 25 ug/ml kanamycin. Kanamycin sensitive transformants that contained the desired construct were identified by restriction site mapping of their respective cosmid DNA. Several clones were identified that lacked restriction sites for Sal I, Bgl II or Sfi I, restriction sites which are contained in the parent vector. This was further confirmed by the absence of hybridization of these DNAs to a radiolabelled Hind III-Bgl II of pSV2NEO. One of these clones was grown up and used in cosmid library construction.

4.2.5 Construction of Cosmid Libraries for Primary Transfection

Wild-type CHO genomic DNA was used to construct a cosmid library in pWE15FTMV. High molecular weight wild-type CHO DNA was partially digested with Sau 3A (New England Biolabs) and DNA fragments of 35-42 kB in size were isolated on a 10% to 40% sucrose gradient (DiLella and Woo, 1987). These fragments were ligated to a 10-fold molar excess of Bam H1 dephosphorylated pWE15FTMV vector. Ligated DNA was packaged using Gigapack gold (Stratagene) and used to infect

ED8767 *Escherichia coli* . Approximately 450,000 cosmid transformants were pooled and grown up in LB medium containing 50 ug/ml ampicillin for DNA isolation.

4.2.6 **DNA Isolation**

High molecular weight genomic DNA from mammalian cells was isolated from exponentially growing cells by the procedure of Gross-Bellard, Oudet and Chambon (1978) and is described in Chapter 3.

Small quantities of cosmid or cosmid vector DNA were purified using the alkaline lysis mini-prep DNA isolation method as described in Sambrook *et al.* (1989).

Cosmid vector DNA was isolated from amplified bacteria cultures using the alkaline lysis method described in Sambrook *et al.* (1989). Vector DNA used for the construction of libraries was further purified by CsCl density gradient centrifugation (Sambrook *et al.*, 1989).

Pooled cosmid library DNA for primary transfections was purified from unamplified cultures using a modified alkaline lysis protocol (Sambrook *et al.*, 1989). After lysis, a large scale phenol:chloroform extraction was performed, followed by precipitation with isopropanol. The precipitate was pelleted at 8000g for 20 min. at 4° C, washed once with 70% ethanol and resuspended in 0.5 ml TE. DNA at this stage was suitable for transfection.

Cosmid DNA from cosmid clone pools of 3 were purified

from 200 mls of unamplified cultures using Qiagen columns by the method described in the accompanying manual (Qiagen, California). This method yielded approximately 50-100 ug of cosmid DNA suitable for transfection. Individual positive cosmid clone DNAs were purified in a similar manner.

4.2.7 Transfection

Transfection of genomic DNA into recipient Mtx RII Oua^R 2-4 cells was carried out by the Polybrene procedure as previously described (Underhill and Flintoff, 1989b.). A similar protocol was used with DNA from cosmid libraries except that the DNA was not passed through a 21 gauge needle prior to transfection. For cosmid library transfections, 10 ug of library DNA per 1-2 X 10^6 cells were transfected into the Mtx RII Oua^R 2-4 cells. Approximately 2-4 X 10^7 recipient cells were used in each transfection. After transfection with this DNA, cells able to grow on low levels of folinic acid (2 X 10^{-9}) were selected by plating in selective medium as previously described (Underhill and Flintoff, 1989b.). Cells transfected with DNA from the cosmid library were initially selected for growth in normal medium containing 1.2 mg/ml G418 (approximately 50% active). After 2-3 weeks G418 resistant colonies were pooled and replated in selective medium. Cells transfected with DNA from primary cosmid transfectants were selected in a similar manner, except single colonies were picked after G418

selection and tested for sensitivity to methotrexate.

For testing cosmid pools or individual cosmid clones for methotrexate uptake function, transfections were done as follows. Approximately 20 ug of cosmid DNA was transfected, using the polybrene procedure outlined above, into 2 T-75 tissue culture flasks seeded 24 hours before with 1 X 10^5 Mtx RII 2-4 Oua^R cells. Following transfection cells were allowed to recover in normal medium for 36-48 hours. Cells were selected either for the ability to grow on low levels of folinic acid, 2 nM, or in medium supplemented with 600 ug/ml active G418.

Isolates were categorized according to the transfected DNA used to generate them, such that transfectants generated with wild-type CHO cosmid library DNA were designated, WETA-1, WETA-2, etc, or WETB-1, WETB-2, etc.; secondary transfectants from WETA3 DNA, WETA3-1, WETA3-2, etc (Appendix 2).

4.2.8 Southern Blots

High-molecular weight DNA or cosmid DNA was digested with a sixfold excess of either Eco Rl, Bgl II, or Hind III (Pharmacia, Inc.) according to the conditions of the supplier. The digested DNA was separated on a 0.8% agarose gel, and transferred onto nitrocellulose paper by the method of Southern (1975). Southern blots prepared from cosmid DNA or DNA isolated from transfectants were probed with the

following sequences. Cosmids from human secondary transfectant libraries were hybridized with either nick translated sheared (avg. size 50 kB) CHO or human G2 DNA as described in Chapter 3. WET transfectants and their corresponding cosmid clones were hybridized with labeled FTMV fragment or the Hind III-Bgl II fragment encompassing the SV40 origin of replication from plasmid pSV2neo (Southern and Berg, 1982). In each case, these vector sequences were labeled by random priming (Feinberg and Vogelstein, 1983) with [^{32}P]dCTP (ICN Pharmaceuticals; 3200 Ci/mmol) and [^{32}P]dGTP (ICN; 3200 Ci/mmol) to a specific activity of 5-10 X 10^{8} cpm/ug. DNA was added to the prehybridization cocktail to a final concentration of 2-4 ng/ml. After 48 hr at 42°C, blots were washed 3 times with 2X SSC (0.15M sodium chloride, 0.015M sodium citrate)-0.1% SDS at room temperature and 3 times with 0.1X SSC-0.1% SDS at 52°C. Blots were exposed to X-ray film at -70°C with intensifying screens.

4.2.9 Cosmid Library Construction and Screening

4.2.9.1 Cosmid Libraries from HT2-2 DNA

DNA from a Mtx sensitive secondary transfectant containing human specific sequences (HT2-2) was partially digested with Sau 3A and cosmid libraries were prepared as described above using pWE15. Libraries were plated onto LB agar plates supplemented with 50 ug/ml ampicillin at a

density of approximately 15-25 X 10^3 colonies per 150 mm petri plate. Colonies were allowed to grow up for 10-12 hours, at which time they were transferred to nitrocellulose filters. The filters were incubated for an additional 6-8 hrs at 37° C on LB agar plates with 50 ug/ml ampicillin and finally transferred to LB agar plates containing 180 ug/ml chloramphenicol for 20 hr. Colonies were lysed and DNA fixed to the filter, by a 5 min treatment with 0.5 M NaOH-1.5 M NaCl, followed by a 15 min incubation in 0.75 M Tris-HCl (pH 7.5)-1.5 M NaCl. Filters were baked and probed with nick translated human DNA as outlined in Chapter 3. Secondary and tertiary rounds of screening were conducted similarly but at low density (100-400 colonies per 82 mm filter) to purify positive clones.

4.2.9.2 Construction and screening of cosmid libraries from cosmid transfectants

Cosmid libraries were constructed as previously mentioned in pWELSV, except that libraries from the primary transfectant were prepared by a partial Eco RI digest of genomic DNA and ligated to Eco RI cleaved dephosphorylated pWELSV. For the isolation of cosmid clones containing a functional neo gene, these libraries were plated out at high density, 2-3X10^5 ampicillin resistant colonies/ 100mm petri plate, on 25 ug/ml kanamycin. Individual colonies, usually 2-10 colonies per 100 mm plate, were subsequently

transferred to LB agar plates supplemented with either 50 ug/ml kanamycin or 50 ug/ml ampicillin. After approximately 20-22 hours the colonies on the ampicllin LB plates were transferred to nitrocellulose filters. Filters were processed as described above and probed with the Hind III-Bgl II fragment of pSV2neo using conditions similar to that described for Southern blots.

4.2.10 Methotrexate Uptake

The time dependent intracellular accumulation of 0.20 or 0.25 uM ^{3}H-labelled Mtx within the various cell lines was determined as previously described (Underhill and Flintoff 1989a), except that measurements were taken on monolayer cultures rather than in suspension. Uptake values were normalized to picomoles per milligram of protein. Protein content was determined by the method of Bradford (1976) using the BioRad Protein Microassay.

4.3 RESULTS

4.3.1 Properties of Transfection Recipient

The CHO Mtx RII Oua^{R} 2-4 mutant cell line is approximately 50 times more resistant to Mtx than wild-type CHO cells, and is deficient in Mtx uptake and fully competent in folic acid uptake (Underhill and Flintoff, 1989a). The inability of this cell line to transport Mtx is

reflected in its high requirement for folinic acid for growth in the absence of exogeneous folic acid. These cells have been found to require approximately 250 times the level of folinic acid as wild-type CHO cells to maintain similar levels of growth (Underhill and Flintoff, 1989a). This dependence on high levels of folinic acid for growth by this cell line has been used to isolate cells that have regained the ability to survive on low levels of folinic acid (Underhill and Flintoff, 1989a). These isolates appear at a low frequency ($< 10^{-5}$), are Mtx resistant, and as such do not take-up Mtx. Using DNA-mediated gene transfer as a vehicle for transfer of genetic material it has been demonstrated that the methotrexate uptake-deficient phenotype of Mtx RII Oua^R 2-4 mutant could be rescued by transfection with DNA from either wild-type CHO or human G2 cells (Underhill and Flintoff, 1989b). In addition, the Mtx uptake competent phenotype could be transferred to Mtx RII 2-4 Oua^R cells by transfection with DNA from primary Mtx sensitive transfectants. Furthermore, a common human specific fragment was detected within the primary transfectants generated with human DNA and their respective secondary transfectants. The conservation of this fragment throughout this series of transfectants suggested that this fragment may be tightly linked to part of the gene that complements the Mtx resistant phenotype of this mutant.

