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(54) **Title:** PROTECTION OF HUMAN BONE MARROW FROM HIGH DOSE ANTIFOLATE THERAPY USING MUTATED HUMAN DIHYDROFOLATE REDUCTASE DNA

(57) **Abstract**

This invention provides a DNA vector which comprises DNA encoding a mutant, antifolate resistant, dihydrofolate reductase inserted into a site within the vector, the presence of which site is not essential for replication of the vector. This invention also provides bone marrow cells which comprise the above vector. Finally, this invention provides a method for reducing the toxic effects of antifolate therapy in a subject which comprises replacing the subject's hematopoietic cells with hematopoietic cells which comprise the above-described vector so as to reduce the toxic effects of antifolate therapy in the subject.

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PROTECTION OF HUMAN BONE MARROW FROM HIGH DOSE ANTIFOLATE THERAPY USING MUTATED HUMAN DIHYDROFOLATE REDUCTASE DNA

The invention described herein was made in the course of work under grant number CA-08010 from the National Institute of Health, U.S. Department of Health and Human Services. The U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced by arabic numerals within parentheses. Full citations for these publications may be found at the end of each series of experiments. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Dihydrofolate Reductase and Methotrexate

Dihydrofolate reductase (DHFR, 5,6,7,8-tetrahydrofolate: NADP + oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, an essential carrier of one-carbon units in the biosynthesis of thymidylate, purine nucleotides, serine and methyl compounds (Blakly,1969, Figure 1). It is an essential enzyme in both eukaryotes and prokaryotes.

In rapidly dividing cells, the inhibition of DHFR results in the depletion of cellular tetrahydrofolates, inhibition of DNA synthesis, and cell death. Folate analogs such as methotrexate (MTX) inhibit DHFR and are

thus used as antineoplastic agents. A powerful inhibitor of DHFR, methotrexate (MTX) (Figure 2A), has become a widely used antineoplastic agent in the clinic (Bleyer et al., 1978).

The clinical value of MTX treatment is limited by two problems that represent the major obstacles to the effective treatment of neoplastic disease with MTX as well as with most other antineoplastic agents: the rapid development of resistance in tumors and toxicity in normal tissue.

Mechanisms of MTX resistance

Resistance to MTX can be either natural or can be acquired after initial response to the drug. It has long been observed that some types of tumors are responsive to MTX treatment while others are intrinsically resistant to the treatment. One possible explanation is the differences in the ability to transport MTX into the cell (Bertino et al., 1985). An intrinsically poor ability to transport the drug into the cell may confer natural resistance to MTX. After entering the cell MTX is converted intracellularly to polyglutamylated forms by the folylpolyglutamyl synthetase. Compared with the non-polyglutamylated form of MTX, the polyglutamylated form is less ready to be effluxed from the cell, more likely to retained in the cell, and therefore may be accumulated at higher intracellular concentrations. The polyglutamylated forms also have higher affinity for other enzymes in the 1-carbon folate-dependent pathway (Chabner et al., 1985) and can be stored in the cell to exert its cytotoxicity when the cell enters the S-phase (McGuire et al., 1985). A decrease in polyglutamylation has been correlated with the natural MTX resistance of

certain tumors (Curt et al 1985; Pizzorno et al., 1989; Li et al., 1989).

Amplification of the DHFR gene resulting in increased levels of the enzyme has been identified as one mechanism of acquired MTX resistance (Carman et al., 1984; Horns et al., 1984). Defects in MTX transport and polyglutamylation have also been found responsible for some clinical cases of acquired MTX resistance as well as in experimental systems (Sirotnak et al., 1981, Pizzorno et al., 1988).

Mutations in the target enzyme of DHFR resulting in a decreased affinity for MTX is a mechanism for acquired resistance to MTX (see review by Simonsen, 1986). Several mutant DHFRs have been characterized in the past few years. Some mutant DHFR enzymes have mutations in the active site of the enzyme which results in both reduced affinity for antifolate as well as a decrease in catalytic efficiency of the mutant enzyme as compared to the wild type protein (Thillet et al., 1988; Schweitzer et al., 1989; Prendergast et al., 1989).

The first mutant murine DHFR (mDHFR) was cloned from a MTX resistant cell line 3T6-R400 (Simonsen and Levinson, 1983). The mutant DHFR contained a G to T point mutation at nucleotide 68 resulting in a Leu to Arg change at residue 22. Leu 22 is normally involved in hydrophobic contacts with the substrate or inhibitors and play a critical role in the function of DHFR. The presence of a charged residue at this position considerably reduced the catalytic activity of the enzyme and the binding of MTX. Arg would protrude in the active site more than the wild-type Leu residue and could hinder the positioning of inhibitors either by its steric effect or by allowing the

penetration of water molecules in the active site. The mutant enzyme exhibited a 70 fold reduction in catalytic efficiency and a 7.5 x 10^5 fold increase in MTX Ki as compared to the wild type enzyme (Haber et al., 1981; Thillet et al., 1988). When the cDNA of the mutant enzyme was transfected into the parental cells, the mutant enzyme was able to confer MTX resistance to the transfected cells which also contain the wild type DHFR. The ability of the 3T6 enzyme to act as a dominant selective marker has been demonstrated in murine, hamster and human cells (Simonsen and Levinson, 1983; Isola et al., 1986; Banerjee et al., 1994; see review by Simonsen, 1986). The first mutant human DHFR (hDHFR) was isolated from a MTX resistant HCT-8 human colon carcinoma cell line (Srimatkandada, et al., 1989; Schweitzer, et al., 1989). The phenylalanine at residue 31 of the hDHFR was replaced by a serine in this MTX resistant cell line. The Serine 31 mutant has a 2 fold decrease in catalytic efficiency and a 100 fold increase in MTX Kd. This mutant enzyme was able to confer MTX resistance to cells containing wild-type DHFR when the cDNA of the mutant hDHFR was transfected into these cells (Banerjee, et al., 1994). Molecular modeling studies have shown that Phe at position 31 interacts with the p-aminobenzoyl glutamate portion of MTX or folate (Oefner et al., 1988). Thus substituting a large hydrophobic group with a small hydrophilic group has profound effects on MTX binding. Another Phe occurs at position 34 and is also an active site residue, which makes van der Waals contact with peteridine portion and a part of the p-aminobenzoyl group of the ligands. Site directed mutagenesis at this residue with a Ser substitution generated a hDHFR mutant which has a 3 fold reduction in catalytic efficiency and a 8 x 10^4 increase in MTX Kd (Schweitzer et al., 1989). Other mutations in the DHFR were reported in experimental

systems to facilitate the study of the precise nature of the mutation (see review by Schweitzer et al., 1990).

MTX toxicity

Anorexia, progressive weight loss, bloody diarrhea, and leukopenia are the outstanding features of lethal doses of MTX. The major lesions occur in the intestinal tract and bone marrow. Swelling and cytoplasmic vacuolization of the mucosal cells of the intestinal epithelium is followed by desquamation of epithelial cells, extrusion of plasma into the lumen of the bowel, and leukocytic infiltration of the submucosa. Terminally, the entire intestinal tract exhibits a severe hemorrhagic desquamating enteritis. Degeneration of bone marrow develops rapidly. Proliferation of erythroid precursors is inhibited, and significant proportions of primitive erythroid elements have the appearance of megaloblasts. Rapid pathological alteration in myelopoiesis also occurs, and within a few days the bone marrow becomes aplastic. The disturbance in hematopoiesis is reflected in the circulating blood by a marked granulocytopenia and reticulocytopenia and a moderate lymphopenia (Goodman and Gilman, 1980).

Gene Therapy: Current Status

Gene therapy is defined as the transfer of genetic material into the cells of an organism to treat disease. There are many potential applications of this technique to the treatment of numerous hereditary diseases caused by defects in single genes (see review by Miller, 1990). In addition, gene therapy may be useful for acquired diseases, such as cancer or infectious disease. Achievement of efficient gene transfer and persistent

gene expression is the major focus of current research.

Specific gene therapy has been accomplished in cultured cells by homologous recombination of added DNA with endogenous sequences to target genes to specific sites within the genome (Smithies et al., 1985; Thomas and Capecchi, 1987). While this technique has promise for ultimate application to gene therapy, practical considerations, such as the finite life span of normal somatic cells or the inability to isolate or grow the relevant transplantable cells, presently limit its use.

An alternative to gene replacement is the addition of genes to correct a disease, namely gene addition therapy. Gene addition therapy can engineer a cell to express a new gene which the cell does not normally express. This method is currently the most practical approach to gene therapy due to the development in methodology for highly efficient gene delivery with retroviral vectors (see below). It has been found to be useful in application involving acquired as well as hereditary diseases (Sarver et al., 1990; Sullenger et al., 1990; Gansbacher et al., 1990, see review by Anderson, 1992). One potential problematic aspect of gene addition therapy is the random insertion of genes into the genome which may lead to the inappropriate expression of the inserted gene or the genes near the insertion site.

Different methods of gene transfer have been employed in the past. A method of DNA transfection employs purified DNA co-precipitated with calcium phosphate or dextran sulfate and brought into direct contact with the cells. The precipitated DNA on the cell surface is then endocytosed into the cells by uncharacterized pathways (Wigler et al., 1977). The efficiency of the

transfection is very low, a maximum 1% of the cells will have incorporated the transferred DNA. Usually multiple copies of the gene in tandem repeats are integrated into the host genome, which may result in uncontrollable overexpression of the transfected gene, or interruption of the normal chromosome structure. The host range of the DNA transfection is limited to a small number of cultured cell lines, while a majority of primary culture cells either can not stand the toxicity of the method or is not susceptible to it. A few other techniques have been developed using physical means to introduce genes into cells: protoplast fusion (Schaffner, 1980), in which bacteria containing recombinant DNA are fused with eukaryotic cells, resulting in the transfer of DNA from the cytoplasm of the bacteria into the host cell; lipofection (Felgner et al., 1987), in which the positively charged lipids in liposomes complex with DNA and the lipid-DNA complex fuses with plasma membranes and transfer the DNA into the cells; and electroporation (Potter et al., 1984), in which DNA is electrophoretically transferred across the host cell membrane into the cell though pores open up by the electric field. These techniques broaden the host range of susceptible cells, but still have a very low efficiency of gene transfer. Microinjection, in which DNA is injected directly into the nucleus of the cell, results in stable integration of DNA in a large percentage of injected cells although the method is very time consuming and the number of transformed cells is limited by the cells that can be injected.

Gene transfer procedures based on SV40, polyoma, adenovirus vectors, and vaccinia based vectors lead to efficient but transient expression of the transduced gene. Bovine papillomavirus (BPV) (Sarver et al., 1982;

Dimaio et al., 1982), and Epstein-Barr virus (EBV) (Yates et al., 1984) based vectors have only limited host ranges even though they result in stable expression of the transduced gene carried as an episome in multiple copies per cell. Adeno associated virus (AAV) based vectors (Hermonat and Muzyczka, 1984; Tratschin et al., 1985) integrate into the chromosome of the host cell but the full potential of this system needs to be further explored. These viral vectors have not been shown to transduce hematopoietic stem cells effectively (see review by Karlsson, 1991). A new system for delivering genes to cells, which relies on an antibody molecule and a chain of amino acid units to hook DNA to the outside of adenovirus, has been reported lately (Curiel et al., 1992). But the stability of the expression of the transduced gene and the ability of the system to express the transduced gene in vivo remains to be seen.

In the past several years, retroviral-mediated gene transfer, in which the genes are delivered into the cells by retroviruses, has emerged as superior to other techniques explored in gene therapy.

Retroviral Vector Mediated Gene Transfer

Retroviral life cycle

Retroviruses are animal viruses which contain a viral RNA genome which is replicated through a DNA intermediate. Moloney murine leukemia virus (MoMLV) is an ecotropic murine leukemia retrovirus which replicates well in only mouse and rat cells. The retroviral virion contains two copies of the retroviral RNA genome (Kung et al., 1976; Bender and Davidson, 1976; Bender et al., 1978) associated with the gag and pol gene products in an

icosohedral viral core structure which is surrounded by a lipid bilayer (derived from the previously infected host cell). The viral encoded env gene products are embedded in the lipid bilayer (Varmus and Swanstrom, 1984). The interaction of env protein molecules of the virion particle with a cell surface protein on the target cell membrane results in the penetration of the virus into the cell (Figure 3). After penetration the viral RNA genome is released into the cytoplasm and is reverse transcribed into a double-stranded DNA form by the viral encoded RNA dependent DNA polymerase, the reverse transcriptase (Baltimore, 1970; Temin and Mizutani, 1970). This viral DNA migrates to the nucleus and integrates into one of the host's chromosomes to form the provirus. This integrated provirus is the DNA template responsible for the expression of the viral gag, pol and env genes as well as the virion RNA (Varmus and Swanstrom, 1984). In the infected cell the viral RNA is preferentially packaged into the virion particles. This specificity is mediated by an RNA sequence on the viral RNA called the packaging signal (Mann et al., 1983). Integration of the viral genome into the cell chromosome and the formation of subsequent virus usually has no deleterious affect upon the host cell. Thus cells harboring an unrearranged MoMLV provirus are normal and healthy, and continually secrete progeny virus into the surrounding medium.

MoMLV based retroviral vectors

The MoMLV genome encodes three genes, the gag, pol and env genes, whose protein products are needed *in trans* for the replication of the virus, as well as several DNA and RNA elements required *in cis* for the replication of the virus. These *cis* elements include: the viral long

terminal repeats (LTRs) which are required for transcription, transcription termination and polyadenylation; the viral RNA packaging signal which is required for efficient packaging of the viral RNA into virions; and primer binding sites (PBS) required for reverse transcription of the viral RNA to DNA.

The basic principle of a MoMLV based retroviral vector is to remove the sequences of the genome which are required *in trans* and replace them with foreign sequences of interest, while retaining all *cis* sequences necessary for viral replication. The hybrid DNA is then introduced into specially designed packaging cells, which harbor a retrovirus defective in *cis* function. Its RNA cannot be encapsulated into a virion but it can express all the viral proteins and is therefore able to complement the *trans* functions missing in the incoming hybrid vector DNA. The vector DNA is then reverse transcribed into a corresponding RNA which is encapsulated into a retrovirus virion, infectious but replication defective (Temin, 1986; Gilboa, et al., 1986). Such packaging can generate virus containing vector RNA with a fairly high titer of up to 10^6 infectious units/ml (Armentano et al., 1987; Markowitz et al., 1988a, 1988b). The packaging of a retroviral vector in an amphotropic based packaging cell line allows for the generation of amphotropic virus able to infect a wide range of cell types. Through the efficient viral infection process the foreign gene is inserted into the cell chromosome as if it were a viral gene (Figure 4).

Various retroviral vector designs have been utilized in an attempt to increase the titer of the vector containing virus coming from a packaging cell line as well as to increase the fidelity of expression of the transferred

genes after infection (see review by Gilboa, 1987). Among them is the design of vectors with internal promoters (VIP) (Figure 5A). In these vectors a selectable gene is expressed from the viral LTR promoter. The gene of interest is fused to another DNA fragment containing a promoter which is responsible for its expression. These vectors possess the flexibility of choosing a promoter to express the transduced gene most appropriate for a particular target cell (Enerman and Temin, 1984; Miller, et al., 1984). However, positioning a transcription unit within another active transcription unit often leads to the occlusion of the internal transcription unit (Cullen et al., 1984). Therefore placement of promoters and genes within the retroviral vector LTR initiated transcription unit may reduce their expression. The N2 vector is a VIP type of retroviral vector based upon the MoMLV. It contains the first 418 base pairs of the gag coding sequences, as well as the Neo resistance gene. The neo resistance gene appears to be expressed by a cryptic splicing of the vector RNA (Armentano et al., 1987). The cryptic 3'splice site was provided by the 418 base gag sequence just upstream from the Neo gene (Figure 5B). The double copy vector (DC) used in this study is based on the N2 vector. In DC vectors, the gene of interest, driven by its own promoter, was placed outside the retroviral vector's LTR initiated transcription unit to overcome the possible negative effect of the LTR transcription on the transcription initiated by the internal promoter (see Results).

The two major advantages of retroviral vector mediated gene transfer over other means of gene transfer are its high efficiency and the broad host range. The gene maybe introduced into cells at one copy per cell in a

genetically stable manner without adverse effect on the recipient cell and may efficiently infect a large proportion of the target cells. Retroviral vectors packaged in amphotropic viral particles can potentially infect a wide variety of cell types including human cells.

Gene Transfer in Hematopoietic Tissues

Gene transfer has been conducted in various non-hematopoietic types of cells, such as skin fibroblasts (Palmer et al., 1987), skin keratinocytes (Morgan et al., 1987; Flowers et al., 1990), hepatocytes (Wilson et al., 1988; Anderson et al., 1989), endothelial cells (Zwiebel et al., 1989; Wilson et al., 1989), muscle cells (Wolff et al., 1990), lymphocytes (Rosenberg et al., 1990). These cells, however, normally have a limited lifespan and the expression of the transduced gene is therefore short lived.

Bone marrow as the major hematopoietic organ in adults, is an attractive target for gene therapy. Compared to other target cells mentioned above, bone marrow has obvious advantages as the target of gene therapy: the well developed procedures for bone marrow transplantation, the large number and wide distribution of hematopoietic cells, the existence of many diseases that affect hematopoietic cells, and most importantly, the existence of a small number of pluripotent hematopoietic stem cells (HSC) capable of both self-renewal and differentiation following transplantation into appropriately conditioned recipients. These cells and their progeny will contribute to hematopoietic reconstitution for the lifetime of the recipient.

The frequency of stem cells has been estimated to be approximately 0.001% of nucleated marrow cells (Harrison et al., 1988). Therefore, any method of introduction of exogenous DNA needs to be extremely efficient. To date recombinant retroviral vectors appear to be the most promising technology to transfer DNA into this rare cell type. Retroviral mediated gene transfer demonstrates a

relatively high efficiency of gene transfer, stable integration of the provirus into the host cell genome, and the capacity to carry up to 10 kb of new genetic material (see 1.3).

Gene transfer into bone marrow cells of mouse, primates and human has been reported by many research groups with a variety of retroviral vectors containing genes as diverse as hypoxanthine phosphoribosyltransferase (HPRT), purine nucleoside phosphorylase (PNP), adenosine deaminase (ADA), ß-globin and hematopoietic growth factors as well as drug resistance genes such as NEO^r and MTX^r (Joyner et al., 1983; Williams et al., 1984; Dick et al., 1985; Keller et al., 1985; Gruber et al., 1985; Valerio et al., 1985; McIvor et al., 1987; Williams et al., 1986; Kantoff et al., 1987; Karlsson et al., 1987; Wong et al., 1987; Bender et al., 1989; Corey et al., 1990). The presence and/or the expression of the transferred gene for longer than 4 months posttransplantation in myeloid and lymphoid tissues is generally accepted as evidence for stem cell infection. Sequential transplantation has also been used to demonstrate infection of stem cells with extensive repopulating capability.

Despite the considerable progress in achieving long-term and stable expression of transduced genes in recipients of infected pluripotent stem cells, various difficulties remain to be overcome before gene therapy can be considered a feasible treatment.

The efficiency of infection and expression of any specific vector, for example, is unpredictable without in vitro testing in a proper system. The expression of retroviral vectors in the conventionally used murine

fibroblast cell line NIH3T3 and other cell lines has been repeatedly reported as failing to correlate with the expression in primary hematopoietic cells (Williams et al, 1986; Magli et al., 1987; Belmont et al., 1988; Hock et al., 1989; Li et al., 1992). The expression of certain genes from certain promoters can vary widely in different cell lines or in hematopoietic cells from different species (see review by Apperley and Williams, 1990).

The complicated kinetics of reconstitution of the hematopoietic system also presents a major obstacle for long-term expression of the transduced gene at an adequate level in the hematopoietic cells in vivo. The clonal succession of the normal hematopoiesis, in which the sequential activation of different stem cell clones contribute to hematopoiesis (Lemischka et al., 1986), and the finding that the hematopoietic system consists of stem cell clones which supply progeny for long periods of time as well as those which undergo dramatic temporal changes (Snodgrass and Keller, 1987) made gene therapy difficult to achieve in this organ. 100% of the transplanted cells may have to be infected to ensure permanent correction of the disease phenotype.

One of possible way to overcome this difficulty is to develop a selective scheme to enrich the transduced stem cells in vivo, as well as in vitro. Vectors were developed which contained not only the gene of interest but also a gene conferring a selectable phenotype. A bacterial transposon Tn5 neomycin phosphotransferase gene (NEO), which confers resistance to the drug G418 (Southern and Berg,1982), has been used as a dominant selectable marker in different vector designs by itself or in conjunction with other genes of interest and has

led to successful expression of NEO resistance in hematopoietic cells (Dick et al., 1985; Keller et al., 1985; see review by Williams, 1990). G418 is an aminoglycoside antibiotic, with a structure resembling gentamicin, neomycin and kanamycin. But unlike these related compounds, G418 interferes with the function of 80S ribosomes and blocks protein synthesis in eukaryotic cells (Davies et al., 1980). This aminoglycoside antibiotic can be inactivated by the bacterial phosphotransferase coded by the NEO gene. So far, no mammalian cells have been found naturally resistant to G418 unless the cells are transduced by the NEO gene, a desirable situation for a selection system.

There are limitations to this selection system when applied to gene transfer in hematopoietic cells in vivo. Due to its toxicity to mammalian hosts, G418 can not be used for in vivo selection. Although in vitro selection with G418 for 48 hr before bone marrow transplantation results in the efficient removal of non-transduced cells, the long-term expression of the transduced gene was not improved, suggesting that pre-selection eliminated long-term reconstituting stem cells, either because none of the stem cells were infected or because they were incapable of expressing sufficient neomycin phosphotransferase at the time of selection (Karlsson et al., 1988). Other possible explanations for the failure to sustain expression include mutation or deletion of the transferred DNA sequences (Hauser et al., 1987) or the development of an antibody response to the exogenous protein (St. Louis and Verma, 1988).

Another approach to selection of transduced hematopoietic stem cells would be the use of a selectable gene for which in vivo treatment is possible.

Purpose of Present Invention

Efficient expression of MTX resistance in mammalian cells via gene transfer of altered DHFR cDNAs has important implications for both basic gene transfer studies and eventual clinical applications of the gene transfer technique for gene therapy. The introduction into bone marrow stem cells of an altered murine DHFR gene (3T6 R400) resulting in MTX resistance enabled the selection of transduced hematopoietic cells in vivo. The recipient mice were protected from the lethal bone marrow toxicity induced by MTX, although the enrichment of stem cells under the particular in vivo selection schedule was not obvious (Williams et al., 1987; Corey et al., 1990). Generation of a drug-resistant bone marrow may facilitate the development of aggressive chemotherapeutic regimens that otherwise might lead to lethal bone marrow toxicity (Bertino, 1979).

In an effort to use an altered DHFR gene conferring MTX resistance as a dominant selectable marker and to determine the effect of different promoters on the expression of the mutant DHFR, MoMLV based retroviral vectors carrying the murine mutant DHFR 3T6 were constructed. Five different promoters were used and their expression was compared in NIH 3T3 fibroblast cell lines, three human leukemia cell lines and mouse bone marrow CFU-GM colonies. Retroviral vectors carrying human mutant DHFR S31 and S34 were also constructed and their expression was tested and compared with the murine mutant.

The in vivo expression of mutant murine or human DHFR constructs were tested in mice to determine if protection was conferred to the recipient mice with different MTX

selection schedules which allow the demonstration of long-term as well as short term expression of the MTX resistance phenotype. Serial bone marrow transplantations also were performed. The enrichment of the MTX resistant progenitor cells was tested.

We also tested human mutant *dhfr* with serine mutations at positions 31 or 34, as well as murine mutant *dhfr* with a non-active site mutation at residue 15 where the wild type glycine was changed to tryptophan (G to T change at nt 46), for their use as a selectable marker in Chinese hamster ovary cells.

SUMMARY OF THE INVENTION

This invention provides a DNA vector which comprises DNA encoding a mutant, antifolate resistant, dihydrofolate reductase inserted into a site within the vector, the presence of which site is not essential for replication of the vector.

This invention further provides the above-described DNA vector, wherein the mutant dihydrofolate reductase has substantially the same amino acid sequence as naturally occurring human dihydrofolate reductase.

In an embodiment, the mutant dihydrofolate reductase differs from naturally occurring human dihydrofolate reductase by virtue of the presence of a serine residue at position 31 or 34. In another embodiment, the mutant dihydrofolate reductase differs from naturally occurring human dihydrofolate reductase by virtue of the presence of a tryptophan residue at position 15.

This invention provides the above-described DNA vector, wherein the 5'end of the cDNA encoding a mutant dihydrofolate reductase is operatively linked to a promoter sequence and the 3'end of the cDNA to a polyA sequence.

This invention also provides a human cell which comprises the above-described vector or retroviral vector. The human cell may be a hematopoietic human cell or bone marrow cell.

This invention also provides a method for reducing the toxic effects of antifolate therapy in a subject which comprises replacing the subject's hematopoietic cells

with hematopoietic cells which comprise the above-described vector or retroviral vector so as to reduce the toxic effects of antifolate therapy in the subject.

This invention provides a method for introducing a selectable marker into a mammalian cell which comprises transfecting the cell with DNA encoding a mutant dihydrofolate reductase capable of increasing the antifolate resistance when introduced into a cell.

Finally, this invention provides a method for selecting mammalian cells expressing protein of interest which comprises a. introducing into the cells a DNA molecule comprising DNA encoding the protein of interest and DNA encoding a mutant dihydrofolate reductase capable of increasing the antifolate resistance when introduced into a cell; b. culturing the resulting transfected cells; and c. selecting cells which express mutant dihydrofolate reductase, so as to obtain cells which express the protein of interest.

DETAILED DESCRIPTION OF THE FIGURES

Figure 1. The role of folate coenzymes in the synthesis of thymidylate, purine nucleotides, and methionine. Abbreviations: FH2, 7,8-dihydrofolate; FH4, 5,6,7,8-tetrahydrofolate; CH3FH4, 5-methyltetrahydrofolate; CHOFH4, 5-formyltetrahydrofolate; CHFH4, 5,10-methenyltetrahydrofolate; CH2FH4, 5,10-methylenetetrahydrofolate; S-AM, S-adenosylmethionine.

Figs. 2A-2B: Structure of Methotrexate (MTX) and dihydrofolate (Fig. 2A); Transport, polyglutamation and mechanism of action of MTX (Fig. 2B). Abbreviations: same as Figure 1; FGS, folylpolyglutamyl synthetase; (G)n, polyglutamated forms with various number of the glutamates.

Figure 3. The retroviral life cycle. The retroviral virion contains two molecules of the single stranded (ss) RNA genome. The virus infects a susceptible cell and the RNA enters the cell and is reverse transcribed into double stranded (ds) DNA which migrates to the cell nucleus and is integrated into the chromosome of a host cell to form the provirus. Viral RNA and mRNAs, encoding viral proteins, are transcribed from the integrated provirus. The mRNAs are translated to viral proteins which are used to encapsulate the viral RNAs as the virus buds off from the cell.

Figure 4. The use of a packaging cell line in retroviral vector mediated gene transfer. MoMLV vector virus DNA contains the *cis* elements necessary for retroviral replication such as the LTR sequences and packaging signal sequence, ψ. In the vector DNA the viral genes have been replaced with sequences of interest. A packaging cell line produces the viral gag, pol, and env proteins necessary *in trans* for retroviral replication. These proteins are expressed from templates that lack the MoMLV packaging signal. When a retroviral vector DNA is transfected into and transcribed in the packaging cell, transcripts which contain the viral packaging signal, ψ, are encapsulated by the viral proteins supplied by the packaging cell and are secreted as vector containing virus. Vector containing virus can be used to infect a target cell through the pathway normally used by the retrovirus. This process leads to the stable integration of a vector provirus in the target cell.

Figs. 5A-5B. Retroviral vector designs. All retroviral vectors contain the retroviral LTRs and packaging signal sequence, ψ. Fig. 5A. The vectors with internal promoters (VIP) allow for the expression of two genes, but from different promoters. One gene, often a marker protein encoding gene, is expressed from the viral LTR promoter, and the second gene is expressed from an

internally encoded promoter. Fig. 5B. The N2 retroviral vector contains the first 418 base pairs of the MoMLV gag gene. The marker gene, NEO, is expressed from a spliced RNA which uses the MoMLV splice donor site (SD) and a cryptic splice acceptor site (SA) (Armentano et al., 1987). The unspliced RNA contains the MoMLV packaging signal and represents the vector containing virion RNA.

Figs. 6A-6D. Structure of N2A (Fig. 6A), N2AP (Fig. 6B), DC/AC (Fig. 6C), DC/SV (Fig. 6C), DC/AD/R (Fig. 6D), DC/SV/R (Fig. 6D), DC/TK/R (Fig. 6D) and DC/CMV/R (Fig. 6D). In N2A, the inserted polylinker at U3 region contains ApaI(A), BglII(B), SnaBI(Sn), SacII(S), MluI(M) restriciton sites. The poly(A) signal sequence was cloned in the antiparallel orientation of the LTR transcriptional unit in N2AP. The SV 40 early promoter (SV) or the human ß-actin promoter (AC) was cloned in N2A in the parallel orientation of the LTR transcriptional unit. The SV promoter and the human adenosine deaminase promoter (AD) or the HSV thymidine kinase promoter (TK) or the cytomegalovirus promoter (CMV) was cloned in N2AP in the antiparallel orientation.

Figs. 7A-7E. Structure of DC vectors carrying mutant mDHFR cDNA. Mutant mDHFR was obtained from pFR400 (Simonsen and Levinson, 1983) (Fig. 7A). By inserting different

restriction fragments of pFR400 into the SnaBI site of different vectors in either the parallel or antiparallel orientation, the vectors carrying mutant DHFR cDNA were generated: N2A + PvuII-SacII(parallel)=DC/SV-mDHFR (Fig. 7B); N2A + PvuII-SalI(antiparallel)=DC/SV/R-mDHFR (Fig. 7C); DC/AC + HindIII-NcoI(parallel)=DC/AC-mDHFR (Fig. 7C); DC/AD/R, DC/TK/R and DC/CMV/R + HindIII-NcoI(antiparallel)=DC/AD/R-mDHFR, DC/TK/R-mDHFR and DC/CMV/R-mDHFR (Fig. 7D).

Figs. 8A-8H. Structure of DC vectors carrying mutant hDHFR cDNA. The full length mutant hDHFR cDNAs (S31 or S34) with modified 5'ends were obtained from pKT7HDR (Schweitzer et al., 1989). The NcoI and HindIII fragment (about 800bp) was cloned into the SnaBI site of DC/SV in parallel orientation, and of DC/SV/R and DC/AD/R in antiparallel orientation, generating DC/SV-hDHFR31/34 (Fig. 8B), DC/SV/R-hDHFR31/34 (Fig. 8C) and DC/AD/R-hDHFR31/34 (Fig. 8C). The NcoI and BglII fragment (about 655 bp) was cloned similarly into the SnaBI site of DC/SV in parallel orientation, and of DC/SV/R and DC/AD/R in antiparallel orientation, generating DC/SV-hDHFR31 NB (Fig. 8D), DC/SV/R-hDHFR31 NB (Fig. 8E) and DC/AD/R-hDHFR31 NB (Fig. 8E). The mutant hDHFR cDNA (S31) with non-modified 5'end was obtained from pSV4HDR. The HindIII and BglII fragment was cloned into the SnaBI site of DC/SV in parallel

orientation, and of DC/SV/R and DC/AD/R in antiparallel orientation, generating DC/SV-hDHFR31 HB (Fig. 8G), DC/SV/R-hDHFR31 HB (Fig. 8H) and DC/AD/R-hDHFR31 HB (Fig. 8H).

Figure 9. The reverse transcriptase activity in the 3T3 cell lines transduced by retroviral vectors. Parental and transduced 3T3 cells were passed 4 times in culture over 2-3 weeks before the culture media were collected and centrifuged at 3,000 RPM at 4°C for 10 min. the supernatants were mixed with RT cocktail and the RT assay were performed with the Oligo (dT) primer and α^{32}P-dTTP. The reaction mixture was filtered and washed, and the filter paper was exposed to x-ray film. Parental 3T3 cells and ecotropic producer line were used as negative and positive control respectively. The numbers following the vectors indicate individual virus producer clones from which the infectious supernatants were collected and used in the infection.

Figure 10. Role of the U3 region of the LTR in retroviral replication. The R region of the viral LTRs is present in both ends of the viral RNA. The U5 and U3 region of the viral LTR are only present in one copy in either the 5′ or 3′ end of the viral RNA respectively. Therefore, the single copy of the U5 region serves as the template in reverse transcription for both

copies of the U5 region of the linear DNA form of the viral genome. Similarly, the single copy of the U3 region present in the 3′ end of the viral RNA serves as the template for both the 5′ and 3′ LTR copies of the U3 region in the linear DNA form of the viral genome and the provirus. The proviral DNA encodes a transcription unit which is responsible for the production of the viral RNA. Transcription initiates in the 5′ LTR R region and terminates in the 3′ LTR R region. The primer binding site (PBS) and the poly (A) signal sequence (An) are also illustrated.

Figure 11. Possible messenger RNA species in target cells infected by DC retroviral vectors carrying a foreign gene. In target cells, the gene of interest inserted in the U3 region is duplicated. The gene inserted in the 5′ LTR is outside the retroviral transcriptional unit. The DHFR mRNAs transcripts are initiated from the minigene promoter. The viral RNA and NEO RNA transcripts are initiated from LTR promoter. Higher molecular weight transcripts are possibly initiated from the minigene promoter and read through the proviral DNA.

Figs. 12A-12B. Expression of the mDHFR in NIH 3T3 cells. Total cellular RNA was isolated from 3T3 cells infected with different retroviral vectors carrying mutant mDHFR and fractionated on Oligo(dT) cellulose

columns. The poly (A) fraction was subjected to electrophoresis in an agarose/formaldehyde gel, blotted on a nylon membrane, and hybridized with a ^{32}P-labeled mDHFR probe (Fig. 12A) or a human glyceraldehyde-3-phosphate dehydrogenase (GAPD) probe (Fig. 12B) to control for loading. The number following the vectors indicate individual virus producer clones from which the infectious supernatants were collected and used in the infection. The 1.1 kb and 0.8 kb transcripts are the DHFR transcripts from the internal promoters.

Figs. 13A-13B. Northern analysis of the expression of the mutant mDHFR in NIH 3T3 cells infected with viral supernatants of five individual producer lines of DC/SV-mDHFR and DC/SV/R-mDHFR. The RNA blot was hybridized with a ^{32}P-labeled mDHFR probe (Fig. 13A) or a GAPD probe (Fig. 13B). The number following the vectors indicate individual virus producer clones from which the infectious supernatants were collected and used in the infection. The 1.1 kb and 0.8 kb transcripts are the DHFR transcripts from the internal promoters.

Figs. 14A-14B. RNA blot analysis of the expression of the mutant mDHFR in CEM, K562 and Raji cells infected with different retroviral vectors carrying mutant mDHFR. The RNA blot was hybridized with a ^{32}P-labeled mDHFR probe (Fig. 14A) or a GAPD probe (Fig. 14B). The

number following the vectors indicate individual virus producer clones from which the infectious supernatants were collected and used in the infection. The 1.1 kb and 0.8 kb transcripts are the DHFR transcripts from the internal promoters.

Figs. 15A-15B. Southern analysis of the integrated proviral structure. DNA isolated from Raji, CEM and K562 cells infected with different retroviral vectors carrying mutant mDHFR was digested with DraI, electrophoresed in agarose gels, blotted, and hybridized with a ^{32}P-labled mDHFR probe (Fig. 15A), and a NEO probe (Fig. 15B) as described in Material and Methods. The number following the vectors indicate individual virus producer clones from which the infectious supernatants were collected and used in the infection; Raji:plasmid and K562:plasmid are the positive controls with vector DNA plasmid added to the DNA of noninfected parental cells. The distortion of the DNA bands especially in the K562 panel is due to the high salt content of the DNA prepared using CsCl gradient.

Figure 16. Dose response to MTX of CFU-GM in different selection conditions. The number of CFU-GM colonies per 4 X 10^5 mouse bone marrow cells was counted after culturing for 14 days in 3 different selection medium with various concentrations of MTX. The number of CFU-

GM colonies in the absence of MTX was used as control.

Figure 17. Protocol for DHFR gene transfer to marrow progenitors of the mouse and selection with MTX. Donor marrow (1-2 X 10^7 cells per donor mouse) was cocultured for 48 hr with the pre-irradiated (1500 R, 2 hr earlier) parental packaging line (AM12) or viral producer lines before being transplanted into the recipient mice irradiated with 900 R 24 hr earlier. MTX selection was started either 24 hr later with low-dose schedule or 4 weeks later with delayed high-dose schedule.

Figs. 18A-18B. Survival after BMT and low-dose MTX selection. Irradiated recipients were transplanted with transduced (cocultured with DC/AD/R-mDHFR or DC/SV/R-mDHFR) or untransduced (cocultured with AM12) bone marrow on day 0. The low-dose MTX selection started on day 1. In experiment A, 1.5 mg/kg twice a week was administered ip for the first week and 3 mg/kg twice a week ip for the next few weeks. In experiment B, 2 mg/kg twice a week ip was given for the first week and 5 mg/kg twice a week ip for the next few weeks. Both experiments had 8 to 10 recipient mice in each of the three BMT groups. After day 30, one of the survived animals was sacrificed at intervals to perform CFU-GM assay or for biochemistry test.

Figs. 19A-19B. Changes in hematocrit and white blood cell count (WBC) after BMT and Low-dose MTX selection. The hematocrits in survived recipients of untransduced marrow (control) or transduced marrow (DC/SV/R-mDHFR, DC/AD/R-mDHFR) before the BMT (day 0) and day 13 or day 28 after BMT and MTX selection are shown in Fig. 19A. No animal survived in the control group at day 28. The WBC counts are shown in Fig. 19B. Each BMT group had 8 to 10 animals and the standard deviations are also shown in the figure.

Figs. 20A-20C. The survival in a series of BMT under low-dose MTX selection. The MTX selection schedule of the primary BMT (Fig. 20A) was 1.5 mg/kg twice a week for the first week, 3 mg/kg twice a week for the next few weeks. The secondary (Fig. 20B) and tertiary (Fig. 20C) recipients were selected with MTX 2 mg/kg twice for the first week, 5 mg/kg twice per week for the next few weeks. The survival of the control for the primary BMT (irradiated mice which received untransduced marrow) was used as a reference in the secondary and tertiary BMT survival curves.

Figs. 21A-21B. MTX resistant CFU-GM colonies after in vivo MTX selection following BMT. Bone marrow cells from the primary recipients (Fig. 21A) and from secondary recipients (Fig. 21B) were obtained at intervals during the in vivo MTX selection (2 mg/kg

twice for the first week, and 5 mg/kg twice a week for the remaining weeks after BMT). The CFU-GM colonies per 4 X 10^5 bone marrow were counted after culturing in the absence or presence of 100 nM MTX for 14 days. The percentage of resistant colonies (%) was calculated by dividing the number of MTX resistant colonies with the colonies formed in the absence of MTX. Bone marrow cells from normal mice without BMT and MTX in vivo treatment were used as the control.

Figs. 22A-22B. The survival of BMT and Delayed high-dose MTX selection. The number of recipients surviving the delayed high-dose MTX selection in either the control group (receiving the untransduced marrow) or the group receiving DC/SV/R-mDHFR transduced marrow are shown in Fig. 22A. The high-dose selection started 4 weeks after the BMT with MTX of 100 mg/kg twice a week, i.p. for 4 weeks and increased to 200 mg/kg twice a week, i.p. for 6 weeks. There were 3 recipients in the control group and 5 in the DC/SV/R-mDHFR group.

Figs. 23A-23C. PCR blot of mouse tissues after BMT and MTX selection. Genomic DNA from mouse tissues was prepared 8 weeks after BMT and low-dose MTX selection (Fig. 23A). NEO1 and NEO2 were used as primers in the PCR reaction (40 cycles of 94°C 1 min, 55°C 1 min, and 72°C 1 min). The products were subjected to electropheresis in an agarose

gel, blotted to a nylon membrane, and hybridized with a ^{32}P-labelled NEO probe. The 415 bp hybridization is the specific NEO fragment amplified. DNA from normal mouse tissues were used as control. Genomic DNA extracted from mouse tissues 4 months after BMT with delayed high-dose MTX selection was also analysed by a PCR blot with NEO primers and NEO probe (Fig. 23B). DNA extracts from the mouse receiving untransduced marrow (AM12) were used as controls. The genomic DNA from 3T3 cells transduced by mutant mDHFR were subjected to PCR blot analysis under similar conditions (Fig. 23C). DNA extracts from a AM12 mouse were used as controls.

Figure 24. PCR blot analysis of MTX resistant CFU-GM colonies after BMT and in vivo MTX selection. Bone marrow cells from a normal mouse or recipients of BMT with either untransduced marrow (AM12) or transduced marrow (DC/SV/R-mDHFR, primary or secondary) were used in the CFU-GM assay in the absence or the presence of 100 nM MTX. Genomic DNA was pooled from 5 to 6 CFU-GM colonies resistant to MTX in the recipients of transduced marrow, and from colonies of a normal mouse or the recipients of AM12 marrow grown in the absence of MTX. NEO1 and NEO2 were used as primers in the PCR analysis and a NEO probe was used for hybridization (see legend of Figure 17).

Figs. 25A-25B. Sequence analysis of mouse tissues after BMT and MTX selection. Genomic DNA from peripheral blood cells 8 months after secondary BMT and Low-dose MTX selection (Fig. 25A), and from spleen and liver 5 weeks after primary BMT and low-dose MTX selection (Fig. 25B), was amplified by asymmetric PCR using GT-NC1 and M301 primers. The PCR product was sequenced with a M210 primer by the dideoxy chain termination method. The four lanes of the sequencing gel, read from left to right are A, C, G, T bases. The arrow points to the mutation of A to C in the non-coding strand.

Figs. 26A-26B. Southern analysis of the mouse tissues after BMT and MTX selection. Genomic DNA extracted from tissues of mice receiving transduced marrow (DC/SV/R-mDHFR or DC/AD/R-mDHFR) or untransduced marrow (AM12), or of a normal mouse (NM) was digested with DraI, electrophoresed in agarose gels, blotted and hybridized with a ^{32}P-labelled mDHFR probe (Fig. 26A), and a NEO probe (Fig. 26B). 3T3:plasmid is the positive control with vector DNA plasmid added to the DNA of noninfected parental 3T3 cells. 3T3 cells infected with either of the two DC vectors are used as control for 100% integration of a single copy of the proviral DNA. All tissues were obtained from primary BMT unless otherwise indicated as secondary (2) BMT.

Figure 27. The detection limit of the southern analysis. DNA extracted from 3T3 cells transduced by DC/AD/R-mDHFR was digested with DraI, diluted to the indicated percentage in DNA of the untransduced 3T3, electrophoresed in an agarose gel, blotted, and hybridized with a ^{32}P-labelled NEO probe. The vector plasmid DNA was used as control.

Figs. 28A-28B. RNA and DNA analysis of the expression and the proviral structure of the retroviral vectors carrying the full length mutant hDHFR cDNA. The poly A fraction of total cellular RNA isolated from 3T3 cells infected with retroviral vectors (G418 resistant, gr, with the exception of DC/SV-hDHFR31/mr, see legend of Table 5 for detail) carrying the full length of mutant hDHFR cDNA, was subjected to electrophoresis in agarose/formaldehyde gel, blotted to a nylon membrane, and hybridized with a mixture of ^{32}P-labelled mDHFR and hDHFR cDNA probes (Fig. 28A). The 3T3 cell lines infected with retroviral vectors carrying mDHFR or the vector alone were used as control for the experiment. The 1.2 and 0.9 kb markers indicate the expected length of the hDHFR transcripts. DNA isolated from 3T3 cells transduced by retroviral vectors carrying full length mutant hDHFR cDNA was digested with DraI, was electrophoresed in agarose gels, blotted, and hybridized with a 32P-labelled NEO probe (Fig. 28B). The

parental 3T3 cells and 3T3 cells infected with the vector alone were used as negative controls and the 3T3:plasmid is the positive control with hDHFR carrying vector plasmid DNA added to the DNA of uninfected parental cells.

Figs. 29A-29B. Immunoprecipitation of the DHFR enzyme protein in the 3T3 cells transduced by retroviral vectors carrying full length mutant hDHFR cDNA.

Fig. 29A. A rabbit anti-human polyclonal antibody against DHFR was titered in a CHO cell line that lacks the DHFR (DG44). The ratios at the top indicate the dilution of the antibody against a ^{35}S-labelled cell extract. The DG44-hDHFR is the DG44 cell line that had been transduced by hDHFR. The molecular weight markers are on the right and the arrow points to the 22 kd DHFR enzyme precipitation.

Fig. 29B. Cell extracts from 3T3 cell lines parental or infected with retroviral vector alone or with the vectors carrying the full length mutant hDHFR were incubated with the Ab in a 50 to 1 ratio. Equal amounts of radioactivity of the precipitates were electrophoresed on a 15% SDS polyacrylamide gel. The DG44-hDHFR line was used as control for the experiment. The arrow points to the 22 kd DHFR enzyme precipitated. Gr stands for G418 resistance; mr stands for MTX resistance (see Table 5 legend and the text for detail).

Figs. 30A-30B. RNA analysis of the expression of the retroviral vectors carrying less than full length mutant hDHFR cDNA. The RNA blot of the poly A fraction of the cellular RNA from the 3T3 cell lines transduced by the vectors carrying less than full length of mutant hDHFR cDNA (hDHFR31HB or hDHFR31NB) were hybridized to a ^{32}P-labelled hDHFR probe (Fig. 30A) and a GAPDH probe (Fig. 30B). The numbers under each vector indicate individual virus producer clones from which the infectious supernatant was collected and used in the infection. The conditions in which the infected 3T3 cells were selected are designated as gr for G418 resistant or mr for MTX resistant. The arrows point to the expected 1.1 and 0.8 kb messages from the internal promoters. The two 3T3 cell lines transduced by the mDHFR were used as control though the mDHFR did not hybridized well with the hDHFR probe. The parental 3T3 cell line was used as the negative control.

Figs. 31A-31B. DNA analysis of the proviral structure of the retroviral vectors carrying less than the full length of the mutant hDHFR. Genomic DNA extracted from the 3T3 cell lines infected with the viral vectors selected either with G418 (gr) or MTX (mr) was digested with DraI and separated on an agarose gel, blotted and hybridized to a 32P-labelled NEO probe. The parental 3T3 cell line was used as a negative control

and 3T3:plasmid is the positive control in which the vector plasmid DNA was added to the parental 3T3 DNA. The molecular size markers are on the left. The numbers under different vectors indicate individual virus producer clones from which the infectious supernatant was collected and used in the infection.

Figs. 32A-32B. The survival of BMT with mutant hDHFR and MTX selection. The survival of mice receiving untransduced marrow (control) or transduced marrow (DC/SV-hDHFR31HB) under the low-dose schedule (2 mg/kg, twice a week for the first week, 5 mg/kg twice a week for the next 6 weeks) (Fig. 32A) or the delayed high-dose schedule (no MTX for first 4 weeks, 200 mg/kg twice a week for the next 7 weeks) (Fig. 32B) of MTX selection are shown. The animals surviving 7 weeks of the low-dose selection were subjected to the high-dose selection (200 mg/kg twice a week) for 5 weeks.

Figure 33. PCR blot of the MTX resistant CFU-GM colonies after BMT with mutant hDHFR and in vivo MTX selection. Genomic DNA from the pooled CFU-GM colonies (5 to 6) was amplified with H250 and GT-NC1 primers (40 cycle of 94°C 1 min, 55°C 1 min, and 72°C 1 min), electrophoresed on an agarose gel, blotted to a membrane, and hybridized to a hDHFR cDNA probe. The arrow points to the size of the specific hDHFR fragment

amplified.

Figure 34. pSV5 Expression Vector Containing the *dhfr* Insert Cloned into the NcoI and HindIII Sites. The dhfr insert is placed downstream of the SV40 early promoter and is followed by poly A+ signal containing sequences. Some important restriction sites within the plasmid vector are shown. Vector Construction: Generation of the Ser31 and the Ser34 mutants by site directed mutagenesis has been described (Schweitzer et al., 1989a). The expression vectors containing the mutant *dhfr* cDNAs as well as the wild type were constructed as follows: The plasmid pHD80 containing human *dhfr* cDNA was obtained from G. Attardi, California Inst. of Tech., CA. The pHD80 plasmid was used as a template for amplification of the wild type human dhfr cDNA by the polymerase chain reaction (PCR). Amplification of the Ser31 and Ser34 cDNAs were carried out by the polymerase chain reaction (PCR) using the oligomers DHFR24 and pSV3' and the Ser31 and Ser34 cDNA inserts as templates. The DHFR24 PCR primer for the 5'end of the h-*dhfr* cDNA contains a NcoI site centered at ATG start codon and anneals to the noncoding strand between nucleotides -8 and 24. The pSV3' PCR primer for the 3'end of h-*dhfr* cDNA contains a HindIII site attached to nucleotides 638-609 of the cDNA.

pSV3' 5' CGATCGA GGATCC C AAGCTT ACCTTTT 3' (Sequence ID No. 1)

HindIII

DHFR24 5' ATCATCCCATGGTTGGTTCGCTAAACTGCATCG 3'

------ (Sequence ID No. 2)

NcoI

In vitro amplification using PCR was carried out, and the product was restricted with NcoI and HindIII. The pSV2 plasmid vector was modified to generate NcoI and HindIII sites. The h-dhfr cDNAs were then cloned into the NcoI and HindIII sites of the modified vector. The resulting plasmid expression vector was termed pSV5. For cloning the mouse Arg22 mutant *dhfr* cDNA a similar approach was taken. The cDNA was amplified by PCR from the 3T6R400 *dhfr* cDNA template (present in the vector pFR400 obtained from C. Simonsen and A. Levinson, Genetech, CA) using the primers M5'NcoI (which anneals to the noncoding strand between nucleotides -9 and +15 taking the A of the first ATG as +1) and M3' HindIII (which anneals to the coding strand between nucleotides 610 and 643). The mouse *dhfr* cDNA was of the same length as the human *dhfr* cDNAs. The primers had the following sequences:

M5' NcoI 5' GCTGCCATCCATGGTTCGACCATTG 3'

------ (Sequence ID No. 3)

NcoI

M 3 ' H i n d I I I

5'TCTAAAGCCAGCAAAAGCTTCATGGTCTTATAA 3'
------ (Sequence ID No. 4)
HindIII

After digestion with NcoI and HindIII the Arg22 *dhfr* cDNA was also cloned into the pSV5 vector. This was done so that comparisons between the mutant *dhfr* cDNAs could be carried out after transfection using the same expression vector system. Competent DH5 alpha cells were transformed with the pSV5 plasmids containing the respective inserts. Ampicillin resistant colonies were isolated and analyzed for the presence of the correct insert. For transfection studies, plasmids were isolated from the transformed bacteria using a midi prep kit (Qiagen, Inc., CA). Plasmids were purified by two rounds of phenol chloroform extraction and ethanol precipitation prior to transfection.

Figs. 35A-35B. Southern Blot Analysis of NcoI and HindIII Digested Genomic DNA Isolated from Transfected Cells Hybridized with ^{32}P Labelled Human *dhfr*. Panel A: Lanes 1, 2, and 3 represent DNA isolated from Ser31 transfected cells grown in 100,500 and 1000 nM MTX; lanes 4, 5, and 6 represent DNA from Arg22 transfected cells grown in 100,500 and 1000 nM MTX; lanes 7, 8, and 9 represent DNA from Ser34 transfected cells grown in 100,500 and 1000 nM MTX, respectively; while lane 10 represents DNA from control CHO cells transfected with the *neoR* only (Fig. 35A). Panel B shows

the same samples hybridized with hamster *dhfr* probe (Fig. 25B). Autoradiogram exposure time was 24h. Signal in lane 7, panel A was too weak to show any visible band at 24h; longer exposure (72h) showed a visible band. For Southern analysis, 10 μg of genomic DNA for each cell line was incubated with restriction enzymes HindIII and NcoI in 10 x universal buffer for 18h at 37°C. The restricted DNA was electrophoresed on a 0.8% Tris Acetate EDTA (TAE) agarose gel (SeaKem GTG, FMC) and transferred to Nytran (S&S, MA) overnight in 10 x SSC transfer solution by capillary transfer. The DNA was cross linked to the membrane by UV exposure (UV Stratalinker 1800, Stratagene) and then hybridized to a ^{32}P-labeled human *dhfr* cDNA. Labeling of probe was done by random priming using the random priming kit from Boehringer Mannheim and alpha-^{32}PdCTP (> 3000 Ci/m mole, NEN). Calcium phosphate mediated gene transfer was carried out using the mammalian transfection kit (Stratagene, CA) according to the manufacturers directions. The wild type and mutant *dhfr* containing plasmids (20 μg) were cotransfected with plasmids harboring the neomycin resistance gene in a ratio of 20:1 (i.e., *dhfr:neoR* = 20:1). MTX resistant colonies as well as G-418 resistant colonies were scored after 14 days. A ^{32}P-labeled human *dhfr* probe was used for hybridization, and the blot was washed twice at room temperature

in 1 x SSC/0.1% SDS for 30 min. and once at 55°C in 0.1 x SSC/1.0% SDS for 20 min.