In an attempt to isolate this 9 kB fragment, a cosmid

library was constructed from DNA of a secondary (HT2-2) transfectant in pWE15. Several putative clones were picked and tested for the presence of human-specific fragments by probing isolated cosmids with the human genomic probe. All isolated cosmids contained fragments that hybridized not only to the human probe, but also to a genomic CHO probe (Figure 4.2). This stems from the fact that DNA from CHO and human cells contain cross-hybridizing repeat sequences (Jelinek, W.R. *et. al.* 1980.). Other researchers have described these same difficulties in trying to retrieve human specific sequences within a CHO genomic background (Dulhanty *et al.*, 1988, Schatz *et al.*, 1989).

To circumvent this problem an alternative strategy was developed that did not rely on isolation of complementing sequences by probing with species-specific repeat sequences. Rather, complementing genomic sequences would be identified by their constructed linkage to an unambiguous identifier sequence.

4.3.2 Transfection With DNA Isolated From Cosmid Libraries

A second strategy involved transfection with DNA from a wild-type CHO genomic library in pWE15FTMV. pWE15FTMV was constructed such that inserts would be tightly linked to a unique fragment, FTMV, to facilitate both identification and isolation of insert sequences. This vector also contained the neo gene under control of the SV40 early promoter such

Figure 4.2 Autoradiograms of Southern hybridizations of cosmid clones isolated from an HT2-2 secondary transfectant cosmid library with appropriate radiolabeled probe. DNA (2 ug) from each cosmid clones was digested with Eco RI and separated by electrophoresis on a 0.8% agarose gel. A. Cosmid clones probed with nick translated human genomic DNA, lanes: 1, 2; 2, 4; 3, 5; 4, 6; 5, 7; 6, 9; 7, 10; 8, 11; 9, 12. B. Cosmid clones probed with nick translated CHO genomic DNA, lane designations are the same as in 4.2A. The arrow denotes the position of linearized cosmid vector. ● Positions of molecular weight markers produced by digestion of phage λ with Hind III.


A
B
23.1
9.4
6.6
4.4
2.3
2.0
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9

that CHO cells that carried the intact vector could be selected for in medium that contained sufficient G418 to kill non-transfected cells.

Cosmid DNA isolated from a wild-type CHO cosmid library constructed within pWE15FTMV was transfected into the Mtx RII Oua^R 2-4 mutant and selection was carried out in medium supplemented with G418. After two weeks approximately 20,000 G418 resistant colonies were pooled and replated in selective medium that was supplemented with folinic acid to 2 X 10^{-9} M. A portion of this pool was plated in two independent experiments, and isolates were desginated WETA- and WETB-, respectively. Of the 12 surviving colonies tested in the first experiment, 4 were Mtx sensitive (Table 4.1). In the second plating 3 of 4 isolated colonies were Mtx sensitive (Table 4.1). Consistent with rescue, WETA-3 had a D_{10} for growth in folic acid of 9 nM, compared to 5 nM for wild-type cells and 83 nM for Mtx RII Oua^R 2-4. One representative Mtx sensitive transfectant was tested for the ability to take up Mtx, and as shown in Figure 4.3 this transfectant accumulates labeled Mtx, indicating that the Mtx deficient uptake phenotype of Mtx RII Oua^R 2-4 cells had been rescued.

4.3.3 Identification of Cosmid Sequences Within Transfectants

To confirm that cosmid insert sequences were present

Table 4.1

Frequency of mtx^{s} colonies after transfection[a] with DNA from cosmid libraries, secondary or tertiary transfectants.

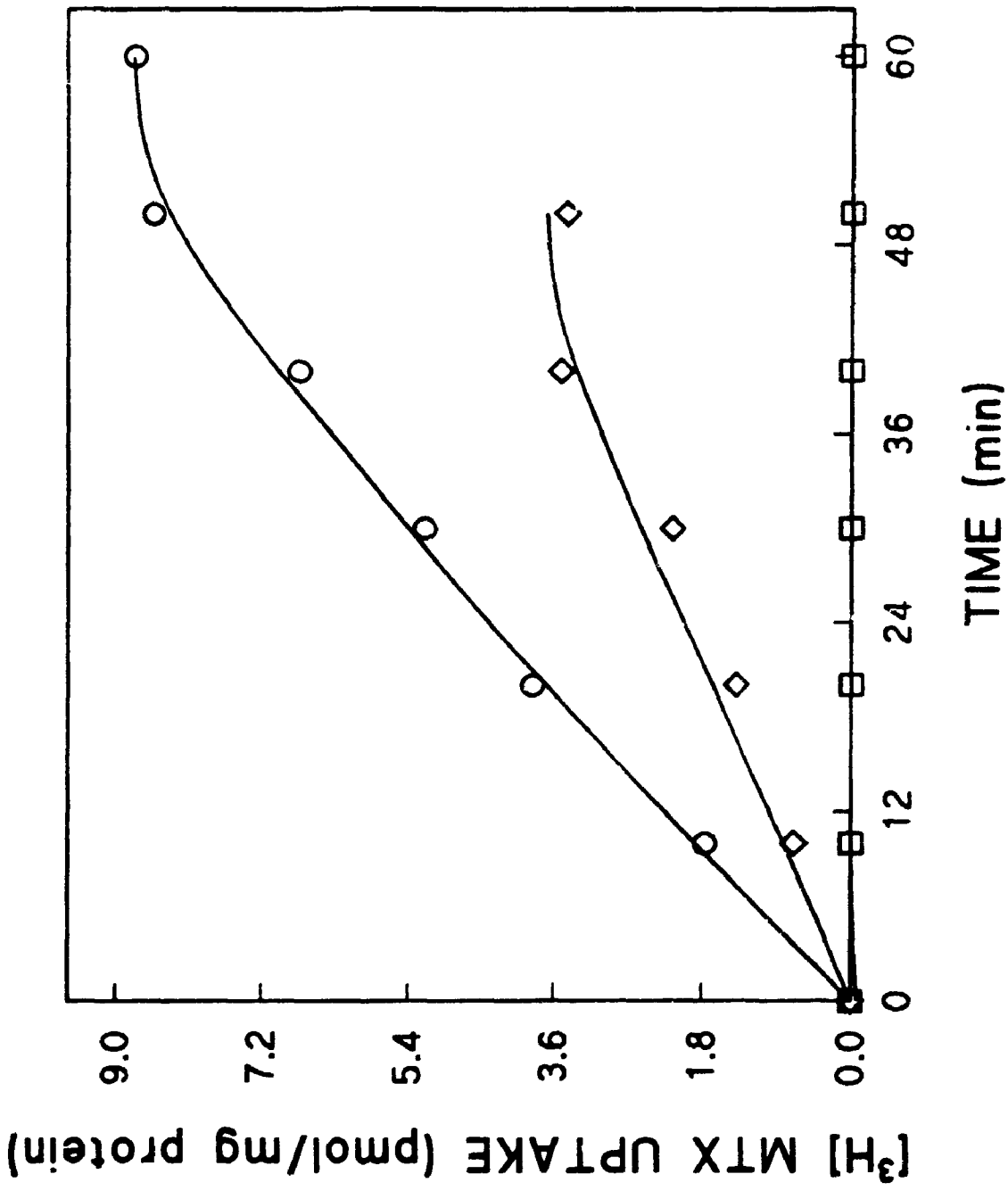
DNA Source	Colonies	No. of colonies tested	No. of Mtx^{s} colonies[b]	Frequency of Mtx^{s} (colonies/30 ug DNA per 10^{6} cells plated)
No DNA	31	10	0	
Primary transfection				
Cosmid Library[c]	12	12	4	0.32
Cosmid Library[c]	4	4	3	0.24
Mtx RII Oua^{R} 2-4	9	9	0	<0.03
Secondary Transfection[c]				
WETA-3	9	9	1	0.02
WETA-5	8	8	0	
Tertiary Transfection[c]				
WETA3-5	7	7	0	

[a] Transfections were carried out as described in the Materials and Methods, 30 ug of DNA was used per 5 X 10^{5} cells for genomic transfections, 10 ug of DNA per 2 X 10^{6} cells for cosmid library transfections and 20-30 ug of DNA per 2 X 10^{6} cells for secondary and tertiary transfections.

[b] Mtx^{S} isolates are defined as having Mtx sensitivity similar to that of wild-type CHO cells with a D_{10} of 2 X 10^{-8} M.