Figs. 36A-36B. Northern Blot Analysis of Total Cellular RNA from Transfected and Control CHO Cells. Panel A shows ethidium bromide staining of the nylon filter after transfer (Fig. 36A). Panel B shows the result of hybridization of the filter with a ^{32}P labelled h-*dhfr* probe (Fig. 36B). Lane 1 represents RNA from control CHO cells; lane 2 represents RNA from Ser31 transfected CHO cells; lane 3 represents RNA from Ser34 transfected cells; and lane 4 represents RNA from Arg 22 transfected cells. The presence of the smaller message(s) in lanes 2, 3, and 4 (between 0.6 and 0.7 Kb) indicate that the transfected Ser31 and Ser34 as well as the Arg22 *dhfr* cDNAs are expressed. Total amount of RNA loaded in each lane was 50 µg. RNA was isolated from cells derived from a single colony growing at 100 nM MTX for the Ser31 and Ser34 as well as the Arg22 transfectants. For the control CHO cells RNA was isolated from a culture expanded from a single colony growing in 750 µg/ml of G-418. For Northern analysis, total cellular RNA was extracted from cells by the guanidinium thiocyanate-phenol method (Chomczynski and Sacchi, 1987) according to the manufacturers instructions (RNAzol, Cinna Biotecx, TX). The RNA was electrophoresed on agarose formaldehyde gel using 1 x MOPS

buffer. The RNA was then transferred to Nytran (S&S, MA) by capillary transfer overnight and hybridized to a radiolabelled full length human *dhfr* cDNA. After hybridization at 42°C overnight all blots were washed for 30 min. at room temperature in 1 x SSC/0.1% SDS and then for 20 min. at 55°C in 0.1 x SSC/0.1% SDS.

Figure 37. Northern blot analysis of RNA isolated from untransfected cells and from cells transfected with the Trp15 mutant murine *dhfr*.

Figure 38. Southern blot analysis of DNA isolated from untransfected cells and from cells transfected with the Trp15 mutant murine *dhfr*.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a DNA vector which comprises DNA encoding a mutant, antifolate resistant, dihydrofolate reductase inserted into a site within the vector, the presence of which site is not essential for replication of the vector.

This invention further provides the above-described DNA vector, wherein the mutant dihydrofolate reductase has substantially the same amino acid sequence as naturally occurring human dihydrofolate reductase.

In an embodiment, the mutant dihydrofolate reductase differs from naturally occurring human dihydrofolate reductase by virtue of the presence of a serine residue at position 31 or 34.

In another embodiment, the mutant dihydrofolate reductase differs from naturally occurring human dihydrofolate reductase by virtue of the presence of a tryptophan residue at position 15.

This invention also provides the above-described DNA vector, wherein the 5'end of the DNA encoding a mutant dihydrofolate reductase is operatively linked to a promoter sequence and the 3'end of the cDNA to a polyA sequence.

In an embodiment, the promoter sequence is an SV40 promoter.

This invention also provides a plasmid which comprises the above-described vector.

In an embodiment, the plasmid is designated pSV5-Ser31 h-DHFR (pSV5-Ser31). This plasmid contains DNA encoding a mutant dihydrofolate reductase with a serine residue at position 31. This plasmid also contains SV40 promoter and poly A sequences. Plasmid, pSV5-Ser31 h-DHFR was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. on April 9, 1993 under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The plasmid was accorded ATCC accession number 75441.

In another embodiment, the plasmid is designated pSV5-Ser34 h-DHFR (pSV5-Ser34). This plasmid contains DNA encoding a mutant dihydrofolate reductase with a serine residue at position 34. This plasmid also contains SV40 promoter and poly A sequences. Plasmid, pSV5-Ser34 h-DHFR was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. on April 9, 1993 under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The plasmid was accorded ATCC accession number 69276.

In another embodiment, the above-described vector is a retroviral DNA vector. In a further embodiment, the retroviral vector comprises DNA from a retrovirus corresponding to a 5′ long terminal repeat, a 3′ long terminal repeat and a packaging signal. In a still further embodiment, the site at which the DNA encoding a mutant dihydrofolate reductase inserted is in the 3′ long terminal repeat of the retroviral vector.

This invention further provides plasmids which comprises the above-described retroviral vectors.

In an embodiment, the plasmid which comprises a retroviral vector which comprises DNA encoding a mutant dihydrofolate reductase capable of increasing the antifolate resistance when introduced into a cell is designated pDC SV S31 h-DHFR. This plasmid contain DNA which codes for a mutant dihydrofolate reductase with serine at position 31. Plasmid, pDC SV S31 h-DHFR was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. on April 9, 1993 under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The plasmid was accorded ATCC accession number 75440.

This invention also provides a mammalian retroviral producer cell which comprises the above-described vectors or plasmids.

This invention further provides human cell which comprises the above-described vectors or plasmids. In an embodiment, the human cell is a hematopoietic cell. In another embodiment, the human cell is a bone marrow cell.

This invention also provides a method for reducing the toxic effects of antifolate therapy in a subject which comprises replacing the subject's hematopoietic cells with hematopoietic cells which comprised the above-described vectors or plasmids so as to reduce the toxic effects of antifolate therapy in the subject. In an embodiment, the antifolate is methotrexate.

This invention also provides a method for introducing a selectable marker into a mammalian cell which comprises transfecting the cell with DNA encoding a mutant dihydrofolate reductase capable of increasing the antifolate resistance when introduced into a cell.

Finally this invention provides a method for selecting mammalian cells expressing protein of interest which comprises: a.introducing into the cells a DNA molecule comprising DNA encoding the protein of interest and DNA encoding a mutant dihydrofolate reductase capable of increasing the antifolate resistance when introduced into a cell; b. culturing the resulting transfected cells; and c. selecting cells which express mutant dihydrofolate reductase, so as to obtain cells which express the protein of interest. In an embodiment, the DNA molecule of step (a) of the above method is part of a retroviral vector.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

First Series of Experiments

MATERIAL AND METHODS

Materials

Chemicals

Common laboratory chemicals were of the highest purity commercially available and were obtained from Mallinckrodt, Fisher, Sigma, or Bio-Rad. The following is a list of special reagents used:

From Boehringer Mannheim: DNA markers, RNAase, dNTPs, ATP, Proteinase K, Klenow fragment, T4 DNA ligase, Reverse transcriptase, PMSF, Bovine serum albumin (pentax fraction V)

From GIBCO: Trypan-blue, G418, Penicillin-streptomycin, Trypsin

From Lederle: Methotrexate

From Pharmacia: Protein A sepharose CL-4B, Poly A, Oligo dT

From Sigma: Thymidine phosphorylase, Polybrene, Diethyl pyrocarbonate, Lysozyme, Ampicillin, Tetracycline, Dithiothreitol, ß-Mercaptoethanol, Sarkosyl, Dimethyl sulfoxide, Hypoxanthine, Xanthine, Mycophenolic acid, Hygromycin B, MOPS, Ethidium bromide, Salmon sperm DNA, Polyvinylpyrolidone, Sodium-deoxycholate, Leupeptin

From United States Biochemicals: T7 DNA polymerase (Sequenase version 2.0)

Medium

From central medium laboratory of SKI unless otherwise indicated: IMDM, DME, RPMI, PBS, fetal bovine serum (Hy Clone Laboratory)

Restriction Enzymes

From Boehringer Mannheim: AluI, ApaI, BamHI, BglII, DraI, EcoRI, HindIII, MluI, NcoI, PvuII, SacI, SacII, SalI, SmaI, SnaBI, XbaI, XhoI

Radioactive Isotopes

From Amersham: α-^{32}P-dCTP, ^{32}P-dTTP, α-^{32}P-dATP, ^{35}S-Met, stored at -20°C

Others

Bacteria: E.coli (JM109, Stratagene®)

Cell lines: NIH3T3, murine fibroblast cell line; CEM, human T lymphoblastoid cell line (Foley et al., 1965); K562, human multipotential, hematopoietic malignant cell line (Lozzio and Lozzio,1975); Raji, Burkitt lymphoma derived lymphoblast-like cell line (Pulvertaft, 1964); CHO, Chinese hamster ovary line (Puck, T.T., 1958); DG44, CHO line lacking DHFR activity (Urlaub and Chasin, 1980); E86, ecotropic retroviral packaging cell line (Markowitz et al., 1988a); AM12, amphotropic packaging cell line (Markowitz et al., 1988b); WEHI-3B, murine myelomonocytic leukemia cell line (Ralph and Nakoinz, 1977).

Antibody: Rabbit anti-human DHFR polyclonal antibody

prepared by Dr. Srimatkandada

Animal: mouse (CBA/J 7-11 week old, male)

Retroviral Vector Construction (General Techniques)
Plasmid DNA preparation

A) miniscale plasmid DNA preparation: modified from Holmes and Quigley (1981). Bacteria were grown in 2 ml L-Broth (1% Bacto tryptone, 0.5% Bacto Yeast, 0.5% NaCl) containing 50 μg/ml Ampicillin in a 4 ml snapcap polypropylene tube overnight at 37°C in an incubator shaker (220-230 RPM), transferred to a 1.5 ml eppendorf tube and pelleted for 2 min in a microfuge. The pellet was resuspended in 200 μl of lysis solution (8% Sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris-Cl, pH 8.0, and with freshly added Lysozyme 0.75 mg/ml). The suspension was boiled for 1 min and centrifuged for 10 min at room temperature in a microfuge. The pellet was removed with a toothpick and the DNA was precipitated from the supernatant by addition of 2.5 M sodium acetate to a 0.25 M final concentration and 250 μl isopropanol. After incubation for 10 min at -20°C, the DNA was pelleted by spinning in a microfuge for 10 min. The pellet was washed with 70% ethanol and resuspended in 30 μl TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with 1 μg /μl RNAse and incubated at 37°C for 30 min. The DNA solution at this stage may be used for restriction digestion and gel analysis.

B) Large scale plasmid DNA preparation: The alkaline extraction procedure (Birnboim and Dolly, 1979) with some modifications was followed to prepare plasmid DNA from bacteria.

250 ml L-Broth with 50 μg/ml Ampicillin was inoculated with 2.5 ml (1/100) of 5-6 hr bacterial culture (as described in 2.2.1 A) and incubated overnight at 37°C in a shaker (220-230 RPM). The bacterial culture was transferred to a 250 ml plastic bottle and centrifuged at 6000 RPM for 10 min at 4°C. The pellet was resuspended in 5 ml ice-cold Alkaline Lysis Solution I (ALSI: 50 mM Glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA), containing 5 mg/ml lysozyme freshly dissolved and was transferred into a 50 ml plastic tube. 10 ml ALS II (0.2 N NaOH, 1% SDS, freshly made) was added and the mixture chilled on ice for 10 min before the addition of 7.5 ml of ice-cold ALS III (5 M potassium acetate, pH 4.8). The mixture was chilled on ice for another 30 min after thorough mixing. The mixture was then spun at 16,000 RPM for 40 min at 4°C. The DNA was precipitated out from the supernatant by the addition of 12 ml isopropanol and incubation at room temperature for 15 min, and then by centrifugation at 16,000 RPM for 15 min at room temperature. The DNA pellet was resuspended in 5 ml TE with 20 μg/ml RNAse and incubated at 37°C for 30 min.

A Quiagen column-500 (Quiagen Inc) was used to purify the plasmid DNA according to the protocol provided by manufacturer with some modifications. 5.5 ml of 5 M NaCl and 2.5 ml of 1 M MOPS, pH 7.0, was added to the DNA solution prepared from a bacteria culture (maximum 500 ml) in 33 ml total volume. The mixture was passed at a maximum flow rate of 3 ml/min through the Quiagen pack-500 column pre-equilibrated with 5 ml Quiagen Buffer A (400 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0). The column was then washed with 20 ml Quiagen Buffer C (1M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0). The plasmid DNA was eluded from the column with 5 ml Quiagen Buffer F (1.5 M NaCl, 50 mM MOPS and 15% ethanol, pH 7.5) at a

maximum flow rate of 2 ml/min. The purified plasmid DNA was precipitated from the eluate by the addition of 4 ml (4/5 volume) of isopropanol, freezing at -20°C for 30 min, and centrifugation (10,000 RPM for 15 min at 4°C). The DNA pellet was resuspended in 1 ml TE and phenol extraction was performed twice followed once by phenol-chloroform extraction (in 1:1 ratio). Traces of phenol and chloroform were removed by ethanol precipitation (250 mM sodium acetate and 2.5 volumes of ethanol). After incubation at -70°C for 30 min the DNA was pelleted by spinning in a microfuge for 15 min at 4°C. The pellet was washed with 70% ethanol and resuspended in TE.

Restriction enzyme digestion

A) miniscale digestion with restriction enzymes
0.2-1 µg of DNA in 17 µl water was mixed with 2 µl 10 x digestion buffer in a sterile Eppendorf tube. 0.5 to 1 µl of restriction enzyme was added to the mixture. The mixture was incubated at 37°C for 1 hr before addition of EDTA to a final concentration of 10 mM to stop the reaction. The DNA solution can be used directly to analyze the digestion pattern on a minigel (see 2.2.3).

When more than one restriction enzyme was used in the digestion, the enzymes were added to the digestion mixture at the same time provided the ionic strength of the digestion buffers recommended for each enzyme was the same. If not, the enzyme requiring low ionic strength digestion condition was added to the mixture first. After the first digestion was completed, the ionic strength in the mixture was increased according to the requirement of the second enzyme and so on. In case of some enzymes with altered cleavage sequence specificity under non-optimal conditions ("star" activity, Polisky, B., et al., 1975),

heat-inactivation (65°C, 20 min) was performed to inactivate the enzyme after the first reaction was completed before increasing the ionic strength for the next enzyme.

B) Large-scale restriction digestion

For purposes of preparation or in Southern analysis, more than 10 μg DNA was usually used for digestion. DNA was digested at a final concentration of less than 0.1 μg/μl, with 1/10 volume of 10 X digestion buffer and with 4-5 units of restriction enzyme per μg of DNA. The reaction was usually performed overnight at 37°C and terminated by addition of 10 mM EDTA.

Purification of DNA by gel electrophoresis

A) Preparation of agarose gel (Maniatis et al, 1982)

The agarose powder was added to electrophoresis buffer (0.5 X TBE: 45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA, pH 8.0) at a final concentration of 1%. The mixture was heated in a microwave oven until the agarose dissolved. After cooling to 50°C, ethidium bromide (from a stock solution of 10 mg/ml in water, stored at 4°C in a light-proof bottle) was added to a final concentration of 0.5 μg/ml. The solution was poured into the gel mold (50-60 ml for minigel of 4 x 6", 200-300 ml for larger gel of 6 x 10") and kept at room temperature for 30 min until the agarose was solidified.

B) Agarose gel electrophoresis

The gel was immersed in electrophoresis buffer (0.5 x TBE) in the electrophoresis tank to which a power supply was connected. When the electrophoresis was performed using the large gel, the buffer was recirculated by a pump. For separation of DNA fragments generated by

large-scale restriction digestion, phenol-chloroform extraction and ethanol precipitation (see 2.2.1 B) were carried out before the DNA was loaded on the gel with 1/6 volume of 6 x loading buffer (0.25% bromophenol blue, 0.25% xylenecyanol, 30% glycerol in water).

The DNA fragments were visualized by fluorescence of the ethidium bromide (Sharp et al, 1973) which emits at 590 nm in the red- orange region of the visible spectrum when irradiated with UV light of 360 nm wavelength. The desired DNA fragment was cut out with a razor blade and the DNA was electroeluted from the agarose gel by the following method.

C) Electro-elution of the separated DNA fragment
An electro-elutor (Model Uea, International Biotechnologies Ins.) was used to elute the DNA fragment from the agarose gel. The gel slices were placed in the wells connected to channels filled with 100 µl 10 M ammonium acetate. The power supply was connected to the electroelutor box filled with 0.5 X TBE buffer. After the elution, the DNA fragment trapped in the channel was carefully removed and was precipitated by addition of ethanol, freezing at -20°C followed by centrifugation. The DNA pellet was resuspended in TE and another round ethanol precipitation with 0.25 M sodium acetate was carried out to remove traces of the ammonium acetate salt from the DNA solution.

D) Quantitation of DNA was done either by measuring the absorption at 260 nm (The DNA concentration in the solution was calculated by the following formula: OD at 260 nm x 50 x dilution factor = [DNA] µg/ml) or by comparing the density of the red-orange fluorescence of the ethidium bromide bound to the DNA fragment on a

minigel with the density of a DNA marker of known quantity.

Klenow and ligation

Blunt end ligation was used in retroviral construction. A Klenow reaction was conducted to fill the recessed 3′ ends and to degrade the protruding 3′ ends (Wartell and Reznikoff, 1980). The Klenow fragment of DNA polymerase I (E.coli) was used in the reaction, which has the 5′ to 3′ DNA polymerase activity and the 3′ to 5′ exonuclease activity. The reaction mixture contained 0.2 mM of each deoxynucleotide, 1/10 volume of 10 x Klenow buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM DTT, pH 7.5), 1-5 μg of the purified DNA fragment, and 1-5 U Klenow enzyme in a total volume of 200 μl. After incubation at room temperature for 30 to 60 min, the reaction was stopped by adding EDTA to 10 mM. Then, phenol-chloroform extraction and ethanol precipitation were performed to remove the unincorporated nucleotides as well as the protein before proceeding to the ligation reaction.

The ligation reaction was catalyzed by T4 DNA ligase (Weiss et al., 1968). The reaction mixture contained 1 mM ATP, 1/10 volume of the 10 x ligation buffer (250 mM Tris-HCl, 100 mM $MgCl_2$, 50 mM DTT, pH 7.5), the insert and the vector DNA resuspended in water in a molar ratio of 20-40 to 1 (up to 1 μg DNA per 10 μl), and 1 U ligase in a total volume of 10 μl. The reaction was performed at room temperature overnight. The mixture was diluted 3 times to transform bacteria. In some cases two sequential ethanol precipitations first with 3 M ammonium acetate and then with 250 mM sodium acetate were performed before the DNA ligation solution was used for bacterial transformation.

Bacterial transformation

Electroporation was used to transform E.coli (JM109 bacteria strain). Preparation of the competent cells and the electro-transformation was carried out according to the protocol provided by the manufacturer (Bio-Rad).

A) Preparation of cells

One liter of L-broth was inoculated by 10 ml (1/100 volume) of a fresh overnight bacterial culture. The cells were grown at 37°C with shaking (220-230 RPM) for several hours until the OD reading at 600 nm was between 0.5 and 1 (when the cells were in the log phase of growth). The cells were then harvested by chilling the flask on ice for 15 min and centrifuging in a prechilled rotor at 4,000 x g for 15 min at 4°C. The pellets were resuspended in an equal volume of ice-cold sterile water and were centrifuged again at the same setting. The pellets were then resuspended in half volume of ice-cold water and the above centrifugation was repeated. The pellets then were resuspended in 20 ml 10% glycerol and recentrifuged. The cells were resuspended in a final volume of 2-3 ml in 10% glycerol at a concentration of 3 x 10^{10} cells/ml. The suspension was either frozen in aliquots on dry ice and stored at -70°C, or was used immediately for the electro-transformation.

B) Electro-transformation

The cells were thawed at room temperature and kept at 4°C on ice. 40 μl of the cell suspension was mixed with 1 to 2 μl of recombinant DNA from the ligation reaction in a cold 1.5 ml polypropylene tube and incubated on ice for 1 min. The DNA was in a low ionic strength buffer (TE). The Gene Pulser apparatus was set at 25 μF and 2.5 kV and the Pulse Controller at 200 Ω. The electroporation was

performed in a pre-chilled 0.2 cm electroporation cuvette with a single pulse. The time constant was between 4.5 to 5 msec. Immediately after electroporation 1 ml of SOC (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the cuvette and the cells were quickly resuspended and transferred to 4 ml polypropylene tube and incubated at 37°C for 1 hr with 220-230 RPM shaking. The cells were then plated on selective plates (1% Bacto Tryptone, 0.5% Bacto Yeast, 0.5% NaCl, 1.5% Bacto Agar, containing 50 μg/ml Ampicillin).

Diagnosis of the clones containing the correct construct

Bacterial colonies growing on the selective plates were picked with sterile toothpicks. These individual colonies were used to inoculate 2 ml L-Broth and the miniscale plasmid DNA preparation procedure was followed to isolate 30 μl DNA solution (2.2.1 A). 4 to 5 μl of this solution was used in a 20 μl restriction digestion mixture (2.2.2). At least 2 restriction digestion patterns were chosen to determine if the structure of the construct was correct. Usually it was determined first whether the construct contained the insert gene, and then the direction of the insert was determined, which was essential for checking constructs generated by blunt-end ligation.

Freezing and storage of the bacterial colonies

Two bacterial colonies containing the correct construct were grown up individually in 2 ml selective medium (2.2.1A) for 4 to 5 hr and were frozen in 15% glycerol at -70°C in 1 ml aliquots.

The Construction of Retroviral Vectors Carrying a Mutant DHFR cDNA

Construction of retroviral vectors (Figs. 6A-6D)

The Moloney murine leukemia virus-based N2 retroviral vector(Armentano et al., 1987), which contains the bacterial transposon Tn5 neomycin resistance gene (NEO), was modified by the insertion of a 52-bp polylinker (containing the unique restriction sites 5'-ApaI-BglII-SnaBI-ScaII-MluI-3') into the NheI restriction site present in the U3 region of the 3'LTR. The polylinker-modified N2 vector was designated as N2A (Hantzopoulos et al., 1989). The N2A vector was further modified by insertion of a 275 bp poly A fragment into the ApaI restriction site in the anti-parallel orientation of the viral transcriptional unit; the poly A fragment was obtained from plasmid PBC12/CMV/IL2 (Gansbacher et al., 1990) by restriction digestion with SmaI and EcoRI, followed by AluI. The modified vector containing the poly A fragment in the anti-parallel orientation is designated as N2AP. The SV40 early (SV) promoter was cloned into the BglII site of N2A in a parallel orientation, generating the vector DC/SV, and into the MluI site of N2AP in an anti-parallel orientation, generating the vector DC/SV/R (DC stands for double copy, see Result 3.1.1; R stands for reverse). The SV promoter fragment was from pFR400 (Simonsen and Levinson, 1983), digested with KpnI and XbaI. The human ß-actin promoter was cloned into the BglII site of N2A vector, generating the vector DC/AC; the promoter fragment was from p14T-ß17 (Gunning et al., 1987), digested with BamHI and SacI. The human adenosine deaminase (AD) promoter fragment was cloned into the MluI site of N2AP in the anti-parallel orientation, generating the vector DC/AD/R; the AD

promoter fragment was from the 2.2 ADA plasmid (Wiginton et al., 1986). It was digested with SspI/NcoI, followed by Mung bean nuclease and Klenow modification to eliminate the ATG codon in the SspI/NcoI fragment. The Herpes virus thymidine kinase (TK) promoter and the cytomegalovirus (CMV) promoter were cloned similarly into the MluI site of N2AP, generating the vector DC/TK/R and DC/CMV/R. The TK promoter was obtained from the pHSV-106 plasmid (Mcknight and Gavis, 1980), digested with BamHI and BglII. The CMV promoter was obtained from pBC140, digested with HincII and XhoI.

Construction of retroviral vectors carrying murine mutant DHFR (Figs. 7A-7E)

The murine mutant DHFR cDNA was obtained from pFR400, containing the 3T6 DHFR with a T to G point mutation at nucleotide 68 resulting in a Leu to Arg change at residue 22 (Simonsen and Levinson, 1983). In the construct designated as DC/SV-mDHFR, the DHFR cDNA plus SV40 promoter was excised from pFR400 by restriction enzyme PvuII and SacII and was cloned into the SnaBI site in N2A in a parallel orientation. In the construct designated as DC/SV/R-mDHFR, the minigene fragment containing the poly A signal was excised from pFR400 by the restriction enzymes PvuII and SalI and was cloned into the SnaBI site of N2A in an anti-parallel orientation. DHFR minigene fragments in both the DC/SV-mDHFR and DC/SV-mDHFR constructs contained the SV40 promoter.

In the construct designated as DC/AC-mDHFR, the DHFR fragment excised from pFR400 by HindIII and NcoI restriction enzymes was cloned into the SnaBI site of DC/AC in the parallel orientation. In the construct designated as DC/AD/R-mDHFR, DC/TK/R-mDHFR and DC/CMV/R-

mDHFR, the DHFR fragment was cloned into the SnaBI site of DC/AD/R, DC/TK/R and DC/CMV/R in a reverse orientation.

Construction of retroviral vectors carrying mutant human DHFRs (Figs. 8A-8H)

The mutant hDHFRs used in these constructs were S31 and S34 which contain a T to G point mutation at nucleotide 95 or 104 resulting in a Phe to Ser change at residue 31 or 34 respectively (Schweitzer, B.I. et al., 1989).

The full length cDNA of mutant hDHFR including the 560bp coding region and 240 bp 3' untranslated region was obtained from pKT7HDR (Schweitzer, B.I. et al., 1989). The cDNAs were excised by restriction enzyme NcoI and HindIII, were blunt ended by the Klenow reaction and cloned into the SnaBI site of DC/SV in a parallel orientation, and the SnaBI site of DC/SV/R and DC/AD/R in antiparallel orientation. These constructs were named DC/SV-hDHFR31/34, DC/SV/R-hDHFR31/34, and DC/AD/R-hDHFR31/34 respectively.

The mutant hDFHR cDNA (Phe to Ser at residue 31) less than full length, containing the coding region and 95 bp of 3' untranslated region, were obtained from pKT7HDR by restriction digestion with NcoI and BglII. The fragments were Klenowed and cloned into the SnaBI site of DC/SV, DC/SV/R, and DC/AD/R, generating DC/SV-hDHFR31 NB, DC/SV/R-hDHFR31 NB, and DC/AD/R-hDHFR31 NB.

In the pKT7HDR construct, the 5' untranslated region of the cDNA immediately before the starting codon ATG was modified to generate a NcoI site for cloning and mutagenesis experiments (Schweitzer, B.I. et al., 1989).

In order to avoid a possible negative effect of this modification on the expression of the mutant hDHFR, the hDHFR cDNA obtained from pSV4HDR which contains the internal mutation at residue 31 (Phe to Ser) but with an unmodified 5′ end was also used. The pSV4HDR was digested with HindIII and BglII and the fragment was blunt ended by the Klenow reaction and cloned into the SnaBI site of DC/SV, and DC/SV/R, generating the DC/SV-hDHFR31 HB and DC/SV/R-hDHFR31 HB constructs.

Production of Live Virus Packaging

The vector DNA was packaged into the retrovirus by transfecting a packaging cell line with the vector DNA by electroporation using a modified protocol from the manufacturer (Bio-Rad). Cells in log phase (80% confluence) were trypsinized and resuspended in DME medium with 10% FBS at the density of 1.5×10^6 cells/ml at room temperature. 2 μg of vector DNA (in a supercoiled circular form) was mixed with 0.5 ml of the cell suspension in a 0.4 cm sterile electroporation cuvette and incubated at room temperature for 10 min. Electroporation was performed with a gene pulser (Bio-Rad) set at 200 volts and the capacitance extender set at 960 μF. The time constant under these conditions was between 23 and 27 μs. After 10 min incubation at room temperature 2 ml DME with 10% FBS was added to the curvet and the cell suspension plated in a 60 mm petri dish and incubated at 37 °C in a CO_2 incubator for 48 hr before selection was applied. Transfected cells were selected with G418 (0.75 mg/ml). The selection medium was changed every 3 to 4 days. After 8 to 10 days of selection, the G418 resistant colonies were large enough to be isolated by ring-cloning and expanded into producer cell lines.

Both ecotropic and amphotropic producer cell lines were used to package the vector DNA. Ecotropic producer lines were obtained by direct electro-transfection of the E86 ecotropic packaging cell line (Markowitz et al., 1988a). Amphotropic producer lines, however, were obtained either by ecotropic virion infection (see 2.5) of an amphotropic packaging cell line AM12 (Markowitz et al., 1988b) or by direct electro-transfection as described above.

Virus-containing supernatant collection

The viral producer cells were grown in a petri dish till 80% confluence, and the medium was replaced by fresh medium (4 ml for a 60 mm petri dish, 10 ml for 100 mm petri dish). 12 hr later, the medium was collected from the petri dish and centrifuged at 3,000 RPM at 4°C for 10 min. The supernatant was carefully removed from the cell debris pelleted at the bottom of the centrifuge tube, and stored at -70°C.

Viral titering

The viral titer is defined as the number of infectious particles in 1 ml of virus containing supernatant. Because the viral vector carries the Neo gene and $DHFR^r$ gene which would render the infected cell resistant to G418 and MTX, the viral titer can be determined by the number of G418 or MTX resistant colonies resulting from the viral infection of NIH 3T3 cells (see 2.5.1).

The viral supernatant was diluted 1 to 10 and 1 to 1000 in medium containing 8 μg/ml polybrene, and 1 ml of the diluted supernatant was used for the assay. After 2-3 hr incubation and 8 to 10 days of G418 or MTX selection, the number of resistant colonies was counted and the

viral titer calculated. The viral titer for both ecotropic and amphotropic producer cell lines was approximately 4 x 10^4 - 5 x 10^5 NEO and MTX resistant colony forming units/ml.

Freezing and storage of the producer cell lines

Producer cell lines were frozen in DME medium containing 50% FBS and 10% DMSO, and stored in liquid nitrogen.

Target Cell Infection
NIH 3T3 cells

TK$^-$NIH 3T3 fibroblasts were plated at a density of 10^5 cells/ 60 mm petri dish the night before the infection. Supernatants collected from the producer lines were used to infect these cells in the presence of 8 μg polybrene per ml for 2-3 hr at 37°C. Parallel selections in G418 (0.75 mg/ml) and MTX (1-2 x 10^{-7} M) started 24 hour post infection. After 8-10 days the resistant colonies were counted and the G418 resistant colonies were pooled and expanded in drug-free media for use in subsequent experiments. The vector-transduced cell lines were free of replication-competent virus, even after extensive culturing in vitro, as determined by absence of reverse transcriptase activity in the culture medium (Figure 9, see 2.6).

Human leukemia cells

Human leukemia cells (CEM, K562, Raji) were incubated for 3 hours with the amphotropic producer supernatant in the presence of 8 μg polybrene per ml. The 3 hour infection was repeated after overnight incubation in virus-free medium. The selection with G418 (0.75 mg/ml) was started

24 hr after the second infection. After 2-3 weeks in G418 the resistant cells were expanded in drug-free medium for subsequent experiments.

Mouse bone marrow cells

Bone marrow cells from CBA/J 7-11 week old male mice were harvested in IMDM medium and a mononucleated cell suspension was prepared. The bone marrow cells were cocultured with producer cells irradiated with 1500 R 2 hr before the coculturing. The coculture was carried out in IMDM medium containing 20% FCS, 10% WEHI-3B conditioned medium (See 2.13) and 8 μg/ml polybrene for 48 hr in 37°C CO_2 incubator with a 1:1 starting ratio of marrow cells to producer cells. The bone marrow cells were then either used for colony forming assays or transplanted to recipient mice.

Reverse Transcriptase Assay

The activity of the reverse transcriptase was measured by synthesis of poly T in the presence of poly A template (Goff et al., 1981). The sample to be measured was mixed with RT cocktail containing 50 mM Tris-HCl, pH 8.0, 20 mM DTT, 0.6 mM $MnCl_2$, 60 mM NaCl, 0.05% Nonidet P-40, 5 μg/ml Oligo(dT) primer, 10 μg/ml poly (A), 10 μM dTTP, 1 μl α-^{32}P-dTTP (3000 Ci/mMole). The final volume was 50 μl and the incubation was carried out at 37°C for 1-2 hr. The reaction mixture was added to DEAE filter paper which was washed with washing solution (2 x SSC) at room temperature twice for 15 min. The filter paper was then rinsed with ethanol and exposed to x-ray film or counted in a scintillation counter.

DNA Analysis

Genomic DNA preparation from cultured cell lines

Chromosomal DNA was prepared by the guanidinium isothiocyanate extraction procedure and centrifugation through a CsCl cushion (Chirgwin et al., 1979). Confluent cells from two 100 mm petri dishes were washed twice with PBS. 2.5 ml GTC solution (4 M Guanidinium Thiocyanate, 25 mM Sodium Citrate, 0.5% Sarkosyl, 0.1 M ß-Mercaptoethanol, stored in a dark bottle at room temperature) was added to each dish. The cell extracts were pooled after 5-10 min of shaking at room temperature. 2 g of CsCl was added to the 5 ml extract and dissolved by gentle shaking and incubation at 37°C. The cell extract was loaded on top of the 6 ml CsCl solution cushion (5.7 M CsCl, 0.1 M EDTA, pH 7.5) in a 14 x 89 mm centrifuge tube (Beckman). After 16-18 hr centrifugation at 30,000 rpm in room temperature, the DNA layer was saved (about 2 ml in volume) and diluted to 5 ml with water. 10 ml ethanol was added to the solution to precipitate the DNA, which was spooled out with a curved pipet, rinsed in 70% ethanol, and resuspended in 5 ml water overnight at 37°C. The DNA was quantitated by measuring the absorbance at 260 nm (see 2.2.3 D).

Genomic DNA preparation from mouse organs

Genomic DNA from whole animal organs and tissues was prepared by the proteinase K digestion method in a SDS denaturing buffer, followed by phenol-chloroform extraction (Gross-Bellard et al., 1972 and Enrietto et al., 1983). The organs were frozen in liquid nitrogen immediately after removal from the animal and ground to a fine powder with a prechilled mortar and pestle. The powdered tissue was suspended in digestion buffer (100 mM

NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA, pH 8, 0.5% SDS, and freshly added proteinase K at 0.1 mg/ml). The suspension was incubated at 50°C overnight with shaking. Phenol-chloroform extraction was performed, followed by ethanol precipitation with 2.5 M ammonium acetate. The DNA was pelleted by centrifugation at 1700 g for 2 min. The pellet was washed with 70% ethanol, air dried and resuspended in TE buffer.

Genomic DNA preparation from CFU-GM colonies

5-6 CFU-GM colonies (50-200 cells per colony) were aspirated and pooled and diluted 1 to 5 with PBS in an 0.5 ml eppendorf tube. The cells were centrifuged at 2,000 rpm and washed once with PBS. The cells were then lysed by boiling for 5 min in 20 µl sterile water, and centrifuged in a microfuge (15,000 rpm) for 5 min. The supernatant was used as the source of DNA for PCR amplification (See 2.9.1).

Southern analysis

A) Restriction digestion, gel electrophoresis and membrane transfer

DNA was digested with restriction enzymes (see 2.2.2 B), and fractionated by electrophoresis on a 1% agarose gel (10-15 µg/line, see 2.2.3 A,B) overnight with circulating buffer. The gel was then soaked in alkaline solution (0.2 N NaOH, 0.6 M NaCl) with gentle shaking for 20 min to denature the DNA, rinsed in water, and equilibrated with electro-transfer buffer (25 mM sodium phosphate, pH 6.5) before being transferred to a nylon membrane (Biotrans, ICN) with an electroblotter (Hoefer) for 4-5 hr at room temperature with cooling water circulation. The DNA was UV cross-linked to the membrane with a

UVstratalinker (Stratagene).

B) Synthesis of radio-active probe

The ^{32}P-labeled specific probe was generated by an Oligolabelling Kit (Pharmacia). DNA (25-50 ng) was first denatured by boiling for 2-3 min. It was then immediately chilled on ice and mixed with hexadeoxyribonucleotides of random sequence which anneal to random sites on the DNA and serve as primers for DNA synthesis by the Klenow Fragment of E.coli DNA polymerase I. ^{32}P-dCTP and three other nonlabelled nucleotides were present in the synthesis which was carried out at 37°C for 1 hr. The labelling reaction was stopped by addition of EDTA and the unincorporated nucleotides were separated from the labelled probe by passing the reaction mixture through a prepacked G-50 sephadex column (Boehringer Mannheim).

C) Hybridization and washing

The nylon membrane was prehybridized in Church and Gilbert buffer (1% crystalline BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% SDS) for 5-10 min at 65°C, before the ^{32}P-labeled specific probe was added. The hybridization was carried on overnight at 65°C. The membrane was then washed with wash I buffer (2 x SSC, 0.1% SDS) 30 min twice at room temperature and with wash II buffer (0.2 x SSC, 0.1% SDS) 30 min twice at 65°C. The washing procedure was monitored by a hand held Geiger counter. The membrane was exposed to an X-ray-sensitive film (Kodak XAR5) in the presence of intensifying screens at -70°C.

RNA Analysis

All procedures were performed in autoclaved and 0.1%

diethylpyrocarbonate (DEPC) treated glassware, and sterile disposable plasticware. All solutions were either prepared in DEPC treated water or were treated with DEPC before use. DEPC is a strong inhibitor of RNAse was added to solutions or water at a concentration of 1%, and allowed to stand for 12 hr, and then autoclaved to inactivate the remaining DEPC (Kumar and Lindberg 1972). Gloves were used for all of the experiments.

Total cellular RNA preparation

Total cellular RNA was prepared by the guanidinium isothiocyanate extraction procedure and centrifugation through a CsCl cushion (see 2.7.1). The RNA pellets after the 16-18 hr centrifugation were washed gently with 70% ethanol, air dried and resuspended in 300 µl water. Ethanol precipitation with 0.5 M NaCl was performed and the RNA was either used immediately or stored in ethanol in -20°C.

Poly(A) selection for mRNA

Total cellular RNA from 5 x 10^7 cells was incubated with Oligo(dT) cellulose (Collaborative Research Incorporated) for 2-3 hr with gentle shaking at room temperature, at a concentration of 25 mg cellulose/ml of the loading buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS). The RNA-Oligo suspension was packed a in plastic disposable column (Bio-Rad) and washed with Oligo(dT) washing buffer (100 mM NaCl, 10 mM Tri-HCl, pH 7.4, 1 mM EDTA, 0.2% SDS). The poly (A) fractions were eluted at 37°C with elution buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% SDS). Ethanol precipitation was performed to pellet the poly $(A)^+$ RNA. The RNA was quantitated by

measuring the absorbance at 260 nm with the following formula: OD_{260nm} x 30 x dilution factor = [RNA] μg/ml.

Northern analysis

The poly $(A)^+$ RNA fraction was resuspended in sample buffer (50% formamide, 2.2 M formaldehyde, 1 X running buffer, 0.4% bromphenol blue), loaded on agarose/formaldehyde gel (1% agarose, 2.2 M formaldehyde), and subjected to electrophoresis at 40 volts with circulating running buffer (20 mM MOPS, 5 mM NaAc, 1 mM EDTA) overnight at room temperature. The RNA was transferred to a nylon membrane and hybridized to a ^{32}P labelled probe (see 2.7.3).

Polymerase Chain Reaction (PCR) and Sequencing
PCR analysis

PCR analysis (Mullis, K.B. and Faloona F.A., 1987) was carried out in a DNA thermal cycler (Perkin Elmer Cetus), for 40 cycles in 50 ul reaction mixtures containing genomic DNA prepared from mouse tissues or from CFU-GM colonies (see 2.7.2, 2.7.3), 1.25 mM of each dNTP, 1 X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin), 1 μl of each primer (300 ng/μl), and 0.7 μl Taq polymerase (Perkin Elmer Cetus). The sequence of primers used in various experiments are described in Results. The reaction mixture was overlayed with 50 μl of mineral oil to prevent evaporation and the following thermocycle profiles were programmed: one cycle of initial denaturation at 94°C for 3 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min was followed by 40 cycles of 94°C 1 min, 55°C 1 min, and 72°C 1 min. Detection of the products was carried out by agarose gel electrophoresis and by blot hybridization

analysis (see 2.7.4).

Sequencing

Direct sequencing of the PCR products was carried out using mostly single stranded DNA products generated from asymmetric PCR using a ratio of 1 to 50 of the primers (Dicker, A.P. et al., 1989). Sequence analysis was performed by the dideoxy chain termination method (Sanger, F.W. et al., 1977) with α- ^{35}S dATP using a modified T7 DNA polymerase (Sequenase version 2.0, United States Biochemicals) according to the manufacturer's instructions. After completion of the reaction, the products were heated to 95°C for 3 min, cooled on ice and loaded on a prewarmed 6% polyacrylamide urea gel. Electrophoresis was carried out at 60 W for 2 hr after which the gel was dried and exposed to Kodak XAR5 film for 16 hr.

DHFR Protein Immunoprecipitation
Cell labelling and cell extraction

Cells of 80-90% confluence were incubated in Met-free medium for 1 hr in a 37°C CO_2 incubator and the medium was replaced by Met-free medium containing 0.3 mCi/ml ^{35}S-Met (0.5 ml/60 mm petri dish). After incubation for 3-4 hr with occasional shaking, the cells were washed 2 times with PBS, and the cell extracts made by adding 0.6 ml ice cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% Na-Deoxycholate, 0.1% SDS) with freshly added protease inhibitors (PMSF 0.2 mg/ml, Leupeptin 0.05 mg/ml) per 60 mm dish. The lysed cells were sonicated in an ice bath, 30 sec x 2 with a 10 sec interval and centrifuged for 30 min in a microfuge at 4°C. The supernatants (about 0.5 ml) were stored at -70°C or

used fresh.

TCA precipitation

TCA precipitation was used to quantitate the concentration of the labelled protein. 5 µl of cell extract was mixed with 100 µl BSA (1 mg/ml) and 1 ml 10% TCA and incubated on ice for 30 min. The sample was passed through Whatman filter paper (934 AH) pre-equilibrated with 10% TCA. The filter was rinsed twice with 10% TCA and 95% ethanol, air dried and 5 ml scintillation fluid (BCS, Amersham) added and radioactivity measured in a scintillation counter.

Preparation of protein A sepharose-Ab complex

Rabbit anti-human DHFR antibody was used for immunoprecipitations. Protein A sepharose (PAS) was allowed to swell in sepharose buffer (20 mM Tris-HCl, pH 7.5) at a concentration of 6 mg/ml and washed 3 times in the same buffer by centrifugation at 1,700 g for 3 min. The antibody was added to the sepharose suspension at various dilution and the mixture was rotated at 4°C for 2-3 hr. The protein A sepharose-Ab complex was pelleted and washed 3 times with Wash A buffer (20 mM Tris-HC, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). Aliquots (0.5 ml) were pipetted into 1.5 ml eppendorf tubes and spun down.

PAS-Ab-Antigen complex formation and purification

PAS-Ab complex pellets were suspended in the equal amount of labelled cell extracts (see 2.8.2) in a volume of 0.5 ml. The mixtures were rotated at 4°C overnight to allow the Ag-Ab reaction to take place. The PAS-Ab-Ag complex

was pelleted by centrifugation at 4°C for 5 min in a microfuge. The pellet was washed with 1 ml Wash B buffer once (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM EDTA, 0.2% Triton X-100), Wash C buffer 3 times (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1% SDS), and Wash D buffer once (10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100).

SDS polyacrylamide gel electrophoresis

The PAS-Ab-Ag washed pellet was resuspended in sample buffer (250 mM Tris-HCl, pH 6.8, 50% glycerol, 5% SDS, 5% ß-mercaptoethanol, 0.25% bromophenol blue, 20 µl/sample), boiled for 10 min, chilled on ice, spun briefly, and the supernatant was loaded on an acrylamide minigel (7 cm x 8 cm), consisting of the separating gel (15% acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.4% N,N'-Methylene-bis-acrylamide (Bis)) and the stacking gel(3% acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.08% Bis). The gel electrophoresis was run at 200 volts, for 45-50 min in SDS-PAGE buffer (25 mM Tris-HCl, pH 8.3, 0.192 M Glycine, 0.1% SDS). The gel was fixed in 7% Acetic acid and 25% Methanol for 20 min at room temperature with gentle shaking, enhanced with Enhancer (NEN) for 30 min, rinsed with water, dried with a gel drier (Bio-Rad, Model 583) at 60°C for 1 hr, and exposed to X-ray film at -70°C with intensifying screens.

MTX Cytotoxicity Assays

Colony formation assay for 3T3 cells

Parental and the transduced 3T3 cells pooled from the G418 resistant colonies were plated at 10^3 cell/100 mm plate. Various concentrations of MTX were added after 16-18 hr. Drug-containing media was changed every 3-4

days. The resistant colonies were scored 8 to 10 days after MTX treatment.

Cell growth inhibition assay for leukemia cells

Exponentially growing parental and transduced human leukemia cells (CEM, K562, Raji) were exposed to different concentrations of MTX at an initial density of $2x10^5$ cells per ml. The cells were counted after 3 days of treatment with a hemocytometer after trypan-blue staining to exclude dead cells. The fetal bovine serum used in the MTX selection medium was treated with thymidine phosphorylase at 6 units per ml for 5 min at 37°C to reduce the background in the presence of MTX (Li et al., 1990).

Granulocyte-Macrophage (CFU-GM) assay for mouse bone marrow progenitor colonies

Immediately after coculture (see 2.5.3) and at regular intervals after bone marrow transplant(see 2.12), CFU-GM assays were performed. 10^5 marrow cells were plated in a grid petri dish (10 x 35 mm) in 2 ml of methylcellulose IMDM cocktail medium (1% α-methylcellulose, 20% FCS treated with thymidine phosphorylase, 10% WEHI-3B CM, 1% $NaHCO_3$, 1% Na-pyruvate, 1 μM ß-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% essential amino acids, 1.5% nonessential amino acids, 0.5% vitamin C). MTX of different concentrations was added to the cocktail medium. The CFU-GM colonies greater than 50 cells were scored after 12-14 days culture in a 37°C CO_2 incubator.

Bone Marrow Transplantation (BMT)

2×10^6 donor marrow cells suspended in 0.2 ml IMDM, after coculture with an amphotropic virus producing cell line (see 2.5.3) were injected through the tail vein into recipient mice irradiated with 900 rads 24 hr before the BMT. In vivo selection with MTX was performed with different dose schedules (see Chapter 3 Results). A typical MTX treatment protocol was as follows: one day after BMT, MTX was given twice weekly i.p. at 2 mg/kg body weight for the first week and 5 mg/kg for the next 7 weeks. Hematocrit, WBC counts, body weight and overall survival rates were monitored throughout the course of the experiment. The recipient mice were provided with water containing streptomycin (1 g/liter).

Cell Line Maintenance

The murine TK$^-$ 3T3 fibroblast lines, and the E86 and AM12 packaging lines were maintained in DME with 10% FBS. Human leukemia lines CEM, K562 and Raji were maintained in RPMI with 10%, 10%, and 15% FBS, respectively. 100 units/ml penicillin and 100 µg /ml streptomycin were also present in the media. Bone marrow culture for retroviral infection (see 2.5.3) and for the CFU-GM assay (see 2.9.3) was performed in IMDM media.

The E86 and AM12 cell lines were selected for 48 hr in selection media after long term storage to maintain the efficiency of packaging. The E86 cell lines were selected in HXM medium (DME with 10% FBS, and 15 µg/ml Hypoxanthine, 250 µg/ml Xanthine, 25 µg/ml Mycophenolic acid) and the AM12 cell lines were selected in HXMB medium (HXM medium with the addition of 200 µg/ml Hygromycin B).

WEHI-3B cells were grown in IMDM medium with 10% FCS. After 3-4 days growth (initial concentration 3 x 10^5 cells/ml), the cells were spun down at 1,200 rpm and the supernatant was collected (WEHI-3B conditioned medium (CM)) and stored at -20°C.

Data Calculation and Analysis

The IC50s were calculated from cell survival studies in the absence and presence of drug using the Median Effect Equation (Chou and Talalay, 1984).

EXPERIMENTAL RESULTS

Vector Design

The construction of double copy (DC) vectors

The retroviral vectors used to introduce the altered DHFR gene into recipient cells are based on the high titer retroviral vector N2, which in turn was derived from the moloney murine leukemia virus containing the bacterial transposon Tn5 neomycin resistance gene (NEO) under the control of the viral LTR promoter (Armentano et al., 1987). The N2 vector was modified by the insertion of a 52-bp polylinker (containing the unique restriction sites 5'-ApaI-BglII-SnaBI-ScaII-MluI-3') into the U3 region of the 3'LTR. The polylinker-modified N2 vector was designated as N2A (Hantzopoulos et al., 1989). The U3 region of the MoMLV 3'LTR serves as the template for the synthesis of both the 5' and 3' U3 regions of the provirus LTRs (Figure 10, see review by Varmus and Swanstrom, 1982). The foreign sequence inserted at this position will be duplicated to the U3 region of the 5'LTR upstream of the LTR initiated transcription unit. Therefore such vectors were termed double copy or DC vectors because in the integrated provirus the foreign sequences are present in two copies (Hantzopoulus et al., 1989; Sullenger et al., 1990). The N2A vector was further modified by inserting a poly A fragment in the ApaI site in the polylinker to allow insertion of foreign sequence in the anti-parallel orientation of the LTR transcription.

In this study, the SV40, human ß-actin, human ADA, herpes virus TK, and the cytomegalovirus promoters were cloned into the polylinker region in either a parallel or an anti-parallel orientation, generating DC vectors

designated as DC/SV, DC/AC, DC/SV/R, DC/AD/R, DC/TK/R and DC/CMV/R. The design of these vectors is outlined in Figs. 6A-6D and described in detail in Materials and Methods (2.3).

The messenger RNA species that would be transcribed from the integrated provirus of the DC vector carrying foreign sequences are shown in Figure 11.

The construction of retroviral vectors carrying a murine mutant DHFR cDNA.

Figs. 7A-7E shows the cloning strategy used to generate retroviral constructs that contain a murine 3T6 DHFR with a Leu to Arg mutation at residue 22 of the DHFR enzyme. In the DC/SV/R-mDHFR construct, the DHFR cDNA should be transcribed from the SV40 promoter in the parallel orientation with a predicted mRNA length of 1.1 kb. In the DC/SV-mDHFR construct the DHFR is transcribed from the same promoter with similar length message as in DC/SV-mDHFR, but in the antiparallel orientation of the viral transcription unit. In DC/AD/R-mDHFR, DC/TK/R-mDHFR and DC/CMV/R-mDHFR, the DHFR transcriptional unit is in the antiparallel orientation and the length of the predicted transcripts is 0.8 kb.

Vector DNA was converted into corresponding virus by the procedure described in Material and Methods.
Note that for all the constructs described above, the promoter-DHFR fragments were inserted into the 3'LTR of N2A or N2AP so that in the infected cell the promoter-DHFR template will be duplicated and be present in both LTRs of the proviral DNA as discussed above. The importance of this duplication is that the second copy of the promoter-DHFR template present in the 5'LTR is placed

outside the viral transcription unit, therefore avoiding possible adverse effects of the active LTR promoter on the promoter-mDHFR transcriptional unit (Emerman and Temin, 1984). This strategy has been previously shown to greatly facilitate the expression of recombinant genes (Hantzopoulos et al., 1989 and Sullenger et al., 1990). Furthermore, in constructs in which the promoter-DHFR was cloned in antiparallel orientation of the viral LTR transcription, the two copies of the template are transcribed against LTR transcription, further reducing the negative effects of the LTR transcriptional unit (Emerman and Temin,1984, Proudfoot, 1986 and Cullen et al., 1984). A similar design of retroviral vectors has resulted in the enhanced expression of the ß-globin gene (Karlsson et al.,1987).

The viral titers determined by the number of G418 resistant colonies following the infection of virus with both parallel or antiparallel constructs were approximately the same, ranging from $4x10^4$ to $5x10^5$ CFU/ml.

Expression and Resistance Produced by the Mutant DHFR under Control of Different Promoters in NIH 3T3 Cells

MTX resistance of the transduced 3T3 cells

The MTX resistance level of the G418 resistant 3T3 cells transduced by the mutant mDHFR viral constructs was determined by measuring the inhibitory effect of MTX on colony formation of 3T3 cells. MTX concentrations that inhibit 50% of colony formation of the transduced cells (IC50) were compared with the non-transduced parental cells to determine the resistance ratio. The DC/SV-mDHFR and DC/SV/R-mDHFR constructs afforded similar levels of

resistance and conferred the highest level of resistance when compared to constructs with promoters other than SV40 (Table 1, see below).

Although the infection of the 3T3 cells with the mutant DHFR constructs give approximately equal number of resistant colonies in either G418 or MTX selection, only the G418 resistant cells were pooled and tested for the level of MTX resistance (IC50) to avoid the complication of increasing MTX resistance due to other mechanisms that might arise during the process of selection.

RNA analysis of the transduced 3T3 cells

The poly A fraction of the total cellular RNA isolated from NIH3T3 cell lines infected with mutant mDHFR vectors was subjected to electrophoresis on 1% formaldehyde-agarose gels, transferred to a nylon membrane, and hybridized with a 3T6 DHFR probe (Figure 12A). The two low-molecular weight RNA species of 1.1 kb and 0.8 kb are the DHFR transcripts from the internal promoters. The high molecular weight species are transcripts from the viral LTR promoter in the spliced or nonspliced forms or the read-through from the internal promoters (Figure 11). The same RNA blots were rehybridized to a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a quantitative control of the amount of RNA loaded in each lane (Figure 12B).