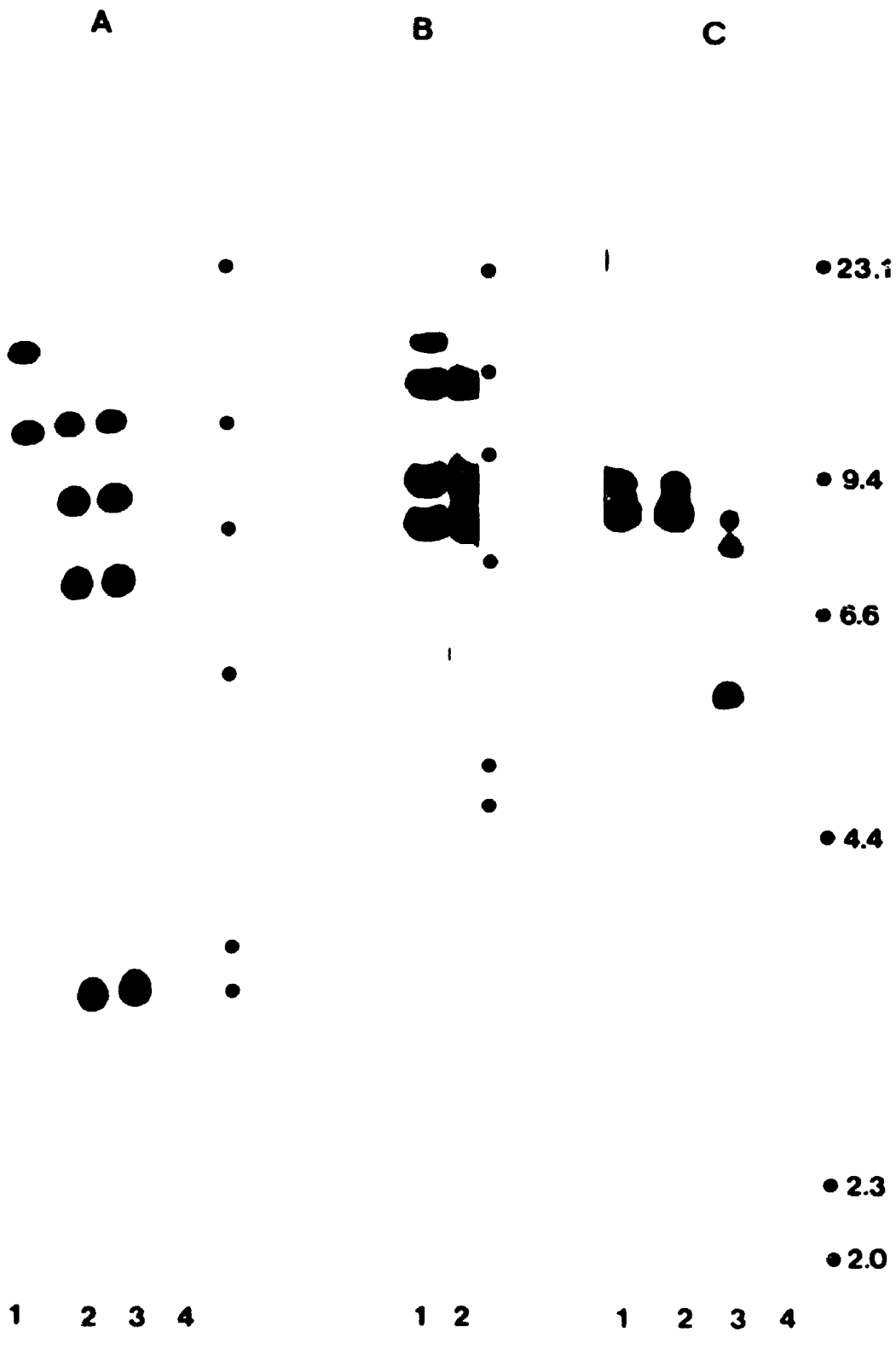
[c] Cosmid transfectants were first selected on G418 and then selected for growth on low folinic acid.

Figure 4.3 Cellular uptake of 0.20 uM [^{3}H]Mtx in primary cosmid transfectants. Cells were incubated at 37°C, and [^{3}H]Mtx uptake measured as described in Materials and Methods. ○ wild-type, □ Mtx RII 2-4, ◇ WETA-3.


[3H] MTX UPTAKE (pmol/mg protein)
9.0
7.2
5.4
3.6
1.8
0.0
0
12
24
36
48
60
TIME (min)

within the cosmid transfectants and possibly involved in rescue of the Mtx resistant phenotype, DNA was isolated from the 8 Mtx sensitive transfectants, digested with Eco RI, transferred to nitrocellulose and probed with radiolabeled FTMV fragment. All Mtx sensitive transfectants contained at least two Eco RI fragments that hybridized to the FTMV probe (Figure 4.4). In addition, six of these independently selected transfectants, WETA-1,3,6 and WETB-1,2,3 had identical hybridization patterns, which indicated that they all derived from the same G418 resistant colony. These six transfectants also contained at least two Eco RI fragments that hybridized to a neo probe (Figure 4.4), a predominant fragment of approximately 8.5 kB in size, similar in size to native pWE15 vector and a 9.0-9.5 kB fragment of lesser intensity . These observations suggested that in transfectants WETA-1,3,6 and WETB-1,2,3 most of the cosmids had integrated randomly into the host genome within the insert, as the vector appeared to remain intact. To determine if this indeed was the case, Southern blots were prepared with Bgl II digested DNA from one representative, WETA-3. Bgl II cuts once within the neo sequence within pWE15, such that distinct cosmid integrates can be distinguished by their banding patterns when probed with radiolabelled FTMV and neo sequences. It is assumed that where the cosmid vector has remained intact in the host genome, the FTMV and neo sequences will be contained on the

Figure 4.4 Autoradiograms of Southern hybridizations of wild-type CHO and Mtx sensitive transfectant DNAs and appropriate radiolabeled probe. DNA (15 ug) from each transfectant line and from wild-type CHO cell line was digested with Eco RI, Bgl II or Hind III and separated by electrophoresis on a 0.8% agarose gel. A. Transfectants isolated after transfection with DNA from a pWE15FTMV cosmid library were digested with Eco RI and probed with the radiolabeled 300 bp FTMV fragment, lanes: 1, WETA-5; 2, WETA-3; 3, WETA-1; 4, wild-type CHO. B. WETA-3 transfectant DNA digested with Bgl II and probed with radiolabelled, lanes: 1, FTMV; 2, Hind III-Bgl II fragment of pSV2neo. C. Mtx sensitive primary transfectants were digested with Eco RI and probed with the radiolabeled Hind III-Bgl II fragment from pSV2neo, lanes: 1, WETA-1; 2, WETA-3; 3, WETA-5; 4, wild-type CHO. ● Positions of molecular weight markers produced by digestion of phage λ with Hind III.


A
B
C
23.1
9.4
6.6
4.4
2.3
2.0
1 2 3 4
1 2
1 2 3 4

same Bgl II fragment, such that neo and FTMV radiolabelled probes should hybridize to identical Bgl II fragments. Using this approach it was determined that WETA-3, in fact, contained three separate Bgl II fragments, each of which hybridzed to both neo and FTMV (Figure 4.4). This suggests that in WETA-3, 3 of the 4 cosmids have integrated into the host genome within the insert and that the cosmid insert should still be tightly linked to either FTMV or neo sequences. These observations strongly suggest that the neo and/or FTMV sequences are tightly linked to the sequences responsible for rescue. Establishment of linkage between these sequences is an absolute requirement for the subsequent molecular cloning of this Mtx uptake gene.

To eliminate extraneous cosmid sequences not involved in complementation, secondary transfectants were generated by transfecting DNA isolated from the primary transfectants. More importantly, the isolation of methotrexate sensitive, G418 resistant secondary transfectants will confirm the identity of the primary transfectants and demonstrate linkage.

4.3.4 Isolation of Secondary Transfectants

Primary transfectants WETA-3 and WETA-5 served as a source of DNA for secondary transfections. Nine G418 resistant colonies were isolated after transfection with DNA from WETA-3, one of these, WETA3-5, was found to be

methotrexate sensitive (Table 4.1) and grow in medium containing low levels of folinic acid. Consistent with the phenotype of WETA3-5, this transfectant was able to accumulate Mtx intracellularly in a manner similar to wild-type CHO cells (Figure 4.5). To determine what cosmid sequences were still present in the WETA-3 generated secondary transfectants, DNA was isolated from both Mtx resistant and senstive transfectants, digested with Eco RI and Southern blots prepared as previously described. Blots were hybridized to either FTMV or neo radiolabelled fragments. WETA3-5 was found to contain no fragments (Figure 4.6) and two Eco RI fragments that hybridized to FTMV and neo probes, respectively. Likewise, some of the Mtx and G418 resistant WETA3 secondaries contained fragments that hybridized to FTMV, while some did not (Figure 4.6). The neo probe hybridized to a prominent band of 7.5-8.0 kB in size (Figure 4.6), slightly smaller than native pWE15FTMV and a band of lesser intensity that appears to correspond to intact vector. These observations verify the authenticity of the primary transfectants, in addition to demonstrating that the neo gene is tightly linked to the complementing sequence.