The high molecular weight RNA species in the DC/SV/R-mDHFR construct was several fold less than the DHFR transcripts from the internal promoter, when compared with the DC/SV-mDHFR construct which had a similar level of transcription for both species. To further investigate the phenomenon, RNA from 3T3 cells infected

with virus-containing supernatant collected from four additional individual producer lines of both DC/SV-mDHFR and DC/SV/R-mDHFR was analyzed (Figs. 13A-13B). A similar pattern of inhibition of the high molecular weight species transcripts in the DC/SV/R-mDHFR constructs was observed, indicating that antiparallel transcription from the internal promoter may have inhibitory effects on the LTR transcription.

Expression and Resistance Produced by the Mutant DHFR under Control of Different Promoters in Human Leukemia Cell Lines

MTX resistance of transduced leukemia cells

The MTX resistance level of the transduced leukemia cells was determined by a cell growth inhibition assay described in Material and Methods. The MTX IC50s were calculated and compared to that of the parental cells (Table 2). Different levels of resistance were observed in different cell lines with different constructs. The DC/TK/R-mDHFR construct produced the highest level of resistance and transcripts in K562 and Raji cells, but not in the CEM cell line. In the CEM cell line the DC/AC-mDHFR produced the highest level (2.1 fold) of MTX resistance.

RNA and DNA analysis of the transduced leukemia cells.

RNA analysis by Northern blotting of the transduced leukemia cell lines by various constructs containing mutant DHFR is shown in Figs. 14A-14B. The mDHFR transcripts from the internal promoter have the expected length of 1.1 and 0.8 kb.

The structure of the proviral DNA in the infected leukemia cells was analyzed by DNA blotting. The DraI restriction enzyme has a unique digestion site in the mDHFR cDNA. Thus only faithful duplication of the mDHFR cDNA sequence in the vector can generate a DNA fragment approximately 4 kb upon digestion of the genomic DNA with DraI, as was observed for all the constructs in the three leukemia cell lines (Figs. 15A-15B). The exact size of the 4 kb fragment varied since it consisted of the vector sequence present between the two draI sites in the U3 regions. This includes one copy of the mDHFR cDNA, one copy of the NEO gene, and one copy of the promoter in the construct (0.4 kb for SV, 0.8 kb for AD and TK, 1.3 kb for AC) and one copy of the additional poly (A) signal sequence (0.6 kb for DC/SV/R, 0.3 kb for DC/AD/R and DC/TK/R) for the antiparallel constructs (see 2.3.1 and Figs. 6A-6D). In Raji cells, additional minor lower molecular weight bands were present. Thee significance of these bands is not known.

Expression and Resistance Produced by the Mutant DHFR under Control of Different Promoters in Murine Bone Marrow CFU-GM Colonies

Conditions for MTX selection in the CFU-GM assay.

The dose-response of CFU-GM to MTX in different selection media was carried out to establish conditions for MTX selection. Five different concentrations of MTX (0, 10, 100, 1,000, and 10,000 nM) were tested in medium containing regular FBS or dialysed FBS or FBS treated with thymidine phosphorylase (TP) (see 2.11). The medium containing 100 nM MTX and TP treated FBS in which untransduced bone marrow cells did not produce background colonies was chosen as the selection condition for the

MTX resistance test (Figure 16).

Comparison of the expression of the mDHFR under control of different promoters in the CFU-GM colony assay.

The MTX resistance level of the bone marrow cells transduced through coculture with the viral producer cells was determined by the CFU-GM colony assay (Table 3). Constructs with the SV promoter and the AD promoter produced a higher percentage of MTX resistant colonies than constructs with the TK or AC promoter. Based on these results the DC/SV/R-mDHFR and DC/AD/R-mDHFR constructs were used in the in vivo bone marrow transplantation studies.

Table 1. MTX resistance level in 3T3 cells infected with mutant mDHFR in retroviral vectors.

NIH 3T3 cells were infected with the 3T6 DHFR in retroviral vectors as indicated. The numbers following the vector indicate different producer lines from which the infectious supernatants were collected. Infection and subsequent G418 selection were performed as described in Material and Methods. The G418 resistant colonies were pooled and expanded in drug-free medium. The G418 resistant cells were incubated with different concentrations of MTX and MTX resistant colonies were counted as described in Material and Methods. The IC_{50} value was calculated (Chou and Talalay, 1984), using 5 to 7 MTX concentrations. The IC_{50} values shown in the table are the average of 3-5 independent experiments.

vector	MTX IC_{50}* (nM)	resistance ratio
none	7.2	1
DC/SV-mDHFR 1.1	79.3	11.1
DC/SV-mDHFR 1.5	73.7	10.3
DC/SV-mDHFR 2.1	147.4	20.6
DC/SV/R-mDHFR 1.1	118.5	16.6
DC/SV/R-mDHFR 1.6	56.3	7.9
DC/SV/R-mDHFR 2.2	135.0	18.9
DC/AD/R-mDHFR 2	17.5	2.5
DC/AD/R-mDHFR 7	11.7	1.6
DC/TK/R-mDHFR 5	13.5	1.9
DC/TK/R-mDHFR 9	20.5	2.9
DC/AC-mDHFR 10	13.5	1.9
DC/CMV/R-mDHFR 1	9.2	1.3
DC/CMV/R-mDHFR 2	12.4	1.7

* Average of 3-5 experiments.

Table 2. MTX resistance level in leukemia cells infected with mutant mDHFR in retroviral vectors.

vector	CEM		K562		Raji	
	IC50+SD (nM)	ratio	IC50+SD (nM)	ratio	IC50+SD (nM)	ratio
none	49.7+ 10.5	1	50.8+ 3.2	1	34.1+ 2.8	1
DC/SV-mDHFR1.1	61.7+ 7.6	1.2	104.9+ 56.2	2.1	61.0+ 1.1	1.8
DC/SV/R-mDHFR1.1	67.8+ 1.7	1.4	123+ 14.6	2.4	97.1+ 7.6	2.8
DC/AD/R-mDHFR2.6	84.5+ 3.1	1.7	94.6+ 1.9	1.9	54.0+ 7.2	1.6
DC/TK/R-mDHFR5.2	49.2+ 4.7	1	172.1+ 70.9	3.4	190+ 132	5.6
DC/AC-mDHFR 10/2.3	106.3+ 3.3	2.1	91.7+ 30.3	1.8	50.3+ 4.9	1.5

CD50s are the average of 3 experiments.

Three human leukemia cell lines (CEM, K562, Raji) were infected with 3T6 DHFR in retroviral vectors. The numbers following the vector indicate different producer lines from which the infectious supernatant was collected. Infection and subsequent G418 selection were performed as described in Material and Methods. The G418 resistant cells were pooled and expanded in drug-free medium before being seeded at a density of $2x10^5$ cells per ml and exposed to different concentrations of MTX. Each concentration was tested in triplicate. Cells were

counted after 3 days of exposure. The IC_{50} values shown are the average of 3 independent experiments.

Table 3. MTX resistant CFU-GM of bone marrow cells infected with mutant mDHFR in retroviral vectors. Mouse bone marrow cells were infected with 3T6 mutant DHFR in retroviral vectors. The numbers following the vector indicate different producer lines cocultured with the marrow cells. Immediately after coculture the CFU-GM assay was performed in the presence or absence of MTX as described in Material and Methods. The percentage of resistant colonies was calculated by dividing the MTX resistant colony number with the colony number in the absence of MTX. Untransduced bone marrow was used as control.

Table 3

vector	No. of colonies -MTX	+MTX (100 nM)	percentage of resistant colonies
control	394	0	0
DC/SV-mDHFR1.1	393	46	11.7
DC/SV/R-mDHFR1.1	386	48	12.4
DC/AD/R-mDHFR2.6	379	51	13.5
DC/TK/R-mDHFR5.2	336	9	2.7
DC/AC-mDHFR10/2.3	300	7	2.3

MTX Resistance Developed in Mice After the Transplantation of the Bone Marrow Cells Transduced by DC/SV/R-mDHFR and DC/AD/R-mDHFR

Protocol for in vivo bone marrow transplantation (BMT) studies (Figure 17).

The donor bone marrow cells were cocultured 48 h with the pre-irradiated producer cell lines: DC/SV/R-mDHFR and DC/AD/R-mDHFR, and AM12, the parental packaging cell line used as a control. After coculture, 2 X 10^6 bone marrow cells were transplanted into each recipient mouse irradiated with 900 R 24 hr before transplantation. MTX treatment was started after the BMT, with the following dose schedules:

A) Low-dose: 48 hr after BMT, MTX of 2 mg per kg body weight, was administered i.p. twice for the first week, and 5 mg per kg body weight twice a week for the rest of the experiment.

B) Delayed high-dose: MTX treatment started 4 week after the BMT at a dose of 200 mg per kg body weight twice a week, i.p.

MTX resistance in mice after gene transfer.

Recipient mice were transplanted with transduced or untransduced marrow cells and treated with MTX under low-dose selection schedule. The survival rates of the recipient mice from two experiments were shown in Figs. 18A-18B. Mice receiving marrow cells cocultured with the AM12 control line did not survive the low-dose selection and died in the first 30 days of the selection, while more than 80% of mice receiving marrow cells transduced

by DC/SV/R-mDHFR or DC/AD/R-mDHFR survived. Mice without BMT after 900R irradiation died within 2 weeks with or without MTX selection, while all irradiated mice with BMT but without MTX selection were alive (data not shown). The changes of the hematocrit and the white blood cell count (WBC) following the transplantation and treatment with MTX are seen in Figs. 19A-19B. At day 13, the hematocrit and the WBC were decreased markedly in the control animals. There was also a decrease in the transduced animals. At day 28 post transplant, complete recovery of the hematocrit and the WBC were noted in the surviving animals with transduced marrow.

Deaths that occurred in the control group (AM12, non-virus producing) or the group receiving transduced marrow were associated with severe anemia, GI bleeding and marked weight loss.

The surviving mice with the transduced marrow were either treated with a lower dose of MTX (5 mg/kg, twice a week) for over 6 months without evidence of toxicity or used as the donor for the second generation transplantation.

The second transplant was carried out 5 weeks after the primary BMT, using marrow from DC/SV/R-mDHFR mice. The secondary recipients were treated as before with low dose MTX, and all recipients survived the selection. A third transplantation was performed 5 months after the second transplant. Marrow from the secondary recipients was used. The tertiary recipients were treated as before with low dose MTX and animals survived for longer than 30 days (Figs. 20A-20C).

Sequential CFU-GM assays were carried out in mice receiving transduced marrow at 20, 34, 45 days after

primary BMT and 30 and 138 days after the secondary BMT. The in vivo MTX selection resulted in an enrichment for MTX resistant colonies, shown by the increased percentage of resistant CFU-GM colonies with the increase of MTX selection time (Figs. 21A-21B).

Recipient mice transplanted with DC/SV/R-mDHFR infected marrow were able to survive the high-dose MTX selection that was started 4 weeks after BMT (Figure 22A). The control group died within 2 weeks of MTX treatment at 100 mg/kg, twice a week, while 3/5 mice in the DC/SV/R-mDHFR group survived 4 weeks of 100 mg/kg twice a week and 6 weeks of 200 mg/kg twice a week MTX. The MTX toxicity on normal mouse without irradiation or BMT was shown in Figure 22B. 4/4 normal mouse died within 4 weeks of MTX treatment at 200 mg/kg twice a week.

Demonstration of the integration of retroviral vector carrying the mutant mDHFR in the recipient mice

Genomic DNA from the spleen, liver, peripheral blood cells (PBC) and bone marrow were extracted as described in the Material and Methods (2.7). Primers NEO1 and NEO2 (Table 4) were used to amplify the NEO gene by PCR in the proviral DNA, generating a 415 bp fragment. The products were analysed by agarose gel electrophoresis, then transferred to a nylon membrane and hybridized to a NEO probe. The presence of NEO sequence in mouse tissues at different time points after BMT with different MTX selection schedules are shown in Figs. 23A-23C.

Table 4. The sequences of primers used for PCR amplification and sequencing.

primer	sequence	anneals to
Neo1	5'-GGAAGCCGGTCTTGTCGATC-3' (Sequence ID No. 5)	NEO(coding)
Neo2	5'-CGAAATCTCGTGATGGCAGG-3' (Sequence ID No. 6)	NEO(non-coding)
M301	5'-TGCCAATTCCGGTTGTTCAAT-3' (Sequence ID No. 7)	mDHFR (coding)
M210	5'-TCTGTCCTTTAAAGGTCG-3' (Sequence ID No. 8)	mDHFR (coding)
H250	5'-GAGGTTCCTTGAGTTCTCTGC-3' (Sequence ID No. 9)	hDHFR (coding)
GT-NC1	5'-CCTCGGCCTCTGAGCTAT-3' (Sequence ID No. 10)	SV40 promoter (NC, nt -50 to -68)

The integration of the proviral DNA in MTX resistant CFU-GM colonies of recipient mice after the primary BMT and the secondary BMT were also analysed by PCR. DNA from 5-6 colonies were extracted and amplified with NEO1 and NEO2 and hybridized to NEO probe (Figure 24).

DNA from PBC of a secondary recipient 8 months after BMT, and from spleen and liver 5 weeks after primary BMT was amplified by asymmetric PCR (Dicker et al., 1989, 2.9.2) with primer GT-NC1, which covered a region spanning part of the SV40 promoter in the vector, and primer M301 in the mDHFR (Table 4). The PCR product was sequenced with primer M210 and revealed the presence of the T to G point mutation in the mDHFR of the PBC in the recipient mouse 8 months after the initial BMT (Figs. 25A-25B).

The direct southern analysis of the genomic DNA extracted from tissues of the recipients, however, did not show the integration of the proviral DNA after repeated trials (Figs. 26A-26B). One of the possible explanations for this result is that only a small percentage of cells in recipient spleen and bone marrow tissues contained the proviral DNA. The detection limit of the southern analysis conducted was between 10% and 3.3% (Figure 27), which would indicate that less than 3.3% of cells in the recipient tissues contained the proviral DNA.

The dose response of normal mice (without irradiation or BMT) to MTX toxicity was shown in B. The animals were injected (ip) with MTX at the indicated weekly doses, which were divided into 2 and administrated twice a week, for 4 weeks. There were 4 mice in each group.

Construction of Retroviral Vectors Carrying Human Mutant DHFR (Figures 8A-8H)

Double copy vectors carrying full length cDNAs of human mutant DHFR (hDHFR) with mutations at residues 31 or 34 (Phe to Ser) under control of the SV40 promoter and the human ADA promoter were constructed and are designated as DC/SV-hDHFR, DC/SV/R-hDHFR, and DC/AD/R-hDHFR (see 2.3.3). The full length cDNA contains 560 bp coding region and 240 bp 3′ non-coding region. The SV40 and ADA promoter were chosen because of the studies described above using the 3T6 mDHFR. The expected length of hDHFR transcripts is 1.2 kb from the parallel promoter and 1.0 kb from the antiparallel promoters.

The same two promoters were used in constructing DC vectors carrying less than full length cDNA of the mutant hDHFR containing the coding region and 95 bp 3′non-coding

region. Constructs carrying the cDNA fragment between NcoI and BglII site from the pKT7HDR, which was modified immediately before the ATG starting codon (see 2.3.3), were prepared and designated as DC/SV-hDHFR(NB), DC/SV/R-hDHFR(NB) and DC/AD/R-hDHFR(NB). Constructs carrying the cDNA fragment between HindIII and BglII site from pSV4HDR without modification before ATG were also prepared and named as DC/SV-hDHFR(HB), DC/SV/R-hDHFR(HB) and DC/AD/R-hDHFR(HB) (Figs. 8A-8H). The expected length of the hDHFR transcript is 1.0 kb from the parallel promoter and 0.8 kb from the antiparallel promoters.

These constructs were packaged to corresponding virus by the procedure described in Material and Methods (2.4).

Expression and MTX Resistance Produced by Viral Constructs Containing Mutant hDHFR cDNAs in 3T3 Cells

The expression of full length cDNA of hDHFR in transduced 3T3 cells

3T3 cells infected with DC/SV-hDHFR, DC/SV/R-hDHFR or DC/AD/R-hDHFR were incubated with either G418 (0.75 mg/ml) or MTX (1.5 X 10^{-7}M) for 8-10 days. Cells infected with DC/SV-hDHFR were able to survive the selection by either G418 or MTX, while cells infected with DC/SV/R-hDHFR or DC/AD/R-hDHFR were able to survive only under the G418 selection but not the MTX selection. The MTX resistance level of these transduced 3T3 cells was determined by measuring the inhibitory effect of MTX on the colony formation of 3T3 cells. The IC50 of the cells transduced by mutant hDHFR were compared to that of the parental 3T3 cells and the cells transduced by wild type hDHFR in DC/SV vector constructs. Only the cells transduced by DC/SV-hDHFR31 had survived MTX selection (DC/SV-hDHFR31/mr, mr indicates MTX resistant) and were

found to be resistant to MTX (Table 5).

Table 5. IC_{50} for MTX in 3T3 cells infected with full length mutant hDHFR cDNA in retroviral vectors. 3T3 cells were infected with the retroviral vectors carrying full length hDHFR (wild type, S31 or S34 mutatation). The infected cells were selected in either G418 (0.75 mg/ml) or MTX (1.5 X 107 M). While cells transduced by all constructs survived the G418 selection, only the DC/SV-hDHFR31 transduced 3T3 cells survived the MTX selection. The resistant cells from each infection and selection (mr stands for MTX resistance) were pooled, expanded in drug-free medium, and plated out in different concentrations of MTX. The resistant colonies were counted and the IC_{50} values calculated, using 5 to 7 MTX concentrations.

Table 5

vector	MTX IC_{50}(nM)
DC/SV-hDHFR	19.0
DC/SV-hDHFR31	24.9
DC/SV-hDHFR31/mr	74.3
DC/SV/R-hDHFR31	10.8
DC/AD/R-hDHFR31	15.2
DC/SV-hDHFR34	13.1
DC/SV/R-hDHFR34	15.5
DC/AD/R-hDHFR34	20.8

Northern analysis of the poly (A) fraction of the total cellular RNA from these transduced 3T3 cell lines is shown in Figure 28A. The cell lines transduced by DC/SV-mDHFR and DC/AD/R-mDHFR were used as controls in the experiment. The length of the mDHFR transcripts in these two constructs are 1.1 and 0.8 kb. The length of hDHFR transcripts from DC/SV-hDHFR, DC/SV/R-hDHFR and DC/AD/R-hDHFR were shorter than the expected length of 1.2 and 1.0 kb with the exception of DC/SV-hDHFR/mr. A full length transcript of 1.2 kb is present in DC/SV-hDHFR/mr as well as the truncated message.

The DNA analysis of these transduced cell lines did not reveal any gross recombination in the proviral DNA (Figure 28B).

Immunoprecipitation of the DHFR enzyme protein with a polyclonal antibody in the transduced cell lines was carried out to determine whether the transcripts were translated to a full length or shorter protein. The antibody was titered on DG44 cells transduced by hDHFR. The DG44 cell line is a CHO cell line lacking endogenous DHFR (Urlaub and Chasin, 1980). The ^{35}S-labelled cell extracts were incubated with the antibody bound to protein A sepharose and the precipitated proteins were separated on 15% SDS polyacrylamide gel. The 22 kd DHFR protein was detected at dilution of 1:50 (antibody:cell extract). The 40 kd band is a non-specific protein precipitation as it is also present in the non-transformed DG44 cells (Figure 29A). The immunoprecipitation of the transduced 3T3 cell lines is shown in Figure 29B. Unlike the DG44 line, 3T3 cells contain endogenous mDHFR to which the polyclonal antibody to hDHFR also cross-reacts. By using the 40 kd non-specific precipitation (band 2) as a control for loading,

the relative ratio of the density of the 22 kd specific precipitation (band 1) to that of the band 2 was calculated, giving a semiquantitative measurement for the DHFR in the transduced cells. The DC/SV-hDHFR/mr which express the full length message at a detectable level produced a higher level of DHFR protein than untransduced 3T3 or 3T3 cell transduced by DC/SV/R vector without hDHFR gene. No smaller proteins were detected, indicating only the full length hDHFR message was able to be translated into the protein which could be precipitated by the antibody.

Expression and resistance produced by the less than full length cDNA of mutant hDHFR

The MTX resistance level of the 3T3 cells transduced by less than full length cDNA of mutant hDHFR was determined by measuring the IC50 of MTX on colony formation of 3T3 cells. The MTX IC50s on DC/SV-hDHFR31(HB), DC/SV-hDHFR31(NB) and DC/AD/R-hDHFR31(NB) were significantly higher than the untransduced parental 3T3 cell line or the 3T3 cell lines transduced by the wild type hDHFR (Table 6).

Table 6. MTX resistance level in 3T3 cells infected with less than full length mutant hDHFR cDNA in retroviral vectors. The 3T3 cells infected with retroviral vectors carrying less than full length mutant hDHFR cDNA (S31) were selected in G418 selection medium, and the resistant colonies were pooled. The inhibitory effect of MTX on the colony formation of these G418 resistant cells was measured as described in Table 5. The IC_{50} values were the average of 3 experiments and the standard deviations are shown. The parental 3T3 cell line and the cell line transduced by wild type hDHFR in retroviral vector

(DC/SV-hDHFR) were used as controls.

Table 6

vector	MTX IC_{50}±SD (nM)	resistance ratio
3T3	16.0±3.0	1
DC/SV-hDHFR	19.0	1.2
DC/SV-hDHFR31(HB)	77.0*±17.7	4.8
DC/SV/R-hDHFR31(HB)	45.5±16.6	2.8
DC/SV-hDHFR31(NB)	67.7*±14.1	4.2
DC/SV/R-hDHFR31(NB)	20.4±4.0	1.3
DC/AD/R-hDHFR31(NB)	54.7*±15.8	3.4

*significantly different from 3T3 parental line

Semiquantitation of the DHFR protein in these cell lines were carried out by immnunoprecipitation (see Material and Methods, and 3.7.1). The DC/SV-hDHFR31(HB), and DC/SV-hDHFR31(NB) and DC/AD/R-hDHFR31(NB) have a higher DHFR content than parental 3T3 cells (Table 7). The DHFR protein content seems to correlate well with the MTX resistance level of these cell lines.

Table 7. Quantitation of DHFR by immunoprecipitation from 3T3 cells infected with mutant hDHFR in retroviral vectors.

The immunoprecipitation of the DHFR enzyme protein from the transduced 3T3 cells is described in Material and Methods (2.10) and in the legend of Figs. 23A-23C. The intensities of the two bands (band1 of 22 kd and band2 of 40 kd) on the X-ray film were measured by Gelscan XL (2.0) (LKB). The ratio of band1 over band2 was calculated to semiquantitate the DHFR enzyme in the cell.

Table 7

vector	band1/band2*
3T3	1.2
DC/SV-hDHFR31(HB)	2.4
DC/SV/R-hDHFR31(HB)	1.0
DC/SV-hDHFR31(NB)	2.4
DC/SV/R-hDHFR31(NB)	1.3
DC/AD/R-hDHFR31(NB)	1.7

*band1: specific DHFR precipitation

band2: non-specific precipitation

The RNA and DNA analysis of the following transduced cell lines were performed: six DC/SV-hDHFR31(HB) lines (infected by viral supernatant collected from three producer lines, and selected by either G418 or MTX), three DC/SV/R-hDHFR31(HB) lines (infected by viral supernatant collected from three producer lines, and surviving the selection of G418), four DC/SV-hDHFR31(NB) lines (infected by viral supernatant of two producer lines, and surviving the selection by either G418 or MTX), one DC/SV/R-hDHFR31(NB) line (infected by viral supernatant of one producer lines, and surviving the G418 selection), and two DC/AD/R-hDHFR31(NB) lines (infected by viral supernatant of two producer lines, and surviving the G418 selection).

Figs. 30A-30B shows the RNA analysis of these cell lines. The DC/SV-mDHFR and DC/AD/R-mDHFR were used as positive controls for the DHFR transcript from the internal promoters and were 1.1 and 0.8 kb respectively. The 3T3 cell line was used as the negative control. The poly A fraction of total cellular RNA was separated on formaldehyde-agarose gel, transferred to a nylon membrane and hybridized with hDHFR31(HB) cDNA probe (A) and rehybridized with GAPDH (B). The 1.1 kb DHFR transcript from the parallel promoter was detected in all six cell lines of DC/SV-hDHFR31(HB) and in the four cell lines of DC/SV-hDHFR31(NB) in less abundance. The 0.8 kb DHFR transcript from the reverse promoter, was detected in DC/AD/R-hDHFR31(NB), but not in DC/SV/R-hDHFR31(HB) nor in DC/SV/R-hDHFR31(NB). Instead a truncated message of 0.4 kb was observed from the reverse promoters, but in less abundance than the truncated message observed with the full length cDNA of hDHFR (Figs. 28A-28B).

The DNA analysis of these cell lines is shown in Figures

31A-31B. The genomic DNA was digested with DraI which cuts once within the hDHFR cDNA, generating a single band of about 4 kb. No gross recombination was observed in any cell line, indicating that the observed truncation of the message occurred at the RNA level rather than at the DNA level.

Expression of Mutant hDHFR in Murine Bone Marrow Cells in CFU-GM Colonies

The MTX resistance level of the bone marrow cells transduced through coculture with DC/SV-hDHFR31(HB) and DC/SV-hDHFR31(NB) constructs was determined by CFU-GM assay and compared with the DC/SV/R-mDHFR. The two hDHFR constructs produced similar level of resistance to MTX as the mDHFR (Table 8). DC/SV-hDHFR31(HB) was chosen for the in vivo bone marrow transplantation studies.

Table 8. MTX resistant CFU-GM of bone marrow cells infected with mutant hDHFR in retroviral vectors.

The CFU-GM assay of the mouse bone marrow cells infected with the mutant hDHFR in retroviral vectors was performed in the absence or the presence of 100 nM MTX. The percentage of resistant colonies was calculated by dividing the number of MTX resistant colonies by the colonies formed in the absence of MTX.

Table 8

vector	No. of colonies -MTX	+MTX (100 nM)	percentage of resistant colonies
control	657	47	7.2
DC/SV/R-mDHFR	691	222	32.1
DC/SV-hDHFR31(HB)	763	234	31.0
DC/SV-hDHFR31(NB)	665	180	27.0

MTX Resistance Developed in Mice After the Transplantation of the Bone Marrow Cells Transduced by Mutant hDHFR

Mice transplanted with the marrow transduced by DC/SV-hDHFR31(HB) survived the MTX selection of both low-dose (A) or delayed high-dose schedules (B), while control mice transplanted with untransduced marrow died (Figs. 32A-32B).