Eight G418 resistant secondary transfectants were selected after transfection with WETA-5 DNA; all of these 8 were found to be methotrexate resistant (table 4.1). The fact that no Mtx sensitive, G418 resistant transfectants

Figure 4.5 Cellular uptake of 0.20 uM ^{3}H-labeled Mtx in secondary transfectants. Cells in 6 well tissue culture plates were washed 3 times with cold uptake buffer and uptake was measured as described in Materials and Methods.
○ , wild-type cells; □ , RII 2-4 Oua^{R}; ▽ , WETA3-5 transfectant.

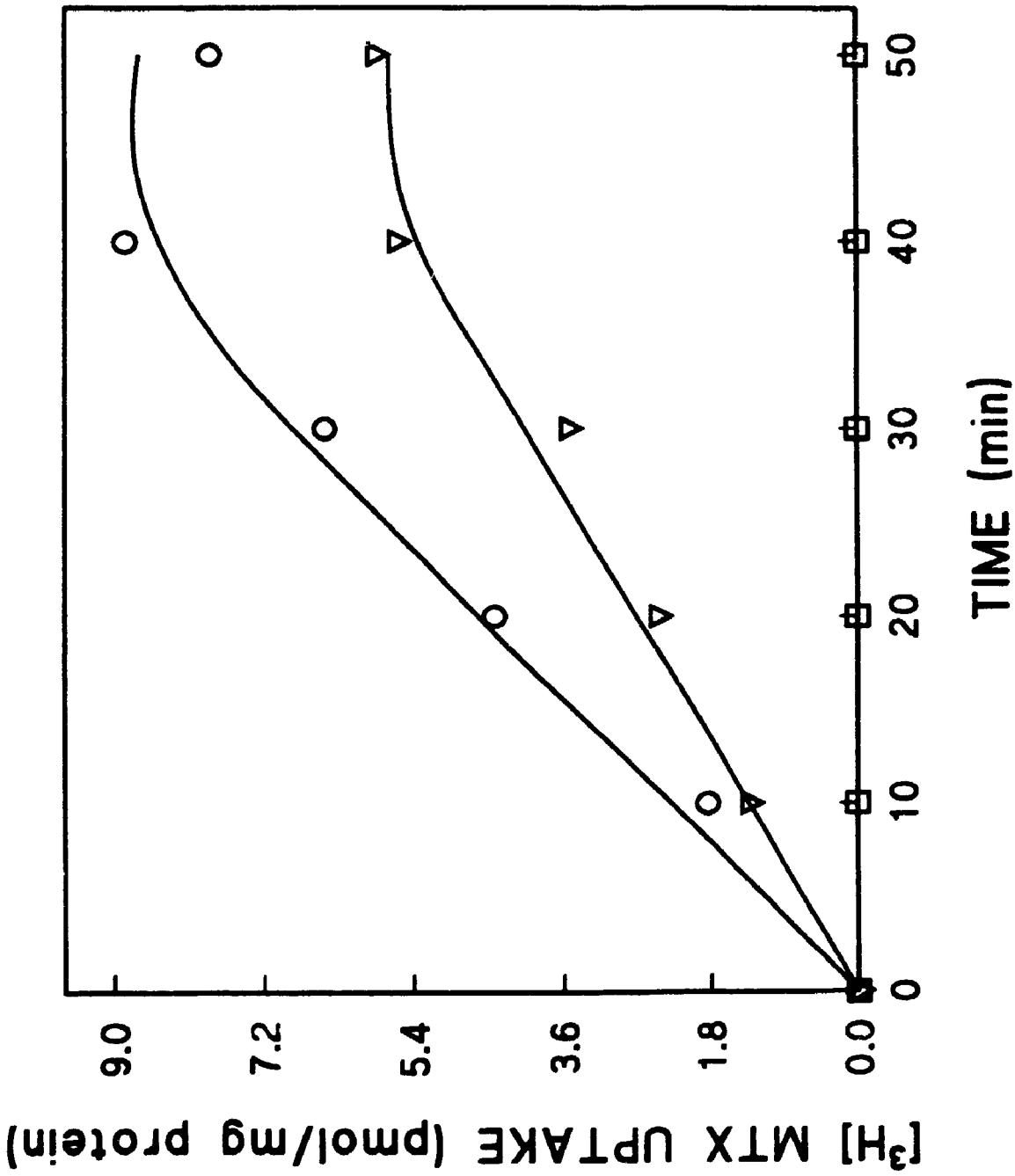
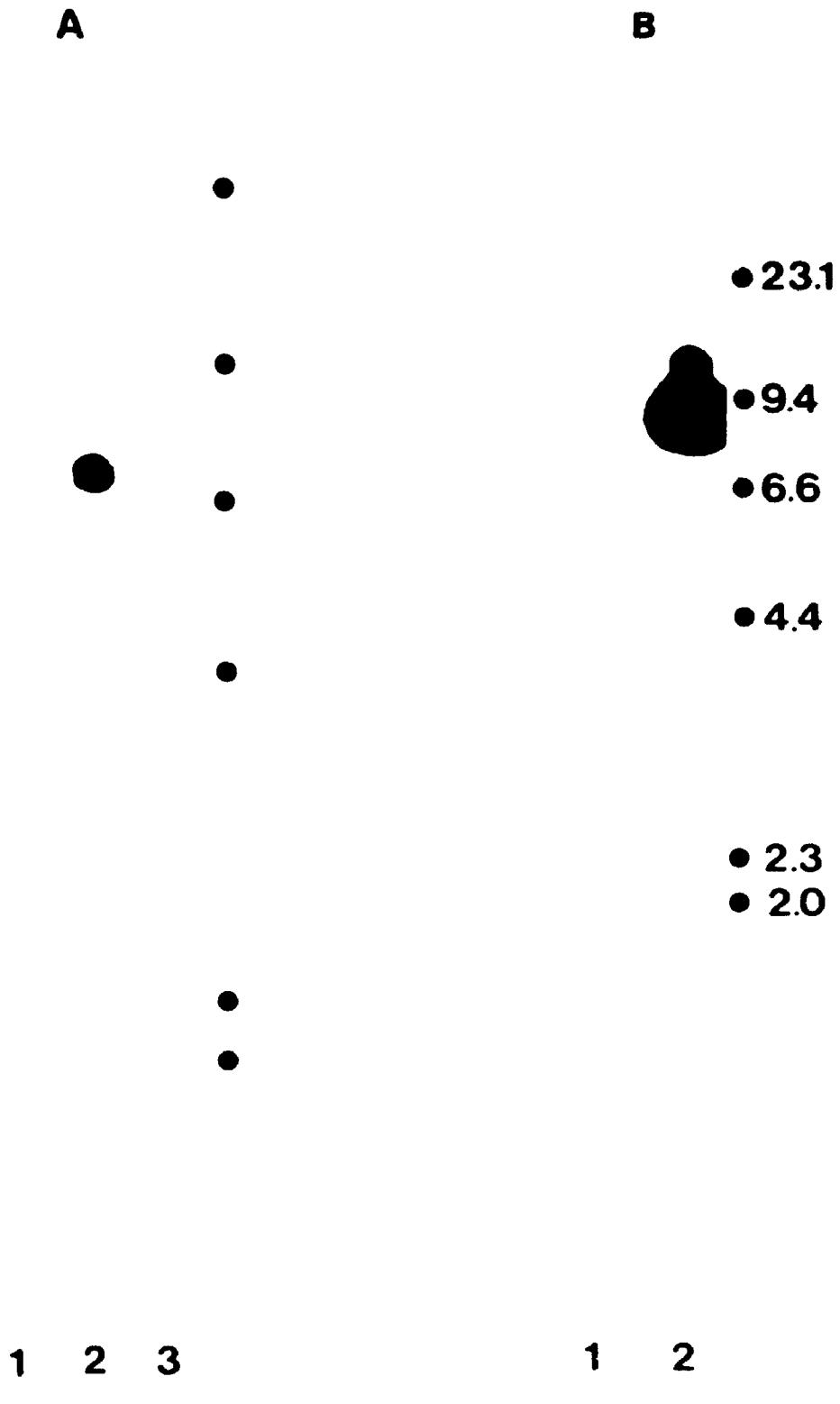

[3H] MTX UPTAKE (pmol/mg protein)
9.0
7.2
5.4
3.6
1.8
0.0
0
10
20
30
40
50
TIME (min)

Figure 4.6 Autoradiogram of Southern hybridizations of wild-type CHO and WETA3 secondary transfectant DNAs with FTMV and neo probes. Approximately 15 ug of DNA from each cell line was digested with Eco RI, electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose and hybridized. A. WETA-3 secondary transfectant DNAs probed with FTMV, lanes: 1, WETA3-5; 2, mtx resistant WETA3-1; 3, wild-type CHO. B WETA3-5 secondary transfectant DNA annealed to radiolabelled Hind III-Bgl II fragment of pSV2neo, lanes: 1, wild-type CHO; 2, WET3A-5. ● Positions of molecular weight markers created by Hind III digestion of phage DNA.


A
B
23.1
9.4
6.6
4.4
2.3
2.0
1 2 3
1 2

were isolated, partly suggests that the complementing sequences may not be tightly linked to the neo sequence. Although to unequivocably confirm this, several more G418 WETA-5 secondary transfectants would have to be isolated and tested.

In order to confirm the identity of the Mtx sensitive secondary WETA3-5, a tertiary transfection was performed with WETA3-5 DNA. Seven G418 resistant colonies were isolated after transfection, however, upon further analysis, none of these were determined to be methotrexate sensitive. The unsuccessful isolation of G418 resistant, methotrexate sensitive tertiary colonies could be for the same reasons as mentioned above. It should be noted that the succesful isolation of secondary and tertiary Mtx sensitive, G418 resistant colonies only establishes that these two functions are linked in the preceding transfectant and has no bearing on linkage in the immediate transfectant.

4.3.5 Isolation of Cosmid Clones From Primary and Secondary Transfectants

As previously mentioned, it had been shown that in WETA-3 a functional neo gene was tightly linked to the sequences that mediated rescue. This observation formed the basis for a strategy designed to recover these sequences from the primary and secondary transfectants. It was determined that cosmid clones containing an intact neo gene

could be isolated by selection for growth on kanamycin, as embedded within the pWE15 neo cassette is the complete neo gene from Tn5 including the promoter sequences necessary for expression in *Escherichia coli* (Southern and Berg, 1982). In this manner, a cosmid cloning protocol was developed for the isolation of functional neo genes, which occasionally would be flanked by sequences which rescue the mutant phenotype. A pWE15 derivative, pWELSV, was designed for this purpose, such that insert sequences could be screened or selected for the presence of neo sequences. It was later determined that pWELSV after transfection or transformation conferred low level resistance to G418 in CHO cells ar o kanamycin in bacteria at a low frequency of approximately 1 X 10^{-5} and 1 X 10^{-7}, respectively. Although in most cases resistance was weak and unstable and true bacteria clones containing a neo gene could be isolated by selection for growth on 50 ug/ml kanamycin or by screening with radiolabelled Hind III-Bgl II fragment from pSV2neo.

Cosmid libraries were prepared in pWELSV from both primary and secondary Mtx sensitive transfectants, WETA-3 and WETA3-5, respectively, and plated onto kanamycin supplemented plates. In an attempt to follow Eco RI fragments identified on Southerns through the cloning procedure, the WETA-3 library was constructed using Eco Rl. Approximately 300 cosmid clones from a WETA-3 cosmid library and 60 from a WETA3-5 library were isolated by

selection on kanamycin. From this number of kanamycin resistant colonies only 12 clones from the WETA-3 library and 4 from the WETA3-5 library were found to contain a *bona fide* neo gene after screening with a radiolabeled neo probe. DNA was isolated from these single cosmid clones and transfected into Mtx RII Oua^R 2-4. These yielded G418 resistant colonies at a frequency comparable to transfection with a control DNA, pWE15. In contrast, no colonies were isolated under low folinic acid selection after transfection with these DNAs. A number of investigators have recently reported success in isolating genes in which only a portion of the gene is contained in any one cosmid (Sekiguchi *et al.*, 1987; Mudgett *et al.*, 1990). In these instances, transfection with pools of cosmids was found necessary for rescue. Mudgett *et al.* (1990) have shown that a functional gene is reconstructed from the introduced cosmids by homologous intercosmid recombination. In order to account for this possibility and hasten screening, subsequent transfections were performed with DNA from pools of 2-3 cosmid clones.

4.3.6 Isolation of Cosmid Clones that Rescue the Mutant Phenotype

Transfection with a pool of 3 cosmids from the WETA-3 cosmid library, 100-1,2,3, yielded 36 G418 resistant colonies (10ug of DNA per 10^5 cells transfected). More importantly, transfection with this pooled DNA produced 9 colonies (10ug of DNA per 10^5 cells transfected) capable of growth in low folinic acid selective medium. This frequency represented an approximate 200-300 fold increase in transfection efficiency, as compared to the frequency of transfection with either genomic or cosmid library DNA. Futhermore, one isolate was examined from this selection and was found to be methotrexate sensitive and G418 resistant. More importantly, transfection with any one of the individual cosmid clones 100-1, 100-2 or 100-3 was sufficient to restore the Mtx sensitive phenotype in the mutant at a frequency similar to that observed with the pooled cosmids (data not shown). These observations strongly suggested that these individual cosmid clones contained the DNA sequences necessary for complementation of the Mtx uptake defect in Mtx RII Oua^R, since sensitivity to Mtx requires that Mtx be transported and accumulated intracellularly.

Restriction endonuclease digestion of DNA from the individual cosmid clones making up pool 100-1,2,3, determined that they overlapped extensively, sharing in

common 41.7 kB of DNA which included 14.5 kB of vector DNA (Figure 4.7). Differences in restriction patterns amongest these clones was limited to the presence or absence of no more than two restriction fragments (Figure 4.7). In addition, the three cosmids contained an Eco RI fragment of approximately 8.5 kB, similar in size to native pWE15, that hybridized to the Hind III-Bgl II fragment of pSV2neo. This vector sequence is flanked by two DNA sequences of 6.6 kB and 20.9 kB that have been found to be common to cosmid clones 100-1, 100-2 and 100-3 (Figure 4.8). At present it is not known which of the flanking DNA sequences contain the complementing sequences. These results have demonstrated that these individual cosmid clones were derived from the primary transfectant and contained the sequences originally transfected into Mtx RII Oua^R 2-4 that restored methotrexate uptake.

No cosmid clones capable of rescuing Mtx RII Oua^R were isolated from the secondary library. Difficulty in isolating cosmid clones from the WETA3-5 secondary library may be the result of weak linkage as evidenced by the difficulty in isolating tertiary Mtx sensitive, G418 resistant transfectants.

4.4 DISCUSSION

DNA-mediated gene transfer has previously been used to isolate genes where little is known about the gene product (Thompson *et al.*, 1990; Troelstra *et al.*, 1990; Weeda *et*

Figure 4.7 Pattern of restriction fragments generated after digestion of cosmid clones 100-1, 2 and 3 with Eco RI and/or Bam Hl. A. Ethidium bromide stained gel of DNA from cosmid clones 100-1, 2, and 3 showing the extent of overlap in the restriction fragments amongest the three clones. Lanes: 2, 5, 8, clones 100-1, 100-2, 100-3 digested with Bam Hl; 3, 6, 9, clones 100-1, 100-2, 100-3 digested with Eco RI; 4, 7, 10, clones digested with Bam HI and Eco RI. Lanes: 1, 11 represent molecular weight markers generated by Hind III digestion of bacteriophage lambda and pWELSV DNA. B. Autoradiogram of the Southern hybridizations of cosmid clones 100-1, 2 and 3 with radiolabeled neo probe, lane assignments are the same as in 4.7A. Positions of molecular weight markers produced by digestion of phage λ with Hind III. A arrow indicates the postion of linearized pWE15 vector. B arrow shows the position of linearized pWELSV.