The integration of the proviral DNA in MTX resistant CFU-GM colonies of recipient mice 5 weeks after the BMT and low-dose MTX selection was analysed by PCR blotting. The DNA pooled from 5 to 6 colonies was amplified using H250 (annealling to hDHFR) and GT-NC1 (annealling to SV40 promoter,) as primers and then was hybridized to a labelled hDHFR probe (Table 4). A 300bp fragment was detected as expected from the PCR reaction (Figure 33).

Experiments are in progress to follow the surviving recipients, to conduct secondary BMT, and to demonstrate

the integration of the mutant hDHFR in the tissues of the recipient mice.

EXPERIMENTAL DISCUSSION

Vector Design and the Expression of the Altered Murine DHFR *in Vitro* from the DC Vectors

Efficient expression of a variant DHFR conferring MTX resistance is a necessary requirement to protect transduced cells from MTX toxicity. In previous studies involving the expression of a mutant mDHFR in various retroviral vectors, the mutant gene was expressed from the viral LTR promoter or from a SV40 promoter situated within the LTR transcriptional unit (Williams et al., 1987, Corey et al., 1990, Kwok et al., 1986, Schuening et al., 1989, Hock and Miller, 1986). The activity of promoters is often reduced however, when placed downstream from an active promoter (Emerman and Temin, 1984). The selection of a gene driven by one promoter may result in the suppression of the expression of another gene driven by the other promoter. Bowtell et al (1988) have also observed that retroviruses carrying two genes, one transcribed from the LTR and the other from the SV40 promoter are poorly transcribed *in vivo*, even in the absence of selection and despite the presence of the provirus in the host hematopoietic cells. It is possible that the suppression and the low expression of the second gene was due to the fact that in both cases the gene driven by the internal promoter was situated within the same transcriptional unit of the LTR promoter, rather than the mere sequential presence of the two transcriptional units. To overcome this suppression, we used a double copy vector which has a 5′ duplication of the recombinant gene inserted at the 3′ LTR (Figs. 6A-6D, 11). The 5′ duplication is outside the LTR transcriptional unit. This vector design has been shown to express the inserted gene at a 10-20 fold higher level

than the vector with the promoter cloned within the LTR transcriptional unit (Hantzopoulos et al., 1989).

To determine the effect of different promoters on the expression of the altered mDHFR gene in different cell types, five different promoters were cloned in the double copy vector and their expression was compared in 3T3 fibroblast cells, three human leukemia cell lines and mouse bone marrow.

In 3T3 cells, the mutant mDHFR is transcribed from the minigene promoters in DC/SV-mDHFR, DC/SV/R-mDHFR, DC/AD/R-mDHFR, DC/TK/R-mDHFR and DC/AC-mDHFR constructs, with the exception of the CMV promoter (Figs. 12A-12B). The MTX resistance level as tested by the colony formation assay correlates well with the mRNA level of transcription from the internal promoter, even though a low level of translation of the mutant enzyme from the polycistronic message cannot be excluded (Kaufman et al., 1987).

The DC/SV/R-DHFR construct which contains the recombinant transcriptional unit in the opposite orientation to viral transcription, has a similar or slightly higher expression of the mutant DHFR from the internal promoter and a 5-10 fold decrease of transcription from the LTR promoter, when compared to DC/SV-DHFR which contains the transcriptional unit in the same orientation as virus transcription (Figure 11, Figs. 12A-12B). The mechanism for the decrease in the viral LTR message level is not known. In a study with a replication-competent MoMLV vector which has a double copy recombinant mutant DHFR under the control of the SV40 promoter, the transcription unit cloned in the opposite orientation of the LTR gave no virus production (Stuhlmann et al., 1989), indicating

transcription in the opposite orientation of the LTR transcription might have an inhibitory effect on viral transcription, message stability or translation efficiency, resulting in no or low viral production. In our study, however, DC/SV-mDHFR and DC/SV/R-mDHFR constructs have similar viral titers, and gave essentially the same number of MTX resistant colonies in infected 3T3 cells. Constructs such as DC/AD/R-mDHFR and DC/TK/R-mDHFR which contain the recombinant transcriptional unit in the opposite orientation of the LTR also do not have reduced viral production. These results indicate the orientation of the transcription of the recombinant gene has very little effect on viral production.

The results from the transduced human leukemia cell lines show that these constructs can give lower but reasonable levels of MTX resistance in human cell lines of hematopoeitic lineage, but there was no correlation with the results obtained in the 3T3 cell line, as regards effectiveness of the various promoter constructs.

The results from the transduced bone marrow cells further indicate that the mouse fibroblast cell lines such as NIH 3T3 cannot be used to assess the effectiveness of retroviral mediated gene transfer, and that the activity of retroviral vector encoded promoters vary in an unpredictable manner and is probably modulated by the transduced cell type.

The lack of correlation of the expression of the mutant DHFR between different cell types is not a total surprise. The SV40, TK, metallothionein (MT), c-fos, CMV and adenovirus E1A promoters were reported to lack or show very low level of expression of the human ADA cDNA

in primary murine hematopoietic cells despite excellent expression in fibroblast and hematopoietic cell lines (Lim et al., 1987; McIvor et al., 1987; Belmont et al., 1988). The present study is the first direct comparison of the expression of a particular gene (altered DHFR) driven by different promoters in the context of the same vector design in four cell lines and in primary hematopoietic cells.

The Expression of the MTX Resistance Phenotype Induced by Altered mDHFR *in Vivo*.

The animal survival data after BMT with low-dose MTX selection clearly demonstrated that the MTX resistant phenotype was present in the animals receiving the bone marrow transduced by altered mDHFR. The hematocrit and white blood cell count of these animals returned to normal 4 weeks after BMT and MTX treatment, while all control animals died of anemia, GI bleeding and marked weight loss within the same time.

It was somewhat surprising that the transduced bone marrow not only protected the animal from the marrow toxicity of MTX but GI toxicity as well. Similar observations were reported from other research groups (Williams et al., 1987; Corey et al., 1990). The prevention of leukopenia by the transduced gene probably contributes to the protection of the GI tract.

The continued expression of the retrovirus-mediated transfer of the altered DHFR and the infection of stem cells with extensive repopulating capability was demonstrated by the serial transplantations of transduced bone marrow cells.

The design of the delayed high-dose MTX treatment schedule takes into consideration the increased MTX tolerance in animals 4 weeks after BMT with a relatively normal cell count compared with the animals just after BMT, so that the long term expression of the MTX resistant phenotype can been measured. The design also allows testing whether the MTX resistance conferred to bone marrow by gene transfer can protect the animal from the toxicity of a dose higher than the regular therapeutic dose of MTX. In normal animals (animals without irradiation or BMT), both the GI toxicity and marrow toxicity contribute to the lethal toxicity of MTX (1.1.2). Our preliminary data showed that the protection was maintained even after challenge with a high dose of MTX.

The integration of the retroviral vector in the hematopoietic cells of the recipient mice is the necessary requirement for the stable and long-term expression of the mutant mDHFR. PCR analysis showed the presence of the NEO gene carried by the viral vector in both the tissues of the recipient mice and in the MTX resistant CFU-GM colonies of the recipient mice marrow. The sequence analysis of the PCR amplification product of the vector DHFR gene product confirmed the existence of the mutant DHFR in the recipient mice.

The negative southern analysis in the primary and secondary BMT recipient is difficult to explain. The obvious conclusion is that less than detectable level of cells (3.3%) in the bone marrow and spleen of the recipient examined were transduced by the mutant mDHFR but somehow conferred the resistance observed *in vivo*. Possible explanations include the following: the products of the enzyme reaction conducted by the mutant mDHFR in

the transduced cells in the presence of MTX might be transported out the transduced cells to rescue other sensitive cells; among the small number of the transduced cells there were progenitor stem cells that could repopulate the sequential transplantation recipients but gave rise to untransduced progenies through DNA sequence deletion. The possibility of a small number of transduced non-multipotential progenitor cells or even mature cells with long life span being carried over in the serial BMT was not excluded. The *in vivo* experiments and the Southern analysis were repeated several times using various controls to exclude the presence of artifacts. The use of a chemotherapeutic agent, 5-fluorouracil (5-FU) to destroy later stage dividing progenitor cells in the donor animal of the sequential BMT might help to exclude the possibility that only the later stage progenitor cells were transduced.

5-FU has also been used in several laboratories in primary gene transfer to enrich the number of primitive cells in cycle since the finding that 5-FU can increase the proportion of stem cells by destroying more mature dividing cells (Hodgson and Bradley, 1979). 5-FU was given to the donor mice several days (1-5 days) before harvesting the bone marrow. Because retroviral integration in the target cell genome requires the division of the host cell, the 5-FU treatment, which results in the depletion of the mature cells thus more primitive cells were forced into cycle at the time of infection, were believed to increase the efficiency of gene transfer into the stem cells (Lerner and Harrison 1990). But 5-FU pretreatment is not an absolute requirement for gene transfer into stem cells (Belmont, 1990).

The use of hematopoietic growth factors such as IL-1, IL-3 and IL-6, to treat the target cells before or in the process of the infection was reported to increase the efficiency of the infection into the stem cells (Lim et al., 1989; Dick et al., 1985). In our experiment, the WEHI conditioned medium, which contains IL-3 and small amounts of other growth factors, was used in the coculture infection to improve the efficiency of infection.

Vector Construction and the Expression of the Human Altered DHFR *in Vitro*

The DC vectors carrying the full length hDHFR cDNA did not express the MTX resistance phenotype effectively except for DC/SV-hDHFR31/mr. Northern analysis demonstrated that the hDHFR messages from the internal promoters were truncated with the exception of DC/SV-hDHFR31/mr. The full length hDHFR cDNA in the DC vectors contained a 250 bp 3' untranslated region, in which there were a few potential poly (A) signal sequences. The shorter length of the message might be due to the early termination of the transcription by the poly (A) signal sequence within the cDNA rather through the poly (A) signal sequence provided by the DC vector. The shorter message, however, was not translated into the hDHFR protein in the host cell (3T3) as demonstrated by immunoprecipitation with a polyclonal antibody against hDHFR.

There was a modification immediately before the ATG codon in the 5'of the full length hDHFR cDNA. The modification was generated to create a NcoI restriction site for cloning and did not show any adverse effect on the expression of the hDHFR in a bacterial expression system

(Schweitzer et al., 1989).

To improve the expression of the hDHFR, we constructed the DC vectors carrying the shorter length of the hDHFR cDNA to exclude the potential poly (A) signal sequences. These constructs indeed expressed the mutant hDHFR more effectively with the exception of the DC/SV/R-hDHFR. The message of expected length was transcribed presumably using the vector poly (A) signal, and the hDHFR protein was translated. The 5′ modification before the ATG starting codon seemed to be a less significant factor than the 3′ poly (A) signals in regulation of the expression of hDHFR.

The expression of the altered hDHFR (S31) was compared with the altered mDHFR (Arg 22) in the CFU-GM assay after the coculture infection of the murine bone marrow cells with the amphotropic producer lines. The percentage of the resistant CFU-GM colonies of two hDHFR constructs tested was similar to the construct DC/SV/R-mDHFR which had been shown to confer MTX resistance *in vitro* and *in vivo*.

The Expression of the MTX Resistance Phenotype induced by Altered hDHFR *in Vivo*

Comparable with the *in vivo* result of the mDHFR gene transfer, 6/9 mice transplanted with marrow transduced by mutant hDHFR (S31) survived the low-dose MTX selection while all mice in the control group died within 3 weeks of the MTX treatment. The surviving mice were then subjected to high dose MTX treatment (200 mg/kg, twice a week) for 5 additional weeks after BMT with the MTX resistant phenotype persisting during the treatment. Another group of mice treated with delayed high-dose MTX

showed a similar result in that the mice with transduced marrow survived, while the control mice died. These results indicate that MTX resistance was conferred to the recipient mice through transplantation of hDHFR transduced marrow.

The presence of the altered hDHFR in the recipient mice bone marrow was demonstrated by PCR blot analysis of the genomic DNA pooled from the MTX resistant CFU-GM colonies. The Southern analysis of the tissues from the recipient mice (data not shown) did not show the integration of the proviral DNA as in the case with the mutant mDHFR gene transfer. Common factors might be responsible for the negative Southern analysis in both cases.

Significance of the Study and Future Work
Vector construct

One of major efforts in the study was an attempt to optimize the design of the retroviral vectors to improve the expression of the transduced gene. DC vector design used in this study seems to have the advantage of achieving equal *in vitro* expression of the two recombinant genes in the vector, i.e. the NEO and the DHFR. The *in vivo* expression of the NEO gene was not tested due to the known cytotoxicity of G418. The expression of DHFR driven by five different promoters was compared in the DC vector construct in different cell lines and murine bone marrow. The result of this study was consistent with the idea that the expression of a gene under control of a certain promoter cannot be predicted in bone marrow cells based on data from a different cell type or in a different context. In cloning the hDHFR cDNA into the retroviral vector, the 3'

untranslated region resulted in a truncated message that was neither translated into hDHFR protein, nor confered MTX resistance. The impact of this finding on optimizing retroviral vector constructs needs to be borne in mind in the future when attempting to improve expression.

The infection efficiency of the DC vector constructs is between 10-30% measured by the MTX resistant CFU-GM colonies after coculture infection. No significant improvement was observed when compared to other MoMLV based vectors carrying mutant DHFR reported before (Hock and Miller, 1986; Kwok et al., 1986; Schuening et al., 1989). This may partly due to the intrinsic character of the MoMLV vector, rather than particular vector construct design. MoMLV induces T-cell lymphomas in new born-NFS mice, while Friend murine leukemia virus (FrLV) induces erythroleukemia. U3 region of the viral LTR were shown to be the primary determinant of the distinct disease specificities of the two virus. A 200 base direct repeat and a short 3′ adjacent GC-rich segment within the U3 region encode the enhancer function for both virus and the exchange of the region resulted in almost complete exchange of the disease specificities of the virus (Li et al., 1987; Golemis et al., 1989). A study done by Holland et al. (1987) showed that replacement of the enhancer segment of the MoMLV with the corresponding fragment of the FrLV improved the expression of the NEO gene carried in the vector in hematopoietic progenitor colonies (GM, BFU-E and GEMM). These results suggest that MoMLV based vectors may not be the ideal vector of choice for expression in hematopoietic cells. The study on Moloney sarcoma virus (MoSV) which transforms fibroblasts *in vitro* and myeloproliferative sarcoma virus (MPSV) which transform HSC and other hematopoietic progenitors *in vivo* as well as fibroblasts *in vitro*

showed similar results. The enhancer region within U3 of MPSV confered the hematopoietic tissue specificity (Stocking et al., 1985). The in vivo study by Corey et al. (1990) demonstrated that there is a small advantage of using Fr/Moloney hybrid LTR for expression in myeloid hematopoietic cells. The enhancer of the FrLV and MPSV can be further studied by deletion and replacement analysis to define the specific function of different segments of the enhancer. This knowledge can be used to modify the DC vector with the enhancer fragment that is shown to improve the hematopoietic tissue specificity.

MTX as a selecting agent, *in vitro* and *in vivo*

MTX resistance as a dominant selectable marker has several advantages over NEO resistance, namely, MTX selection can be conducted *in vivo*; the *in vivo* expression of the MTX resistance is very stable (Williams et al., 1987, Corey et al.,1990 and this study); and the MTX resistance phenotype is readily selectable. In spite of these advantages, the *in vitro* and *in vivo* MTX selection can still be problematic. Unlike G418 which selects for a transduced bacterial gene without background resistance, the thymidine kinase present in mammalian cells can salvage thymidine to synthesize dTMP *de novo* and thus by-pass MTX inhibition, and give rise to background resistance. The use of thymidine phosphorylase to treat the fetal bovine serum in the culture medium in this study successfully reduced the background in the presence of MTX and made the selection system more sensitive.

In vivo MTX selection, on the other hand, was limited by the change in the MTX sensitivity of the hematopoietic cells in the recipient mice after BMT. The MTX *in vivo*

selection used in previous studies (low-dose selection) was only effective before the hematopoietic system recovered from the irradiation and BMT. The delayed high-dose selection developed in this study allowed for the selection of prolonged expression of a MTX resistance phenotype, and indicated that early selection was not required to select for MTX resistance. The hypothesis that MTX resistant bone marrow will enable patients to tolerate higher doses of MTX to treat neoplasms, may be tested with the delayed high-dose MTX selection system.

The study on altered hDHFR (S31) confirmed the report that this variant DHFR can be use as a dominant selectable marker (Schweitzer et al., 1989; Banerjee et al., 1994).

This study shows that MTX resistance offers an attractive choice of a selectable marker in gene transfer studies, as well as an opportunity to protect bone marrow from MTX toxicity in patients treated with this drug for neoplasms or immunosuppression purposes.

The complexity of the hematopoietic system

The discrepancy of the *in vivo* study between the survival data at the phenotipic level and the result of Southern analysis at the molecular level shows the complexity of the hematopoietic system. A better understanding of the system is essential in order to achieve successful gene therapy. Recent advances in purification of hematopoietic stem cells (HSC) and the interaction of HSC with the microenvironment may lead to improved efficiency of gene transfer to HSC.

Besides the known techniques to enrich the HSC in cycle

(the 5-FU pretreatment of the donor and the use of growth factors), techniques to purify hematopoietic stem cells using monoclonal antibodies to stem cell specific antigens (CD34+ for human and other primates, Thy-1 and H-2K for mice etc.) have been developed (Spangrude et al., 1988; Szilvassy et al., 1989; Berenson et al., 1988). Small numbers of the purified cells were reported to be able to reconstitute irradiated animals. Recently, successful engraftment of bone marrow after infusion of purified CD34+ marrow cells into patients has been reported (Berenson et al., 1991). It is also possible to use long term cultures of the hematopoietic cells to improve the efficiency of retroviral infection (Schuening et al., 1989). In addition, immortalized bone marrow stromal lines with selectable phenotypes, which are capable of supporting hematopoiesis in long-term culture, have been developed and may facilitate both the retroviral infection and selection of hematopoietic cells *in vitro* (Williams et al., 1988; Paul, et al., 1991). For example, pre-established irradiated autologous marrow stromal layer was recently shown to enhance cell-free retroviral vector transduction of human bone marrow long-term culture initiating cells (Moore et al., 1992).

Some new growth factors that may play a role in the regulation of hematopoiesis are also under intensive study. C-kit ligand (mast cell growth factor, MGF) was reported to synergistically interact with a number of cytokines, such as IL-3, granulocyte-monocyte colony-stimulating factor (GM-CSF), and directly augment the proliferative capacity of primitive human hematopoietic progenitor cells (Brandt et al., 1992). The leukemia inhibitory factor was shown to be able to improve the survival of hematopoietic stem cells during culture of bone marrow with vector-producing fibroblasts resulting

in a 10 fold increase of infection efficiency, allowing long-term expression of the vector encoded gene (Fletcher et al., 1991). The results generated from these studies indicate that combination of various growth factors can improve the efficiency of the retroviral-mediated gene transfer.

The optimization of the design of retroviral constructs, the perfection of the selection regimen and the improvement in the efficiency of the HSC infection will be the major areas of future efforts to develop MTX resistance in mice by retroviral gene transfer of altered DHFR genes, and form the basis for studies in patients.

CONCLUSIONS

Double copy retroviral vectors were constructed that contained altered cDNA for mDHFR (3T6) and hDHFR (S31) under the control of different promoters.

These constructs were able to express the mutant DHFR and confer different levels of MTX resistance to the infected cells (3T3 fibroblast cell line; CEM, K562, Raji leukemia cell lines; and murine bone marrow cells) *in vitro*.

Studies in irradiated mice transplanted with bone marrow cells infected with retroviral constructs indicated that protection was afforded to MTX cytotoxicity for prolonged periods of time.

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Second Series of Experiments

Transfection of a novel Ser31 and Ser34 Mutant Human Dihydrofolate Reductase cDNA

Two novel mutant human dihydrofolate reductase (DHFR) cDNAs encoding either a Ser31 or a Ser34 mutation in a mammalian expression vector were transfected into Chinese hamster ovary (CHO) cells. Transfection of the Ser31 and Ser34 mutant *dhfrs* into DHFR$^-$ cells converted them into the DHFR$^+$ phenotype. Furthermore, transfection of these mutants into wild type CHO cells made them resistant to high levels of methotrexate (MTX) thus indicating that these variants can act as dominant selectable markers. Southern blot analysis and polymerase chain reaction amplifications confirmed that the transfected plasmids were integrated into the CHO DNA. Gene copy number analysis revealed that both the Ser31 and the Ser34 mutants are amplifiable in increasing concentrations of MTX. These mutated human *dhfr* cDNAs may be worthy of further study as dominant selectable genes for gene therapy.

Dihydrofolate reductase (DHFR E.C.1.5.1.3) catalyzes the reduction of folate and dihydrofolate to tetrahydrofolate, an essential cofactor in the synthesis of purines and thymidylate (Blakley, 1984). Methotrexate (MTX) is a powerful inhibitor of DHFR and is used as an antineoplastic agent in the clinic, although the use of MTX is limited due to emergence of drug resistant tumor cells (Sobrero and Bertino, 1986). Simonsen and Levinson (1983) demonstrated the usefulness of an altered mouse *dhfr* (substitution of Arg for Leu at residue 22) from a MTX resistant 3T6 cell line employing a plasmid expression vector (pFR400) as a dominant selectable

marker in gene transfer studies. This altered enzyme has been used in both *in vitro* and *in vivo* gene transfer studies (Cline et al., 1980; Williams et al., 1987; Carr et al., 1983; Isola and Gordon, 1986; Corey et al., 1990). While the altered 3T6 DHFR is markedly impaired in binding to MTX, it has limitations as a selectable marker in that it has poor catalytic activity (Haber et al., 1981; Thillet et al., 1988).

An earlier report (Srimatkandada et al., 1989) from this laboratory characterized a variant human DHFR from a MTX resistant human colon carcinoma line, HCT-8R. The alteration was shown to be a single amino acid change at position 31 where the wild type Phe was changed to a Ser residue. Molecular modeling studies have shown that Phe at position 31 makes van der Waals interactions with both the pteridine and the p-aminobenzoyl moieties of MTX (Oefner et al., 1987). Thus, substituting a large hydrophobic group for a small hydrophilic group has profound effects on MTX binding. Another Phe occurs at position 34 and is also an active site residue. In a previous report from this laboratory, site directed mutagenesis was used to generate both of these mutants with either the Phe at 31 or 34 changed to Ser (Schweitzer et al., 1989a). Cloning and expression in bacteria of these altered Ser31 and Ser34 cDNAs revealed that these DHFRs exhibited decreased MTX binding properties when compared to the wild type human DHFR. However, unlike the 3T6 altered DHFR, both these enzymes had good catalytic activity (Schweitzer et al., 1989b). In the present communication we report the use of these two altered human *dhfr* cDNAs, the Ser31 and the Ser34 mutants, as dominant selectable markers in CHO cells, and compare the results obtained with the Arg22 3T6 mutant cDNA.

EXPERIMENTAL RESULTS AND DISCUSSION

Vectors

The mammalian expression vectors were constructed as described in the legends of Figure 34. Direct sequence analysis of the PCR amplified SV40 promoter and *dhfr* cDNA verified the sequence of the construct. Sequence analysis revealed that all the cDNAs cloned in the expression vectors contained the desired sequence and did not contain any other mutations (data not shown).

Transfection and Selection

Two types of CHO cells were used in the gene transfer experiments. CHO DG44 cells lacking endogenous DHFR activity cannot grow in F12 without HGT, and colony formation in this medium by cells transfected with the wild type or mutant h-*dhfr* cDNA indicates successful gene transfer. In this DHFR$^-$ cell line the wild type *h-dhfr* (HDR) was able to induce the highest number of colonies as compared to the Ser31, the Ser34, and the Arg22 mutants (Table 9).

Table 9

COLONY FORMATION IN MTX BY TRANSFECTED CHO DG44 CELLS†

nM MTX	HDR	Ser31	Ser34	Arg22	Mock
0	1072 ± 223	955 ± 147	902 ± 98.1	820 ± 93.8	2
100	0	280 ± 35.5‡	153 ± 14.4	122 ± 15.9	0
500	0	115 ± 28.8‡	70 ± 9.5	52 ± 11.1	0
1000	0	23 ± 4.7	32 ± 8.1	43 ± 2.5	0

†Colony formation in F12 without HGT was measured after 14 days in the presence and absence of MTX. An average of four independent determinations. Expressed as colonies per μg DNA per 10^6 cells.

‡$p < 0.05$ as compared to Arg22.

Chinese hamster ovary (CHO) cells lacking DHFR (CHO DG44, obtained from Dr. L. Chasin) were cultivated in complete F12 medium supplemented with 10% fetal bovine serum (FBS). The MTX sensitive parental CHO cells were grown in RPMI-1640 medium supplemented 10% FBS. All MTX selections were carried out in F12 medium without hypoxanthine, glycine, and thymidine (F12 without HGT). All cell culture media were supplemented with L-glutamine and penicillin/streptomycin. For MTX selection, dialyzed FBS was used in place of FBS.

Successful gene transfer was also demonstrated by the emergence of G418 resistant colonies when the neo^r gene was cotransfected. For MTX resistance, 5 x 10^4 cells from each group were plated in 100 mm petri plates and exposed to various concentrations of MTX. Colonies were scored after 14 days. It was observed that the Ser31 mutant gave rise to the highest number of colonies at lower MTX concentrations. The Ser34 and the Arg22 transfections gave higher number of colonies at MTX concentrations above 500 nM.

To study the mutant *h-dhfr* cDNAs as dominant selectable markers the level of MTX resistance in wild type CHO cells (CHO) was determined by selection of transfected cells in various concentrations of MTX. Colonies were scored after 14 days (Table 10).

Table 10

COLONY FORMATION IN MTX BY TRANSFECTED CHO CELLS[†]

nM MTX	HDR	Ser31	Ser34	Arg22
100	0	216 ± 30.5	144 ± 12.8	183 ± 15.2
500	0	66 ± 5.3	73 ± 5.5	120 ± 17.3
1000	0	46 ± 4.7	57 ± 11.1	92 ± 8.1

[†]Colony formation was measured after 14 days of exposure to MTX as described in the experimental section. An average of four independent determinations. Expressed as colonies per μg DNA per 10^6 cells.