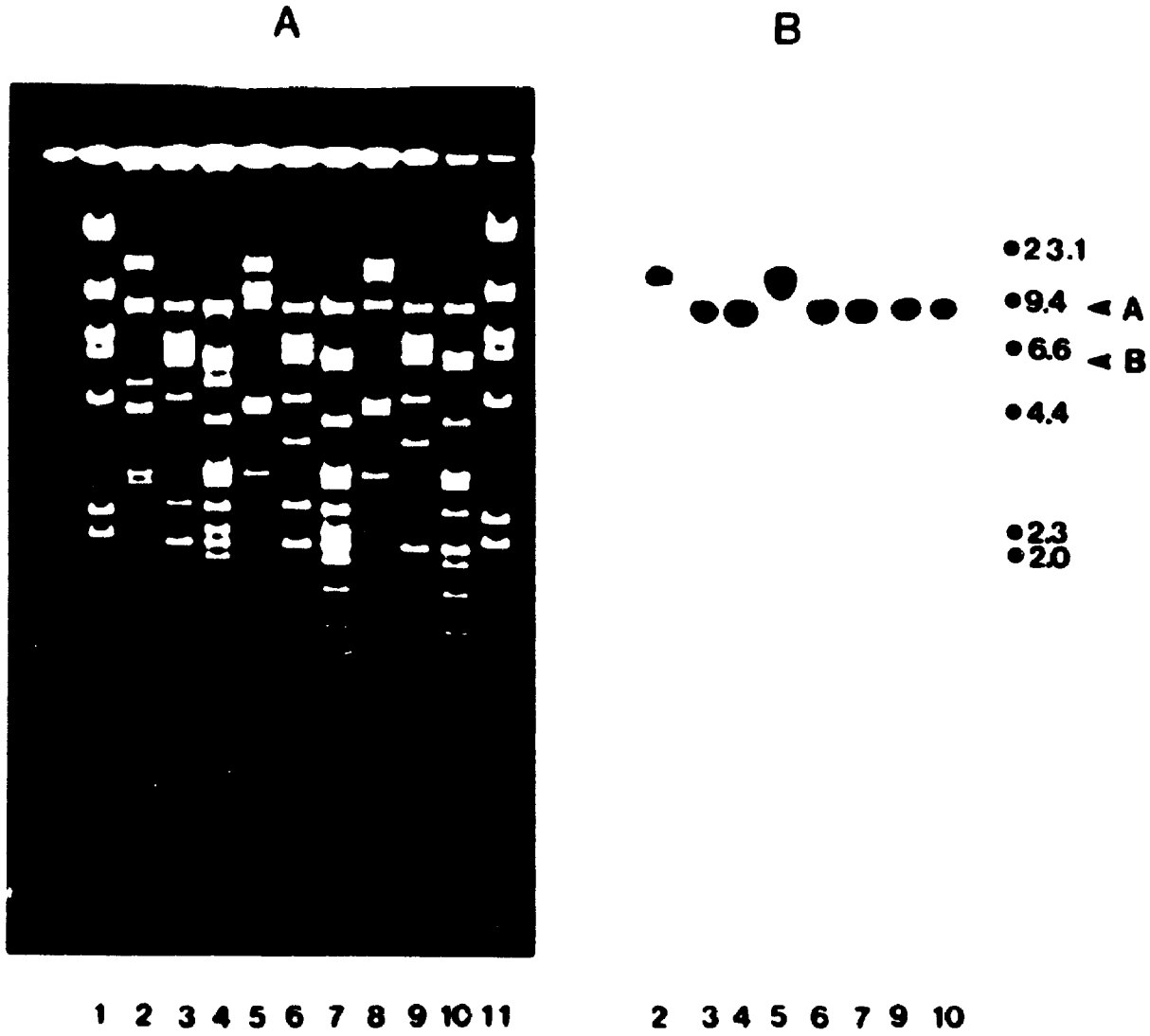
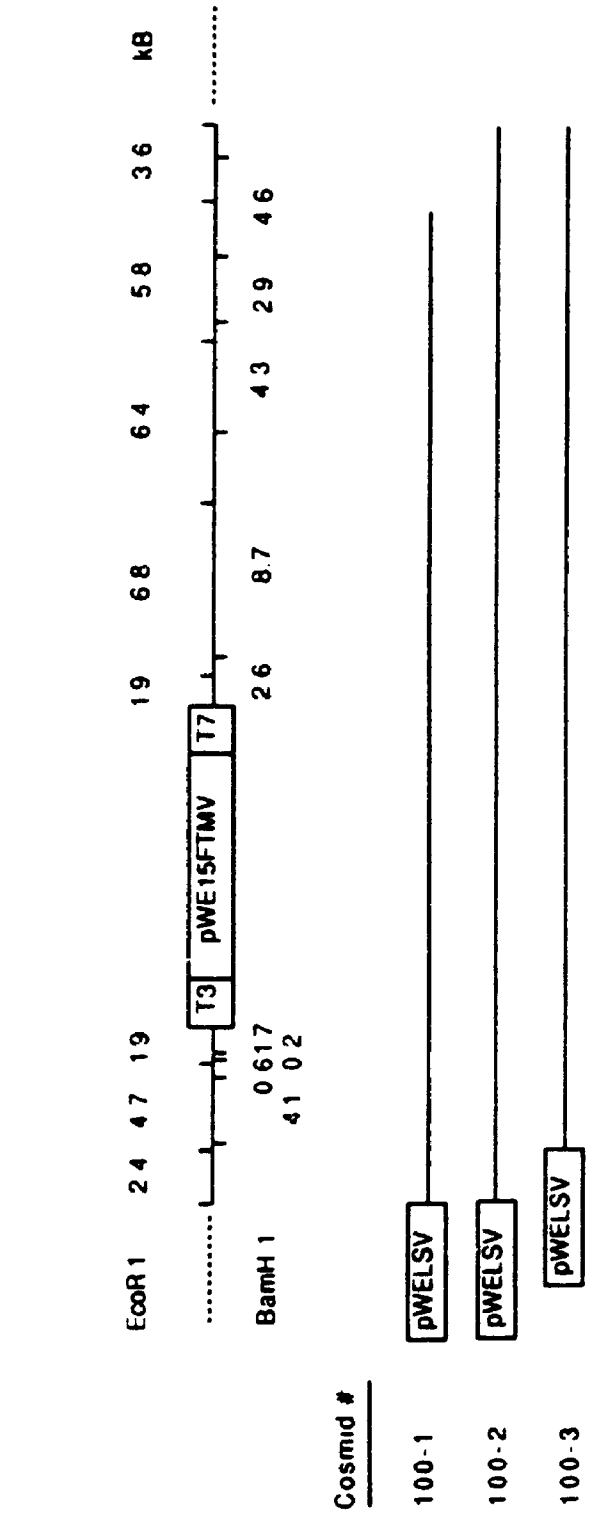

A
B
23.1
9.4
A
6.6
B
4.4
2.3
2.0
1 2 3 4 5 6 7 8 9 10 11
2 3 4 5 6 7 9 10

Figure 4.8 Restriction enzyme site map for WETA-3 derived cosmids. The consensus map of the primary transfectant WETA-3 determined from the overlapping cosmids is shown at the top. The open box diagrams the position of the cosmid vectors in each of the cosmid clones 100-1, 100-2 and 100-3. The numbers above and below the map are site-to-site distances (in kilobases) from single digestions of EcoR 1 (shown on top) and BamH 1 (shown below). The extent of the insert of each cosmid is shown below the map.


EcoR I
24 47 19
19 68 64 58 36
kB
T3
pWE15FTMV
T7
BamH I
0617
4102
26 8.7 43 29 46
Cosmid #
100-1
100-2
100-3
pWELSV
pWELSV
pWELSV

al., 1990; Tanaka *et al.*, 1989; Weber *et. al.*, 1988; Sekiguchi *et. al.*, 1987; Shaham *et. al.*, 1987; Westerveld *et. al.*, 1984; Lowy *et al.*, 1980) but where the genetics of the system are well understood. The success of this procedure hinges on the genetic stability of the recipient being used, ease of selection of transfectants expressing the gene of interest, discrimination of transferred DNA from recipient DNA and a gene no larger than 43 kB for cloning in bacterial systems when using bacterial plasmid and lambda based cosmid vectors. The isolation of an Mtx uptake gene is amenable to such an approach because of the availability of a genetically stable mutant deficient in Mtx uptake and an efficient selection scheme to isolate cells that had regained Mtx uptake. A protocol was thus developed to isolate such an Mtx transport component gene using DMGT (DNA-mediated gene transfer). This protocol was initially shown to be feasible by demonstrating that Mtx competent cells could be isolated from uptake defective mutants after transfection with DNA from either CHO or human cells. Subsequent experiments were designed to facilitate the isolation of DNA sequences that complemented the uptake deficiency in the mutant cell line.

Transfection of DNA from CHO wild-type genomic cosmid libraries into Mtx RII Oua^R 2-4 can rescue the Mtx resistant phenotype via restoration of Mtx uptake. It should be noted, however, that not all colonies isolated after

transfection are Mtx sensitive. Those that are Mtx resistant probably represent spontaneous revertants as their frequency of occurence is similar to the observed spontaneous reversion frequency described previously (Underhill and Flintoff, 1989a.) As expected, transport of Mtx by these transfectants does not always parallel wild-type uptake and this could be due to several factors, some of which may lead to increased or decreased expression of transfected sequences. Furthermore, these uptake properties could be transferred to the Mtx RII Oua^R mutant through a subsequent round of transfection using genomic DNA from an uptake competent primary transfectant. In addition, seven of the eight isolated Mtx sensitive transfectants were found to have originated from a single G418 resistant colony. Mtx sensitivity and uptake in these transfectants is coincident with the presence of transfected sequences. These results taken together confirm that the sequences involved in rescue of the Mtx resistant phenotype of Mtx RII Oua^R 2-4 are tightly associated with vector sequences in the primary transfectant.

A cloning strategy was developed that entailed selection of putative positive cosmid clones from primary and secondary transfectant libraries based on the presence of a functional neo gene and kanamycin selection. A number of kanamycin resistant colonies were isolated and only a subset of these were found to actually harbor a neo gene.

Transfection with a pool of three of these clones rescued the Mtx resistant phenotype of Mtx RII Oua^R 2-4 at high frequency as did transfection with single cosmid clones from the pool. Subsequent restriction endonuclease pattern analysis determined that these three cosmids represented overlapping clones, which originated from the primary transfectant. These data strongly support the existence of a methotrexate uptake gene within these cosmids, but further analysis of uptake function and transfection frequencies of single cosmids would substantiate the validity of these cosmid clones. Hence, the cloning strategy described herein appears to have utility for the relatively rapid isolation of genes by DNA mediated gene transfer.

A strategy based on transfection with pooled cosmid library DNA was designed to overcome some of the problems associated with other DMGT methods which rely on transfection with heterologous DNA, DNA ligated to or cotransfected with an identifiable sequence, and cDNA expression libraries. Cosmid libraries in pWE15FTMV were chosen as a DNA vehicle for DMGT because: one, genomic sequences are tightly associated with the unique identifier sequence FTMV; two, those cells which have been transfected can be selected from the total population of cells; three, the structure of the integrated sequences can in part be determined; four, it is possible to quickly and rapidly screen large cosmid libraries for potentially rescuable

cosmid clones; five, point four makes it feasible to screen primary libraries for cosmid clones that rescue. There are two inherent problems that this strategy shares with others. These are 1) cloning genomic DNA in cosmid vectors, the gene can realistically be no larger than 30 kB and 2) some type of linkage to an identifiable sequence has to be maintained during transfection for subsequent isolation. By adapting the methodology of this cloning procedure to the P1 phage system (Sternberg, 1990), which enables the propagation of 100 kB fragments of DNA in bacteria, it should be possible to overcome the first limitation. Unfortunately, the requirement for linkage in molecular cloning with DMGT is unavoidable.

With the availability of a cloned gene for Mtx uptake it will be possible to address the nature of the defect in Mtx RII Oua^{R} 2-4 in addition to defining the molecular constituents of folate transport.

CHAPTER 5

Discussion

5.1 Overview

The transport of folates across the mammalian plasma membrane appears to be more complicated than originally hypothesized. Much evidence has accumulated recently to support the existence of multiple influx routes for folates. Most of this information came from traditional biochemical strategies which were employed to isolate and characterize the component(s) involved in the intracellular accumulation of extracellular folates. These techniques have been instrumental in identifying proteins that bind folic acid and/or reduced folates, although their functional role has remained somewhat elusive.

Traditionally, genetic analysis of mutants has been very useful in functionally resolving complex multi-component systems in mammalian cells. In addition, mutants provide a basis for comparison in studying normal processes within a cell as they provide a cellular hallmark for the identification of components through correlation of a physical defect with a functional abnormality. In order to resolve some of the intricacies of folate transport in mammalian cells, we have utilized a genetic approach. Firstly, the phenotypic characterization of mutants deficient in Mtx transport sheds light on the cooperative nature of the components that mediate folate transport in CHO cells. Secondly, a strategy has been developed for the isolation of a gene involved in Mtx transport based in part

on the phenotypic properties of the aforementioned mutants.

5.2 Cooperativity in Folate Transport

Recently, a lot of evidence has accumulated to support the presence of distinct components that mediate folic acid and Mtx uptake, although there may be some functional overlap in these various components. The results presented in this thesis confirm these findings and provide further evidence for cooperation between the systems that mediate folate transport.

Initial studies, described in chapter 2, involved the genetic analysis of mutants deficient in Mtx transport, which are briefly summarized here and in table 5.1. In the absence of exogeneous folates, these mutants require 100-250 times the level of folinic acid as wild-type cells to support growth at a similar level. Two classes of mutants were distinguishable by their revertability for growth in folinic acid. Revertants derived from one class were similar to wild-type cells in their ability to grow in medium containing low levels of folinic acid, in their sensitivity to Mtx and in their Mtx kinetic transport parameters. In contrast, partial revertants from a second class were able to grow in medium containing abnormally low concentrations of folic or folinic acid, but retained their Mtx resistant phenotype. Futhermore, mutants of the first class were unable to take-up folic acid while the second

Table 5.1

Summary of the phenotypic uptake properties of the mutants, revertants and pseudorevertants.

Cell Line	$D_{\frac{1}{2}o}$ Mtx (nM)	Mtx Uptake pmol/mg protein @ 30'	Folic Acid Uptake pmol/mg protein @ 30'
WT	20	5.19	0.13
Mtx RII 5-3	800	>.1	>.01
Mtx RII Oua^R 2-4	600	>.1	0.10
Rev Mtx RII 1	20	3.87	0.24
Rev Mtx RII 2	20	3.31	n.d.
Mtx RII Oua^R F1	500	>.1	0.59
Mtx RII Oua^R F2	600	n.d.	n.d.

class of mutants accumulated folic acid to levels similar to wild-type cells. Somatic cell hybrids formed between these two classes were non-complementing. These observations suggested that some, but not all, components may be shared between the transport systems mediating Mtx and folic acid uptake. Practically, this statement can be interpreted in one of two ways: there is a single component with distinct domains that mediates each flux or there are separate components for each flux which interact or cooperate. In light of recent evidence, this data appears to be consistent with the latter interpretation.

It is difficult to imagine that a single route mediates folate uptake within CHO cells after examining the phenotypic properties of the folic acid uptake competent mutant, Mtx RII Oua^{R} 2-4. This mutant appears to have no apparent change in folic acid uptake, while Mtx uptake is severely incapacitated. Secondly, partial revertants selected from this mutant for growth on low folinic acid are unchanged for Mtx uptake while displaying grossly altered folic acid uptake. These observations are consistent with the present hypothesis, that oxidized and reduced folates are taken-up by distinct systems in mammalian cells, although there may be some functional overlap. Folic acid enters the cell predominantly through a high-capacity, low-affinity system which may involve receptor endocytosis. On the other hand, reduced folates are transported by a high

affinity, low capacity classical carrier which requires energy and involves anion exchange.

Conversely, the phenotypic properties of the first class of mutants taken together with the somatic cell hybrid data strongly suggest some type of cooperative role or identity for the components that mediate folic acid and Mtx uptake. Revertants selected from a Mtx and folic acid uptake deficient mutant, Mtx RII 5-3 were observed to have simultaneously regained Mtx and folic acid uptake and futhermore, Mtx uptake kinetics mirrored that of wild-type. These observations argue strongly for some level of cooperation between the factors that mediate the influx of oxidized and reduced folates.

The level of cooperativity between the different uptake systems may in part be influenced by both the folate composition and concentration of the external environment and only experimentally evident at low folate concentrations, below 1 uM. At high folate concentrations, any transfer of folate from the FBP to the transporter is masked by the amount of folate entering the cells through receptor endocytosis (Kamen *et al.*, 1986). At low concentrations of folate, the vast majority of FBP's will be unoccupied, hence receptor endocytosis may be rather inefficient in comparison to transport or transfer to the reduced carrier system. Recent evidence from observations reported by Kamen *et al.* (1991) in a monkey kidney

epithelial cell line, MA104 cells, directly supports this hypothesis. They have found that treatment of MA104 cells with probenicid or low temperatures effectively blocks the transmembrane movement of folate into the cytoplasm without affecting binding to the folate binding protein, receptor or internalization of the folate-receptor complex. Probenecid and low temperatures have been previously shown to effectively inhibit uptake of folates through the reduced folate anion exchange transporter (Henderson and Zevely 1985a). In addition, they noted that the folate receptor appeared to be absolutely necessary for transport of folates at low concentrations, and at these concentrations the apparent requirement for coupling may be exacerbated due to the likely dramatic reduction in transport through receptor endocytosis. Furthermore, it has been postulated that for this model to be realistic, the two molecules that mediate folate uptake must be spatially arranged so that they are contiguous in the plane of the lipid bilayer (Kamen *et al.*, 1991). This appears to contradict recent morphological evidence which suggested the caveola, or uncoated pit, as the vehicle by which folate receptor is internalized by receptor mediated endocytosis (Rothberg *et al.*, 1990). There is no reason to believe that the two mechanisms for transport cannot act in concert and they may have some bearing on the mechanism by which cells become resistant to Mtx by reduced transport.

5.3 Mtx Resistance via Reduced Transport

Cell lines that are Mtx resistant via reduced drug uptake almost invariably require substantially higher concentrations of folinic acid or reduced folates and to a lesser extent folic acid to maintain adequate growth (Sirotnak, 1985; Kano *et al.*, 1986; Underhill and Flintoff, 1989a). The growth of these cell lines is only supported on folate sources that are much higher than physiologically available. So the question arises, how do these cell lines meet their folate requirements in vivo at low folate concentrations and if they cannot meet their folate requirements are they selected against? Sirotnak *et al.* (1985) elegantly showed that when Mtx resistant, Mtx uptake defective L1210 cells were introduced into mice they were unable to form leukemias without administration of high doses of folic acid. The development of resistance afforded the cells with the ability to survive in the presence of Mtx, but in the process they lost the ability to accumulate sufficient amounts of folate to survive. So herein, lies the paradox, the development of resistance via reduced transport appears to be superceded by starvation. Eventually, these cells will be selected against, unless of course there is a second change which enables them to grow on physiological levels of folates while retaining their Mtx resistant phenotype. The Mtx RII 2-4 Oua^R cell line supports this latter hypothesis, in that Mtx resistance can

be retained simultaneously with the ability to accumulate sufficient folates in concentrations which do not even support the growth of wild-type cells. Hence, there are most likely two changes, one that confers the cells with Mtx resistance via reduced uptake and the second which endows the cells with the ability to flourish in physiological levels of folate. The second change may be a natural consequence of tumor progression. It is generally accepted that as cells move from a normal state through tumorgenesis to a neoplastic state there are wholesale changes in gene expression which may lead to expression of alternate folate transport systems (Sirotnak, 1985). This may prove to be useful as a tumor specific cell marker.

5.4 Future Direction

Many questions remain unanswered with regard to folate transport in mammals, some of which are listed below:

1) What contribution do the individual components make to folate transport in neoplastic and normal cells?

2) What role do the two components play in acquired drug resistant to Mtx?

3) What is the nature of the molecular changes which give rise to reduced transport of Mtx?

4) What changes occur to enable Mtx uptake defective cells to survive <u>in vivo</u>?

5) How are the genes for FBP and the Mtx transporter

regulated?

6) Do tumor cells express a subset of folate components normally found only on cells of fetal origin?