The Ser31 and the Ser34 mutants were capable of acting as dominant selectable markers, as was the murine *dhfr* Arg22, in CHO cells. The Ser31 mutant *dhfr* gave rise to the highest number of colonies at 100 nM MTX indicating that at lower MTX doses it was capable of acting as a better selectable mutant. In order to eliminate the possibility that resistance to MTX in these transfected cells was due to other resistance mechanisms, e.g., poor uptake (Sirotnak et al., 1989) and/or defective polyglutamylation of MTX (Pizzorno et al., 1989), another antifolate, trimetrexate (TMTX) which uses an alternate transport mechanism and cannot be polyglutamylated (Kamen et al., 1984), was used to determine resistance. It was observed that cells originally resistant to 1000 nM MTX (cloned from a single colony growing in 1000 nM MTX) were also resistant to TMTX, thus strengthening the conclusion that resistance to MTX was probably due to presence of an altered DHFR in these transfected cells and not due to poor uptake or defective polyglutamylation of MTX.

These results provide evidence that the Ser31 and Ser34 h-*dhfr* mutants can act as dominant selectable markers in CHO cells *in vitro*. Both these mutants convert the DHFR$^-$ to the DHFR$^+$ phenotype when transfected into the DHFR$^-$ DG44 cells. Southern blot analysis, PCR amplification, and direct sequence analysis of the amplified product provide evidence for chromosomal integration of the transfected plasmid expression vectors. Cross resistance to TMTX further strengthens the argument that drug resistance in these cells is due to the expression of the transfected altered DHFRs. The Ser31 and Ser34 mutants are comparable to the Arg22 mutant as dominant selectable markers in CHO cells.

Amplification of Transfected Plasmids

Genomic DNA isolated from cells cloned from a single colony growing at 100 nM MTX (for the Ser31 and Ser34 cDNA transfected cells) was digested with NcoI and HindIII enzymes and analyzed by Southern blotting. The Southern blot demonstrated the presence of unique fragments of approximate size 600 bp generated by restriction with HindIII and NcoI (Figs. 35A-35B). When cells resistant to 100 nM MTX were selected in 500 nM MTX and then in 1000 nM MTX, amplification of the integrated plasmid occurred. The Ser31 and the Ser34 containing plasmids were found to amplify in increasing concentrations of MTX. Gene copy number analysis revealed approximately 5, 35, and 40 copies of the plasmid DNA for the Ser31 transfectants grown in 100 nM, 500 nM, and 1000 nM MTX, respectively, and 2, 5, and 8 copies for the Ser34 transfectants grown in similar MTX concentrations. The Arg22 transfectants also showed amplification of the transfected plasmid in increasing MTX concentrations (15, 20, and 25 copies for 100 nM, 500 nM, and 1000 nM MTX, respectively).

Expression of the Transfected cDNAs

Northern analysis of total cellular RNA isolated from CHO, CHO Ser31, CHO Ser34, and CHO Arg22 cells (the Ser31, Ser34, and the Arg22 transfected cells derived from single colonies growing in 100 nM MTX) showed that the transfected cells contained shorter approximately 600 bp and 700 bp DHFR messages which are not normally present in CHO cells (which have 2.35, 1.8, and 1.3 kb sized messages). The sizes probably result from usage of different poly A+ signals present within the vector plasmid. The two major bands present in all lanes indicated by arrows represent the 28S and the 18S ribosomal RNA species which cross reacts with the probe.

Of all the *dhfr* mutations reported so far, only three have been used in gene transfer studies as candidate dominant selectable markers. McIvor and Simonsen (1990) reported that the altered murine DHFR characterized from the mouse L5178 Y cell line (substitution of Trp for Phe at residue 31) may be of use in gene transfer studies. Hussein et al. (1992) have shown that the Leu to Phe change at codon 22 of the mouse *dhfr* cDNA results in an enzyme also capable of acting in a dominant selectable fashion and may also be of use in gene transfer studies. However, this altered enzyme imparts a low level of MTX resistance (100 nM). The Arg22 and the Trp31 mutants, although they impart a higher level of drug resistance, are catalytically poor enzymes. According to Thillet et al. (1988), mouse DHFR with the Leu to Arg mutation at residue 22 has a 700-fold reduction in catalytic efficiency although it has a 7.5 x 10^5-fold increase in MTX K_i as compared to the wild type mouse DHFR. In contrast the Ser31 mutant human DHFR has only a 2-fold decrease in catalytic efficiency (Schweitzer et al., 1989 a and b) and a 100-fold increase in MTX K_i, while the Ser34 mutant has a 70-fold reduction in catalytic efficiency and a 2 x 10^4 increase in MTX K_i (Schweitzer et al., 1989b). Thus, despite having higher affinities for MTX than the Arg22 enzyme, the Ser31 and the Ser34 DHFRs may function more efficiently in the transfected cells.

The report that the murine Ser31 mutant *dhfr* was not able to behave as a dominant selectable marker in CHO cells (Thillet and Pictet, 1990) is difficult to explain. However, in the same report, the authors were unable to observe MTX resistance in Arg22 *dhfr* transfected CHO cells. This suggests that the experimental conditions were not optimal for the selection of MTX resistant colonies as the Arg22 *dhfr* variant has been reproducibly

shown to be a dominant selectable marker both *in vitro* and *in vivo* (Simonsen and Levinson, 1983; Carr et al., 1983; Williams et al., 1987; Isola and Gordon, 1986; Corey et al., 1990; and the present report). Altered *dhfr* cDNAs may be useful in gene transfer work in two ways, (1) they can act as dominant selectable markers and allow for selection of otherwise nonselectable genes, and (2) they can impart resistance to transfected cells such as bone marrow progenitor cells which can permit high dose antifolate chemotherapy of tumors of non-hematologic origin sensitive to high dose antifolates.

For the first use the ideal mutant *dhfr* should encode an enzyme with high catalytic efficiency and should amplify in increasing levels of MTX. Toward this goal the Ser31 appears to be a superior selectable marker than all the other mutants reported so far (Arg22, Phe22, and Trp31) because it has high catalytic activity, is relatively resistant to MTX which allows for starting selection at fairly high doses of MTX (not possible for the Phe22 mutant), and is readily amplifiable. The Arg22 and the Trp31 mutants on the other hand have a very poor catalytic activity and would be of limited use as a selectable marker, as the production of a relatively little amount of these enzymes would be sufficient to impart resistance and the plasmid would not need to be amplified in order to produce more of the DHFR enzyme. Toward the second goal the Arg22 and the Ser34 mutant *dhfrs* appear equally able to confer MTX resistance.

Conclusions

1) Two mutant human *dhfrs*, the Ser31 and the Ser34, have been shown to act in a dominant selectable manner in transfected CHO cells. Selection of transfected cells in increasing concentrations of

MTX is possible using these mutants. The Ser31 mutant at lower MTX concentrations generates a higher number of colonies than either the human Ser34 or the murine Arg22 mutant.

2) The Ser31 and the Ser34 mutant human *dhfr* cDNAs are amplifiable in increasing concentrations of MTX. This should allow amplification of cotransfected nonselectable genes.

Transfection of a tryptophan-15 mutant murine *dhfr* cDNA into CHO and mouse marrow progenitor cells

Materials and Methods

CHO DG44 and wild type CHO cells were obtained from Dr. L. Chasin, Columbia University, New York as described above and in Urlaub et al., 1983. The murine *dhfr* cDNA containing the point mutation at nucleotide 46 (amino acid residue 15) was excised from the bacterial expression vector pKT7 (Schweitzer et al., 1989) with the enzymes NcoI and HindIII and cloned into the same restriction sites in the mammalian expression vector pSV5, as described above for the Ser31 and Ser34 mutants. Calcium-phosphate mediated transfections of pSV5Trp15 and pWLNeo into both CHO DG44 and CHO S cells were performed using the mammalian transfection kit obtained from Stratagene (LaJolla, California) according to the manufacturer's instructions. Selection of successful transfectants for the DG44 cells were carried out in F12 media lacking hypoxanthine, glycine and thymidine (HGT) as described above. For selection of transfected CHO S cells in various MTX concentrations, 10% dialyzed fetal calf serum was used. G-418 resistant cell which were selected from each batch of transfection (in 750μg/ml of drug) served as controls. Comparisons of colony

formation in selection media were made between the murine Trp15 and the Arg 22 mutants and the human Ser31 mutant DHFR cDNA. For Northern blot analysis 10μg of total RNA isolated from transfected clones were electrophoresed on a 1.0% agarose/2M formaldehyde gel. For Southern blot analysis 10μg of genomic DNA was digested with NcoI and HindIII and electrophoresed on a 0.8% agarose /TBE gel. All hybridizations were carried out at 42°C with formamide in the hybridization solution. Murine *dhfr* cDNA containing the point mutation at nt 46 (G to T) was labeled with alpha ^{32}p-dCTP by the random primer method using a random priming kit (Boehringer Mannheim, IN) to a specific activity of 10^8 cpm/μg DNA. All blots were washed for 30 min at 37°C in 1XSSC/1.0%SDS and for another 60 min. at 55°C in 0.1XSSC/1.0%SDS. The blots were then exposed to x-ray film for autoradiography. DOTAP (Boehringer Mannheim, IN) mediated transfection of pSV5Trp15, pSV5Arg22 and PSV5Ser31 plasmids into murine bone marrow cells was performed according to the manufacturer's instructions. Bone marrow cells from CBAJ (7-11 weeks) mice were harvested in Iscove's modified Dulbecco's medium (IMDM) and a mononuclear cell suspension was prepared by Ficoll hypaque separation. 10μg plasmid DNA mixed with the DOTAP transfection reagent was then added to $2x10^6$ mononuclear cells in IMDM and 20% fetal bovine serum and incubated for 38 hours at 37°C. After incubation, the bone marrow cells were harvested and used for the CFU-GM assay in the presence and absence of MTX as described above.

Results and Discussion

Transfection of Trp15 mutant murine DHFR cDNA into DHFR-DG44 cells converted them to the DHFR$^+$ phenotype which suggested that the altered enzyme was catalytically functional (Table 11). Furthermore, the Trp15 *dhfr* cDNA

was capable of acting as a dominant selectable marker in CHO S cells, as MTX resistant colonies were obtained after transfection and selection in increasing concentrations of MTX (Table 12). Northern blot analyses of RNA isolated from cells obtained from individual MTX resistant colonies revealed that the transfected Trp15 cDNA was expressed at high levels (Fig. 37). Southern blot analysis indicated that the plasmid was incorporated into genomic DNA of the transfected cells. The transfected mutant *dhfr* cDNA was amplified readily by increasing the selection pressure i.e. the MTX concentration within a period of eight weeks as shown by increase in intensity of the bands on the Southern blot (Fig. 38). Quantitation of the gene copy number showed that it increased by approximately 5 and 23 fold over a period of four weeks after selection in 5μM and 15μM MTX respectively.

Other studies from our laboratory have shown that the murine mutant *dhfr* has a markedly reduced affinity for MTX but is catalytically as efficient as the wild type enzyme (Dicker et al., 1993). It has been demonstrated that the Trp15 variant *dhfr* is less stable than the wild type protein, suggesting that there may be a need to compensate for this instability by increasing copy number so that overall enzyme function and hence the resistance phenotype remains the same. This may explain the amplifiability of the Trp15 mutant cDNA in response to increasing selection pressure. In the in vitro CFU-GM assay in presence of 10^{-7}M MTX, the Trp15 cDNA transfected bone marrow cells gave rise to resistance colonies and were comparable to the resistance levels of those imparted by the Arg 22 and the Ser31 cDNAs (Table 13). Like the Ser31 and the Ser34 mutants, the Trp15 mutant cDNA also appears to be a useful dominant selectable

marker in gene transfer studies.

Our studies herein used a murine mutant *dhfr*. In the murine system the triplet code for the amino acid glycine is GGG. To encode tryptophan (TGG) only a single base change of G to T is required. In the human dhfr however the triplet code for glycine at residue 15 is GGC while that for tryptophan is TGG. Thus, a two base change is required to encode tryptophan instead of glycine. As a two base change is less likely than a single base change, isolation of a naturally occurring Trp15 mutant version in antifolate resistant human *dhfr* is less likely. cDNA encoding the human Trp 15 *dhfr* mutation may be constructed by Polymerase Chain Reaction (PCR) amplified mutagenesis using a mutation specific oligonucleotide primer.

Table 11. COLONY FORMATION IN HGT BY TRANSFECTED CHO DG44 CELLS [1]

Mock	2*
HDR	1072
Arg22	620
Ser31	955
Trp15	980

[1]colonies expressed as per μg DNA/10^6 cells

*these were nonviable colonies as they did not propagate upon subcloning in selection media lacking HGT.

Table 12. COLONY FORMATION BY TRANSFECTED CHO S CELLS IN MTX[1]

nM MTX	HDR	Arg22	Ser31	Trp15
100	0	183	216	130
500	0	120	66	85
1000	0	92	46	52

[1]colonies expressed as per μg DNA/10^6 cells

Table 13. CFU-GM ASSAY IN THE PRESENCE AND ABSENCE OF MTX

Bone Marrow cells infected with	Number of Colonies		% resistant colonies
	-MTX	+MTX	
Control no DNA	336	0	0%
pSV5Trp15	339	27	8%
pSV5Arg22	390	23	6%
PSV5Ser31	382	36	9%

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(iii) NUMBER OF SEQUENCES: 10

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(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Not Yet Known
(B) FILING DATE: 13-APR-1994
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: 08/049,284
(B) FILING DATE: 13-APR-1993

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(C) TELEX: 422523 COOP UI

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGATCGAGGA TCCCAAGCTT ACCTTTT 27

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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ATCATCCCAT GGTTGGTTCG CTAAACTGCA TCG 33

(2) INFORMATION FOR SEQ ID NO:3:

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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TCTAAAGCCA GCAAAAGCTT CATGGTCTTA TAA 33

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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GGAAGCCGGT CTTGTCGATC 20

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(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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CGAAATCTCG TGATGGCAGG 20

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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TGCCAATTCC GGTTGTTCAA T 21

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCTGTCCTTT AAAGGTCG 18

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAGGTTCCTT GAGTTCTCTG C 21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCTCGGCCTC TGAGCTAT 18

What is claimed is:

1. A DNA vector which comprises DNA encoding a mutant, antifolate resistant, dihydrofolate reductase inserted into a site within the vector, the presence of which site is not essential for replication of the vector.

2. A DNA vector of claim 1, wherein the mutant dihydrofolate reductase has substantially the same amino acid sequence as naturally occurring human dihydrofolate reductase.

3. A DNA vector of claim 2, wherein the mutant dihydrofolate reductase differs from naturally occurring human dihydrofolate reductase by virtue of the presence of a serine residue at position 31 or 34.

4. A DNA vector of claim 2, wherein the mutant dihydrofolate reductase differs from naturally occurring human dihydrofolate reductase by virtue of the presence of a tryptophan residue at position 15.

5. A DNA vector of claim 1, wherein the DNA encoding the mutant dihydrofolate reductase is operatively linked at its 5′ end to a promoter sequence and at its 3′end to a polyA sequence.

6. A DNA vector of claim 5, wherein the promoter sequence is an SV40 promoter.

7. A plasmid which comprises the vector of claim 1.

8. The plasmid of claim 7 designated pSV5-Ser31 h-DHFR (ATCC Accession No. 75441).

9. The plasmid of claim 8 designated pSV5-Ser34 h-DHFR (ATCC Accession No. 69276).

10. A retroviral DNA vector of claim 1.

11. A retroviral vector of claim 10, wherein the vector comprises DNA from a retrovirus corresponding to a 5′ long terminal repeat, a 3′ long terminal repeat, and a packaging signal.

12. A retroviral vector of claim 11, wherein the site at which the DNA encoding the mutant dihydrofolate reductase inserted is in the 3′ long terminal repeat.

13. A plasmid which comprises the retroviral vector of claim 12.

14. The plasmid of claim 13 designated pDC SV S31 h-DHFR (ATCC Accession No. 75440).

15. A mammalian retroviral producer cell which comprises the vector claim 1 or the plasmid of claim 13 or 14.

16. A human cell which comprises the vector of claim 1 or the plasmid of claim 13 or 14.

17. A hematopoietic human cell of claim 16.

18. A bone marrow cell of claim 17.

19. A method for reducing the toxic effects of antifolate therapy in a subject which comprises replacing the subject's hematopoietic cells with hematopoietic cells of claim 17 so as to reduce the toxic effects of antifolate therapy in the subject.

20. A method of claim 19, wherein the antifolate is methotrexate.

FIGURE 1

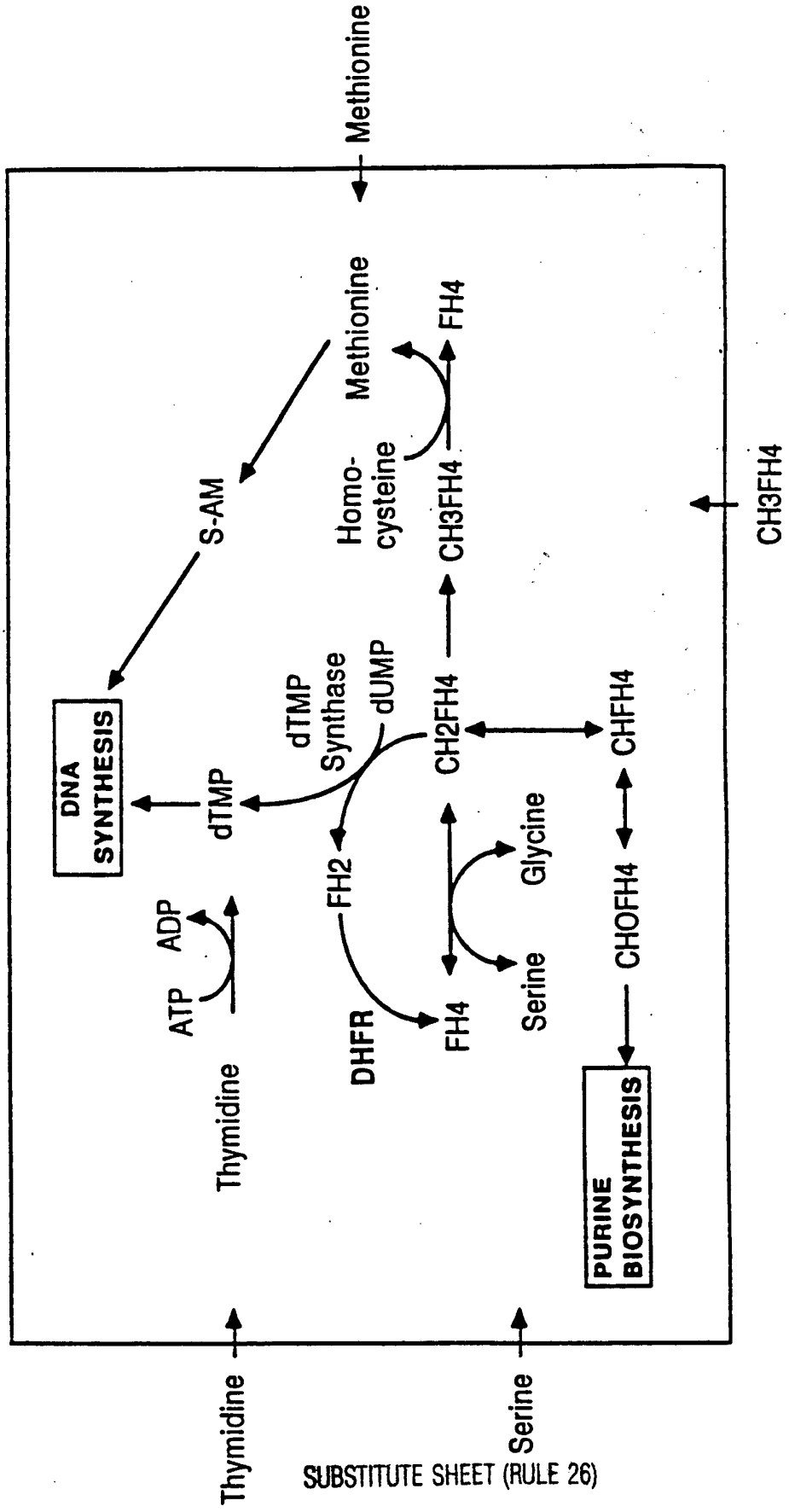
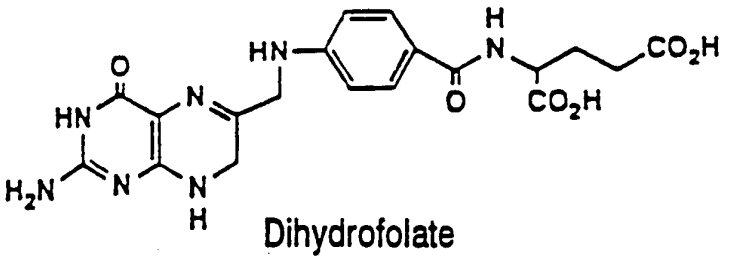
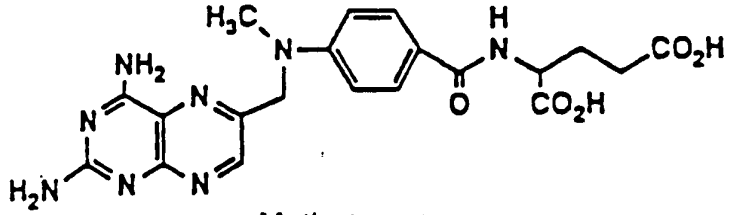

Methionine
Methionine
FH4
Homo-
cysteine
CH3FH4
S-AM
CH3FH4
DNA
SYNTHESIS
dTMP
Synthase
dUMP
CH2FH4
CHFH4
dTMP
FH2
Glycine
ADP
ATP
Serine
DHFR
FH4
CHOFH4
Thymidine
PURINE
BIOSYNTHESIS
Thymidine
Serine

FIGURE 2A



Dihydrofolate



Methotrexate

FIGURE 2B

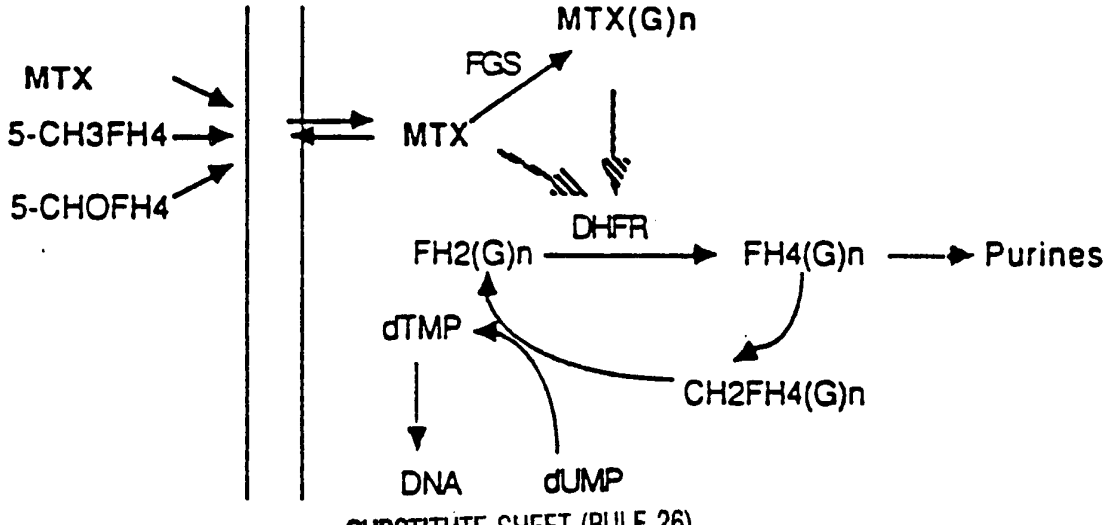


FIGURE 3

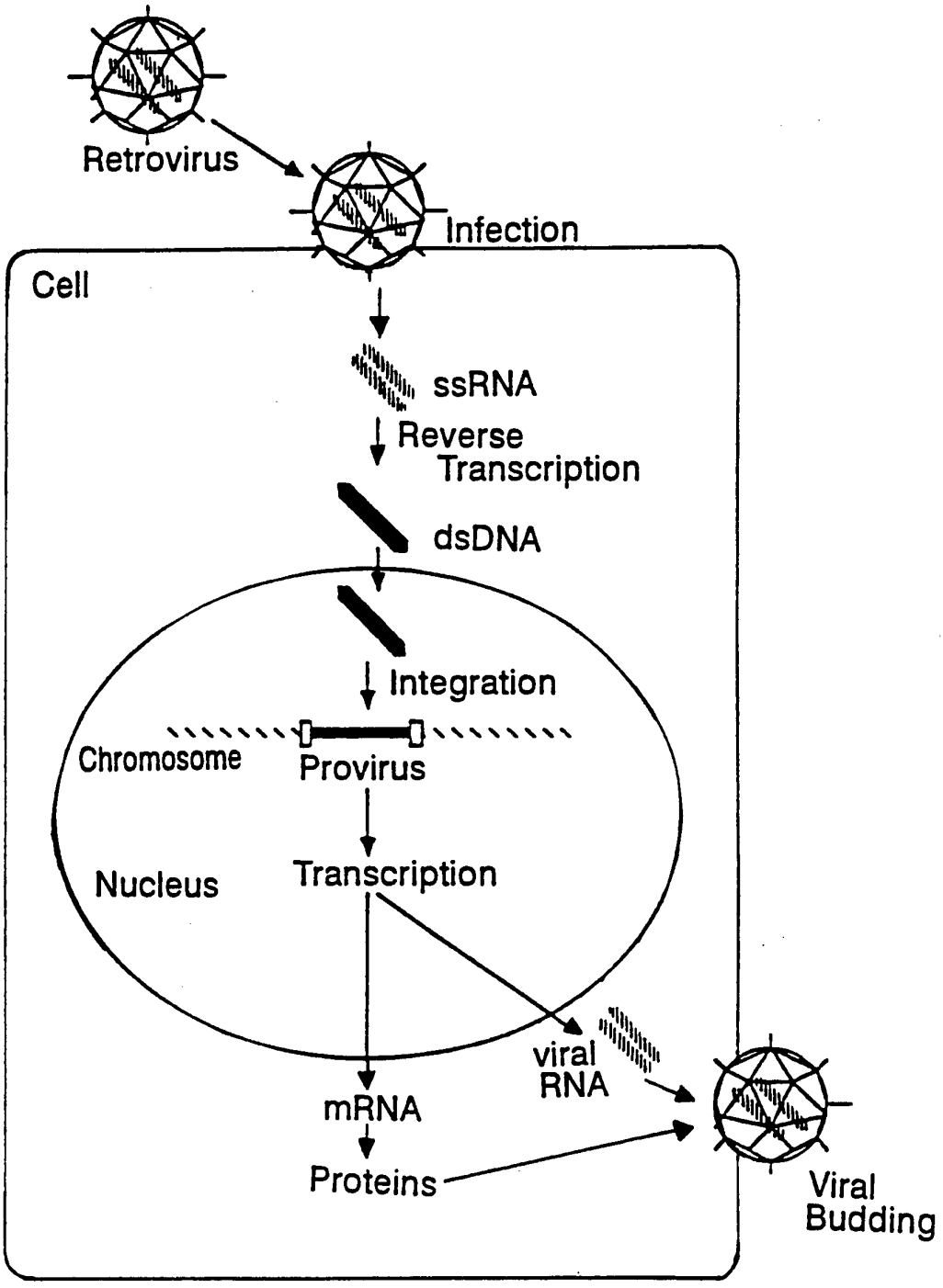

Retrovirus
Infection
Cell
ssRNA
Reverse
Transcription
dsDNA
Integration
Chromosome
Provirus
Nucleus
Transcription
viral
RNA
mRNA
Proteins
Viral
Budding