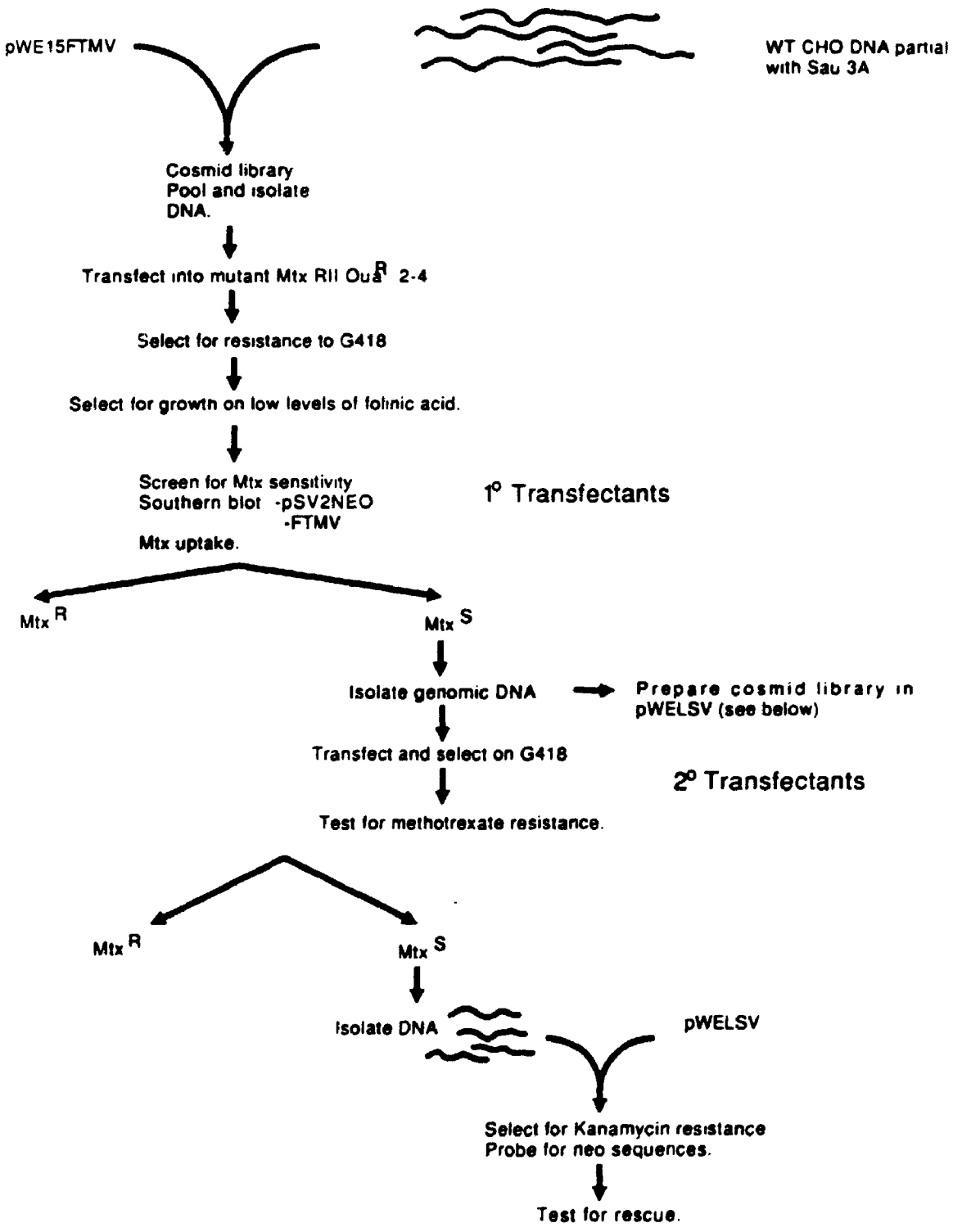
7) What is the gene sequence and structure of the Mtx transporter and is it in any way similar to FBP?

The analysis of mutants deficient in Mtx transport has made significant contributions to our understanding of how folates are transported in mammalian cells.

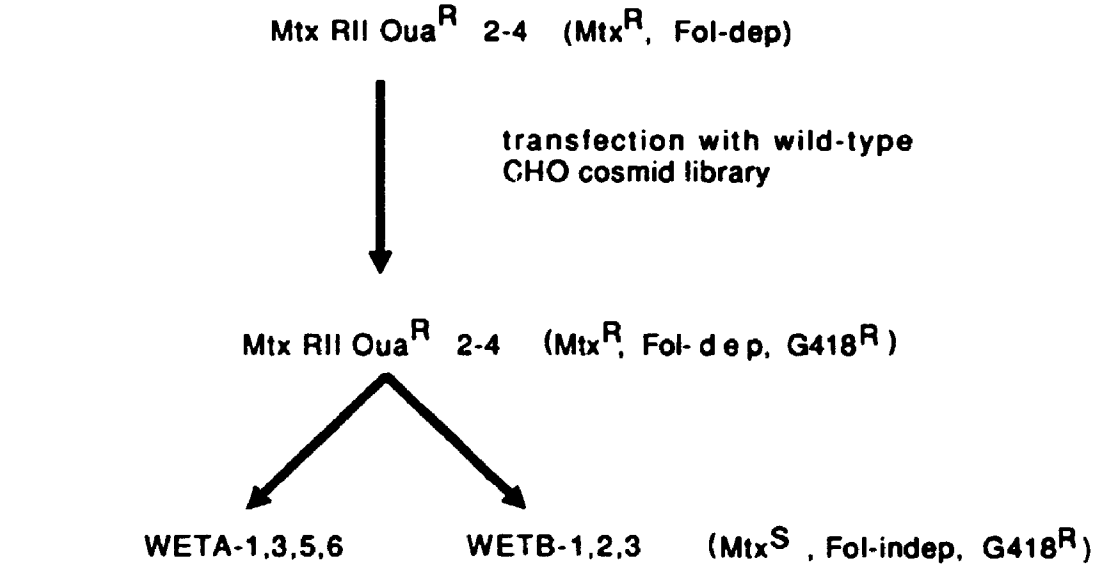
With the molecular probes in hand for both the FBP and reduced folate transporter (described herein) it will be possible to discern the role each folate transport system plays in cellular maintenance and drug resistance.

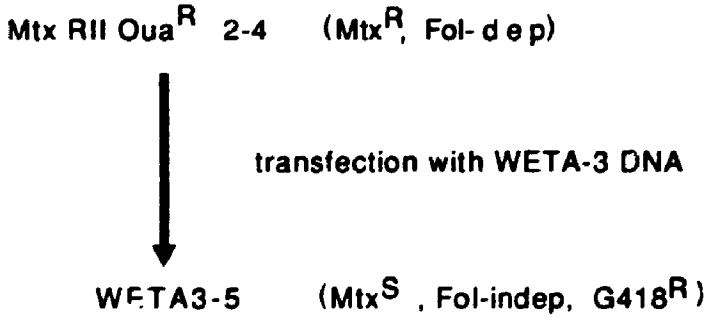
APPENDICES

Appendix 1: Schematic outline of the experimental steps involved in isolating and identifying cosmid insert sequences which complement the methotrexate resistant phenotype of Mtx RII Oua^R 2-4 cell line. Briefly, a wild-type CHO cosmid library was prepared in pWE15FTMV, DNA was isolated and transfected into the Mtx RII Oua^R 2-4 mutant cell line. Transfectants were initially selected by resistance to G418 and subsequently selected for the ability to grow on low levels of folinic acid. Primary transfectants which were isolated after low folinic acid selection were tested for Mtx resistance, uptake of Mtx and the presence of vector specific sequences (FTMV and neo). DNA was isolated from Mtx sensitive primary transfectants and used for the construction of a cosmid library in pWELSV and also as a source of DNA for transfections into the Mtx RII Oua^R 2-4 mutant. Mtx sensitive secondary transfectants were isolated in a analogous manner to that of the primary transfectants and genomic DNA from these secondary transfectants was used to make a cosmid library in pWELSV. Cosmid libraries made from primary and secondary transfectants were selected on kanamycin and then screened for the presence of neo sequences. Those cosmid clones that were found to contain neo sequences from pWE15 were tested for the ability to rescue the mutant.


pWE15FTMV
WT CHO DNA partial with Sau 3A
Cosmid library Pool and isolate DNA.
Transfect into mutant Mtx RII Oua R 2-4
Select for resistance to G418
Select for growth on low levels of folinic acid.
Screen for Mtx sensitivity
Southern blot -pSV2NEO
-FTMV
Mtx uptake.
1° Transfectants
Mtx R
Mtx S
Isolate genomic DNA
Prepare cosmid library in pWELSV (see below)
Transfect and select on G418
2° Transfectants
Test for methotrexate resistance.
Mtx R
Mtx S
Isolate DNA
pWELSV
Select for Kanamycin resistance
Probe for neo sequences.
Test for rescue.

Appendix 2: Pedigree of the cell lines that were generated by transfection with DNA from cosmid libraries. The genotypes of each line are contained within parentheses. The genotype designations are: Mtx^S, methotrexate sensitive; Mtx^R, methotrexate resistant; Fol-dep, requires high levels of folinic acid for growth; Fol-indep, able to grow in medium supplemented with low levels of folinic acid (2×10^{-5}); $G418^R$, G418 resistant.


Mtx RII Oua^R 2-4 (Mtx^R, Fol-dep)
transfection with wild-type
CHO cosmid library
Mtx RII Oua^R 2-4 (Mtx^R, Fol-dep, G418^R)
WETA-1,3,5,6
WETB-1,2,3
(Mtx^S, Fol-indep, G418^R)


Mtx RII Oua^R 2-4 (Mtx^R, Fol-dep)
transfection with WETA-3 DNA
WETA3-5
(Mtx^S, Fol-indep, G418^R)

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