FIGURE 4

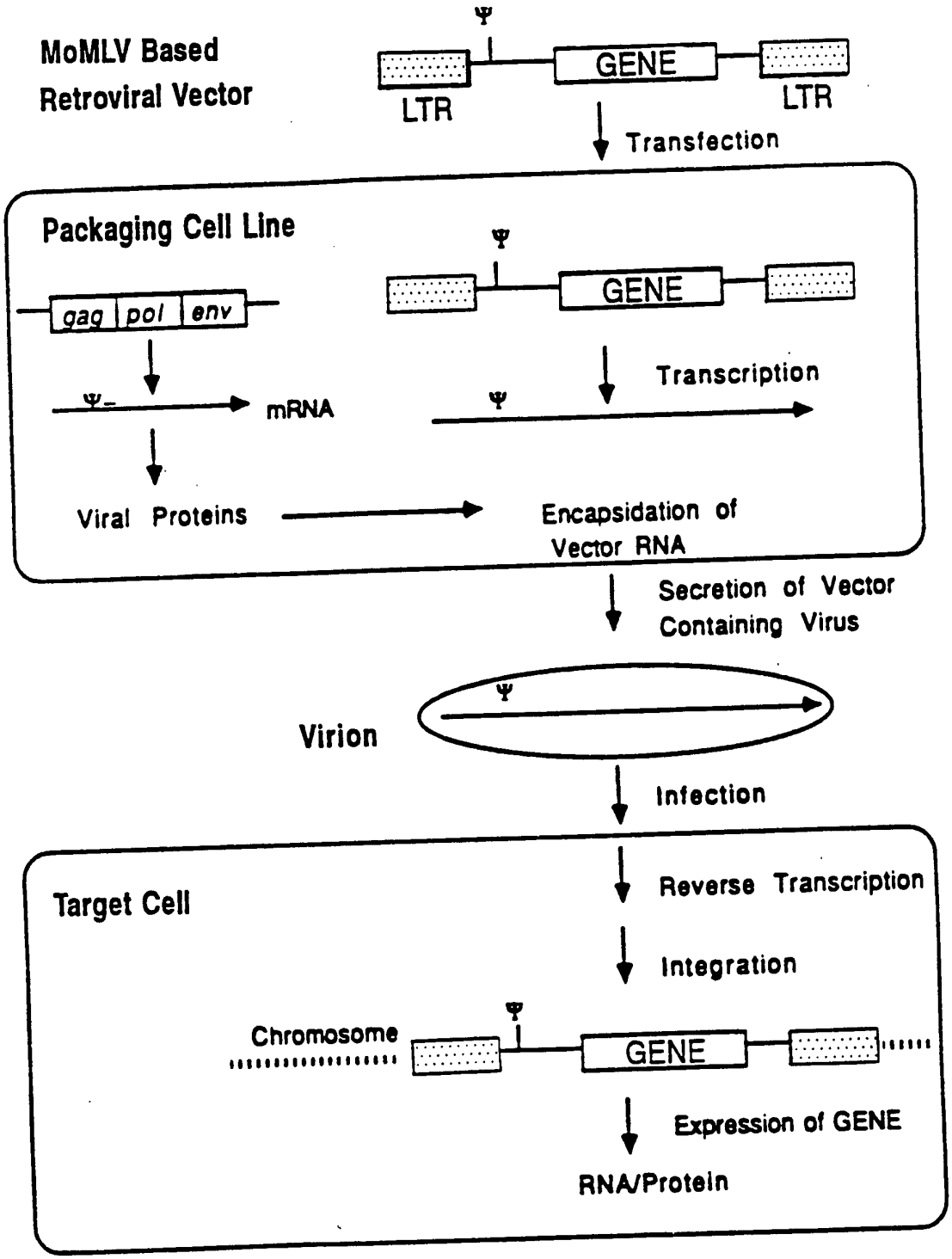

MoMLV Based
Retroviral Vector
Ψ
GENE
LTR
LTR
Transfection
Packaging Cell Line
gag
pol
env
Ψ
mRNA
Ψ
GENE
Transcription
Viral Proteins
Encapsidation of
Vector RNA
Secretion of Vector
Containing Virus
Virion
Ψ
Infection
Target Cell
Reverse Transcription
Integration
Chromosome
Ψ
GENE
Expression of GENE
RNA/Protein

FIGURE 5A

Vectors with Internal Promoters (VIP)

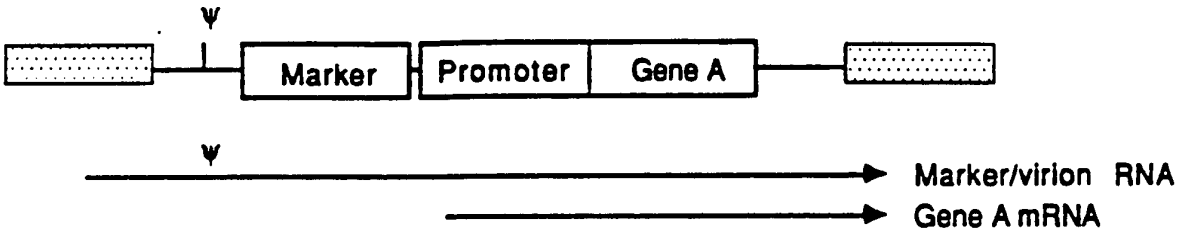


FIGURE 5B

N2 Retroviral Vector

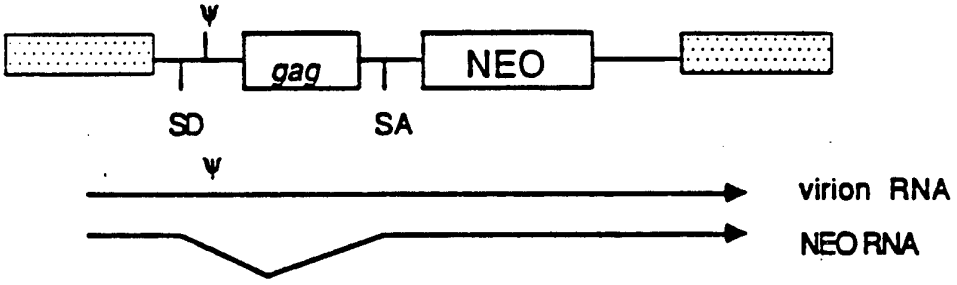
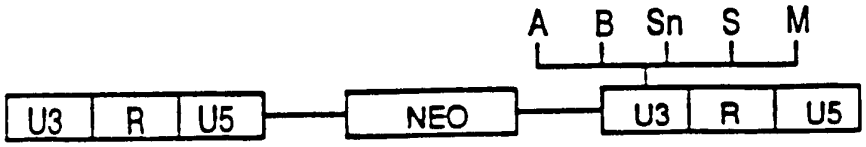
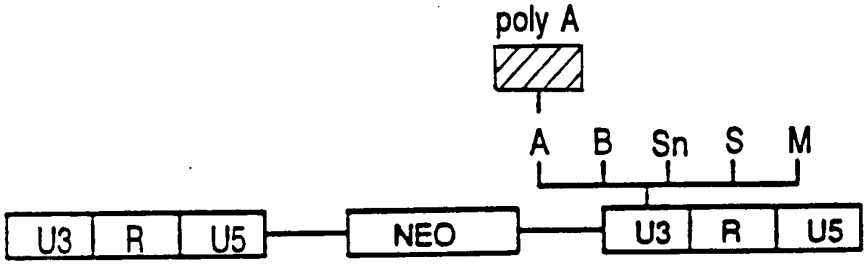


FIGURE 6A



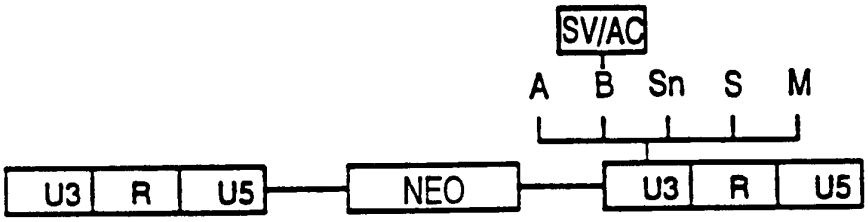
N2A

FIGURE 6B



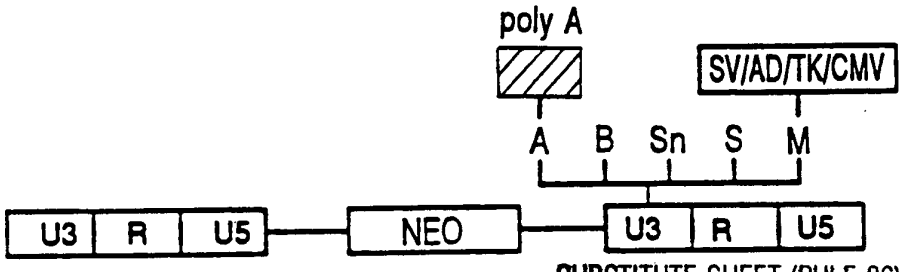
N2AP

FIGURE 6C



DC/SV
DC/AC

FIGURE 6D



DC/SV/R
DC/AD/R
DC/TK/R
DC/CMV/R

FIGURE 7A

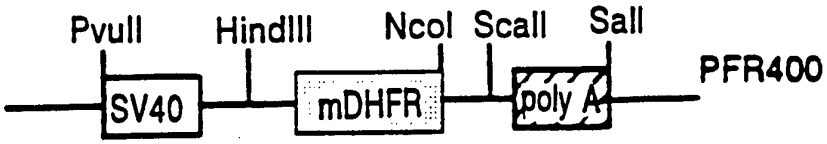


FIGURE 7B

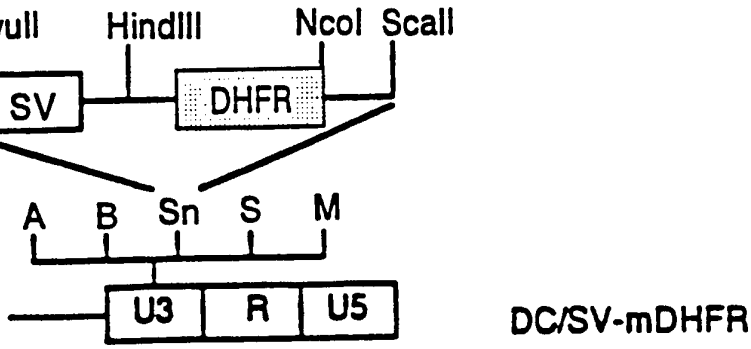


FIGURE 7C

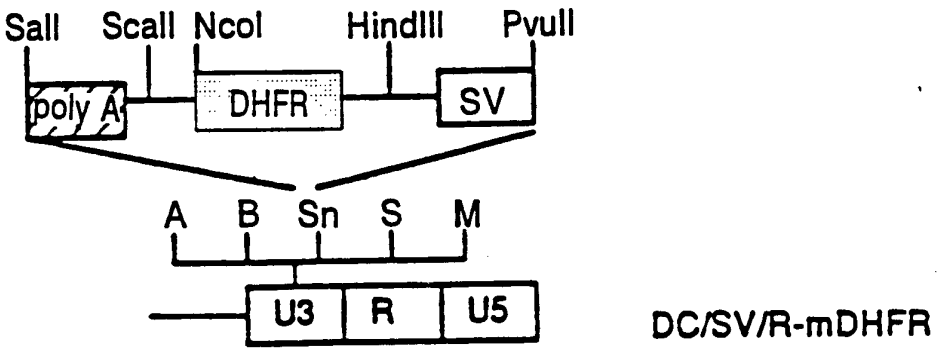
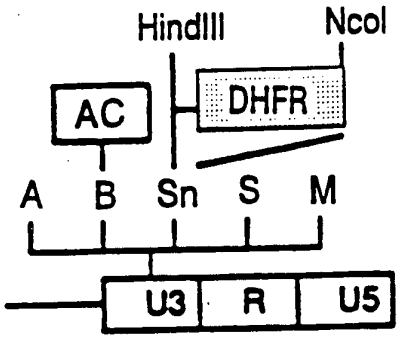
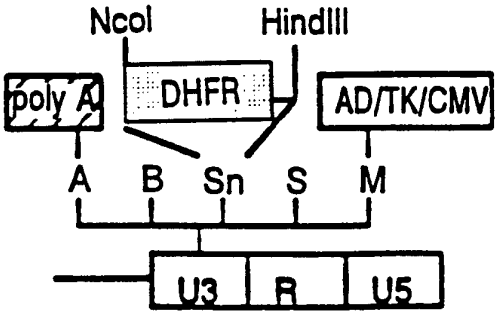


FIGURE 7D



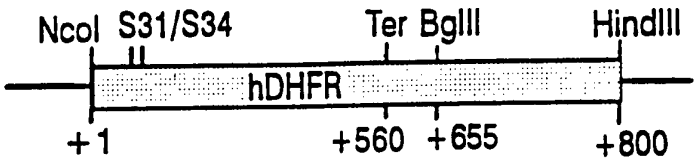
DC/AC-mDHFR

FIGURE 7E



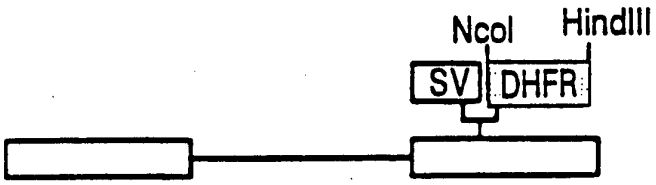
DC/AD/R-mDHFR
DC/TK/R-mDHFR
DC/CMV/R-mDHFR

FIGURE 8A



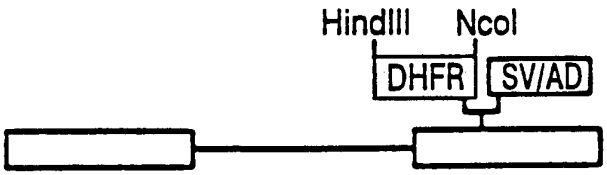
pKT7HDR

FIGURE 8B



DC/SV-hDHFR31/34

FIGURE 8C

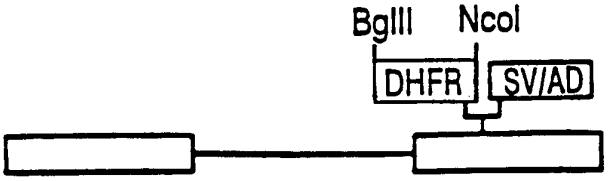


DC/SV/R-hDHFR31/34
DC/AD/R-hDHFR31/34

FIGURE 8D

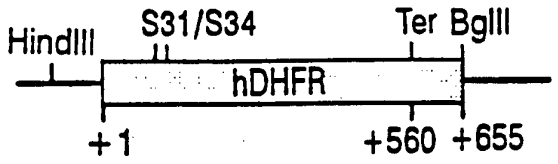
DC/SV-hDHFR31 NB

FIGURE 8E



DC/SV/R-hDHFR31 NB
DC/AD/R-hDHFR31 NB

FIGURE 8F

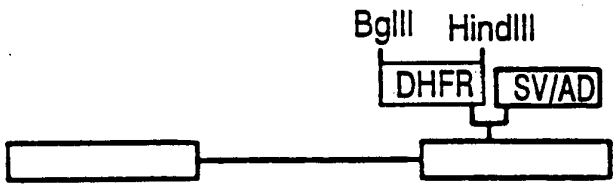


pSV4HDR

FIGURE 8G

DC/SV-hDHFR31 HB

FIGURE 8H



DC/SV/R-hDHFR31 HB
DC/AD/R-hDHFR31 HB

FIGURE 9

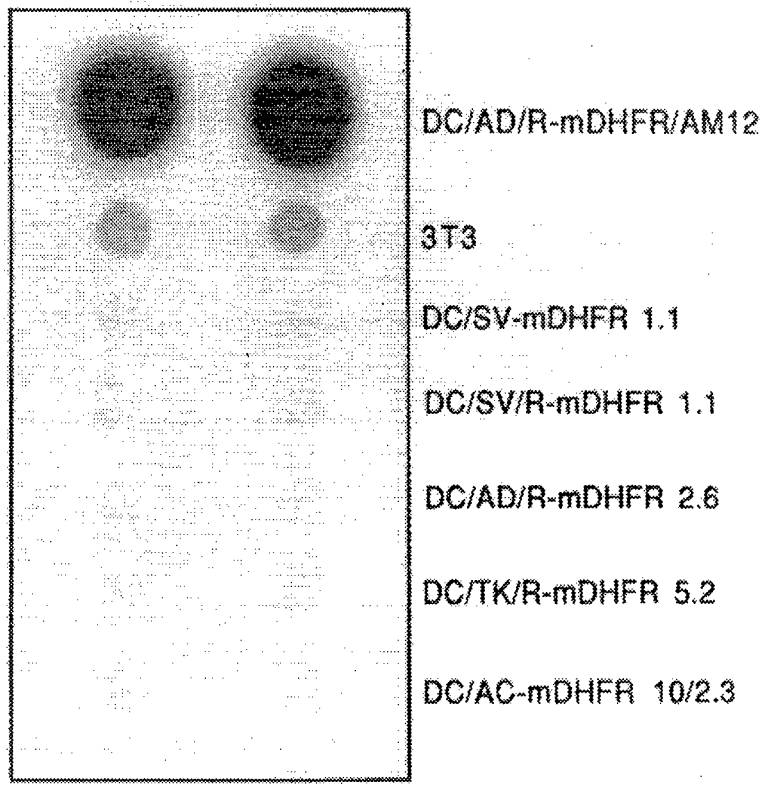

DC/AD/R-mDHFR/AM12
3T3
DC/SV-mDHFR 1.1
DC/SV/R-mDHFR 1.1
DC/AD/R-mDHFR 2.6
DC/TK/R-mDHFR 5.2
DC/AC-mDHFR 10/2.3

FIGURE 10

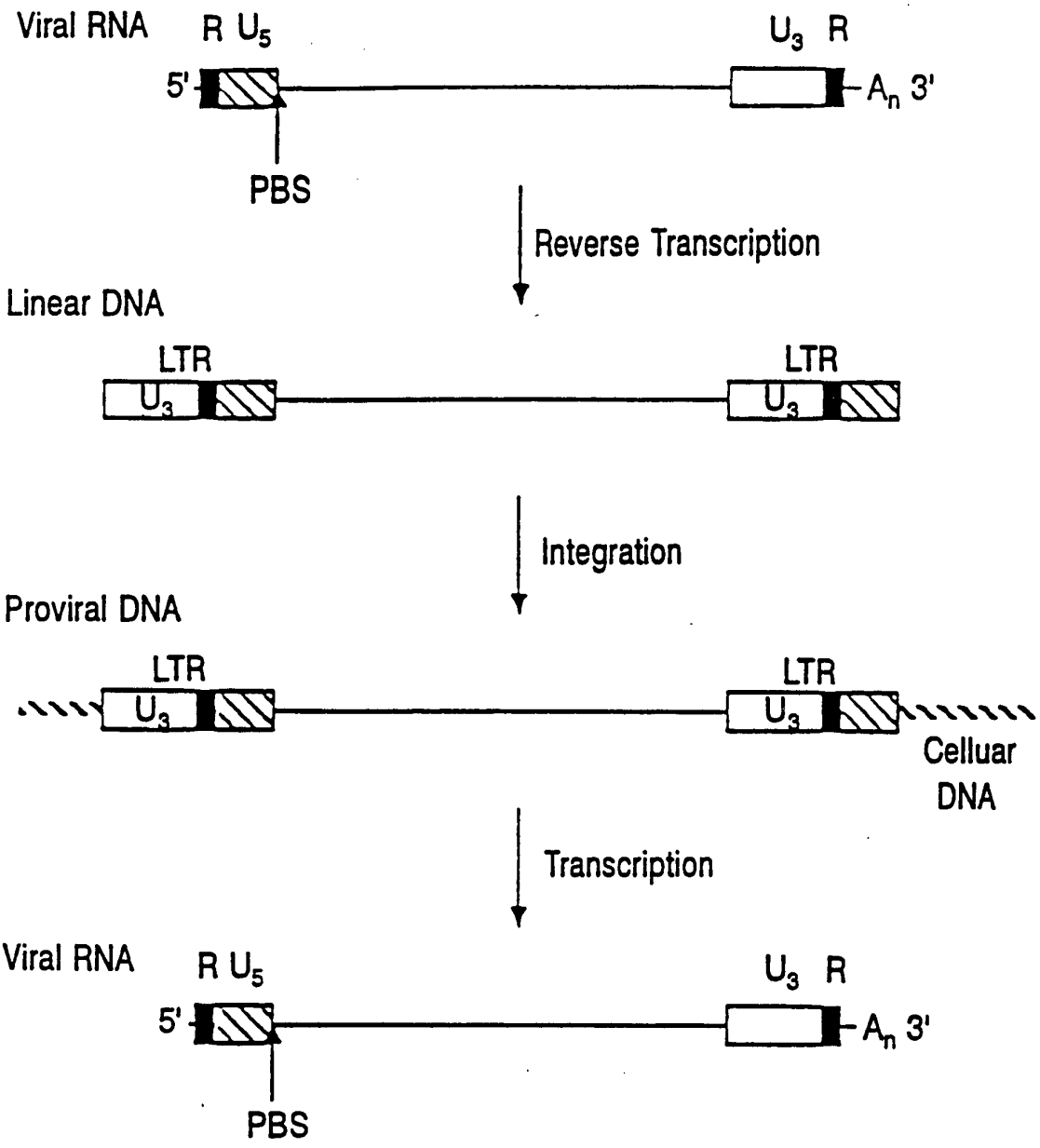

Viral RNA
R U5
U3 R
5'
An 3'
PBS
Reverse Transcription
Linear DNA
LTR
U3
LTR
U3
Integration
Proviral DNA
LTR
U3
LTR
U3
Cellular DNA
Transcription
Viral RNA
R U5
U3 R
5'
An 3'
PBS

FIGURE 11

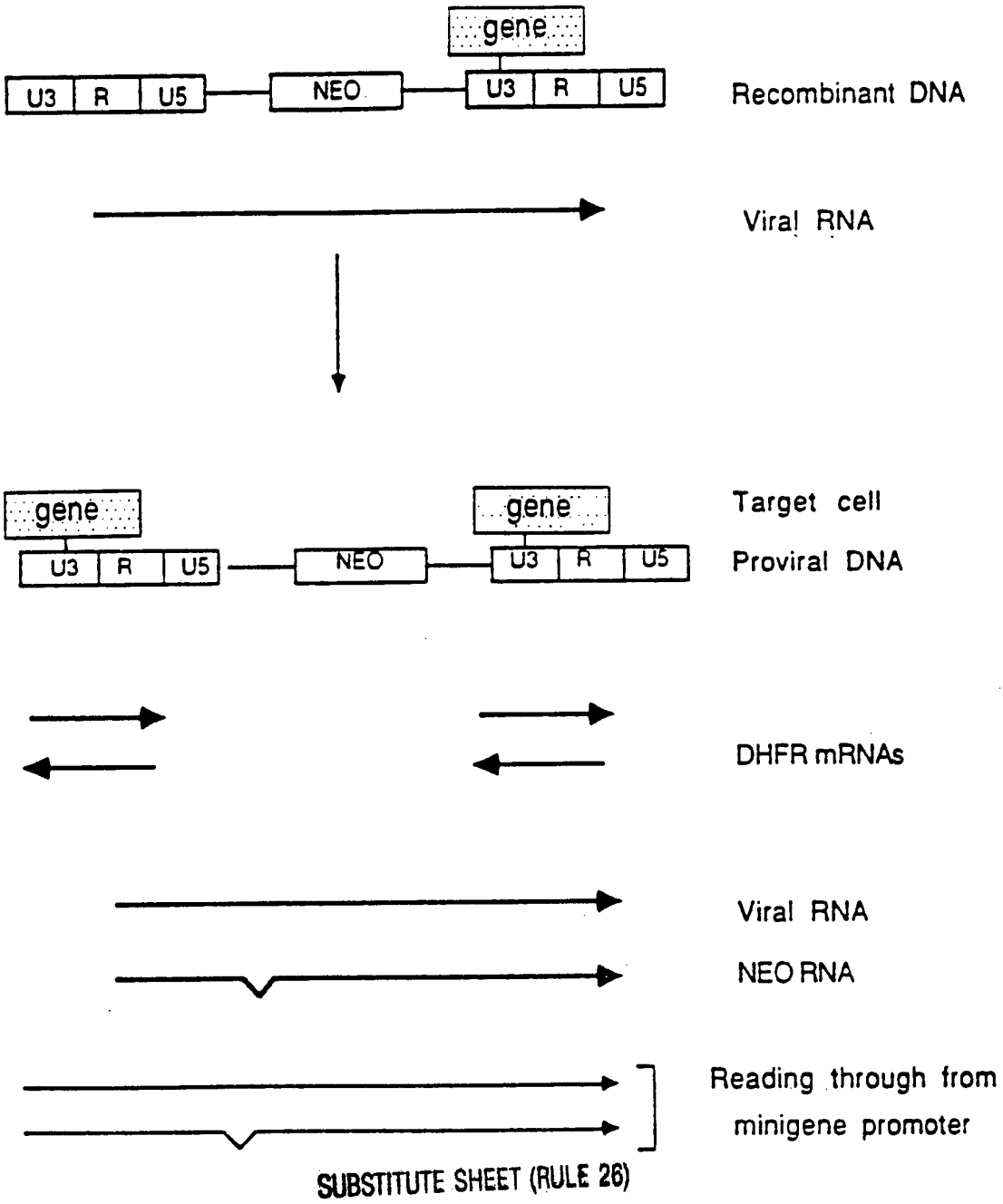

gene
U3
R
U5
NEO
U3
R
U5
Recombinant DNA
Viral RNA
gene
gene
U3
R
U5
NEO
U3
R
U5
Target cell
Proviral DNA
DHFR mRNAs
Viral RNA
NEO RNA
Reading through from
minigene promoter

FIGURE 12A

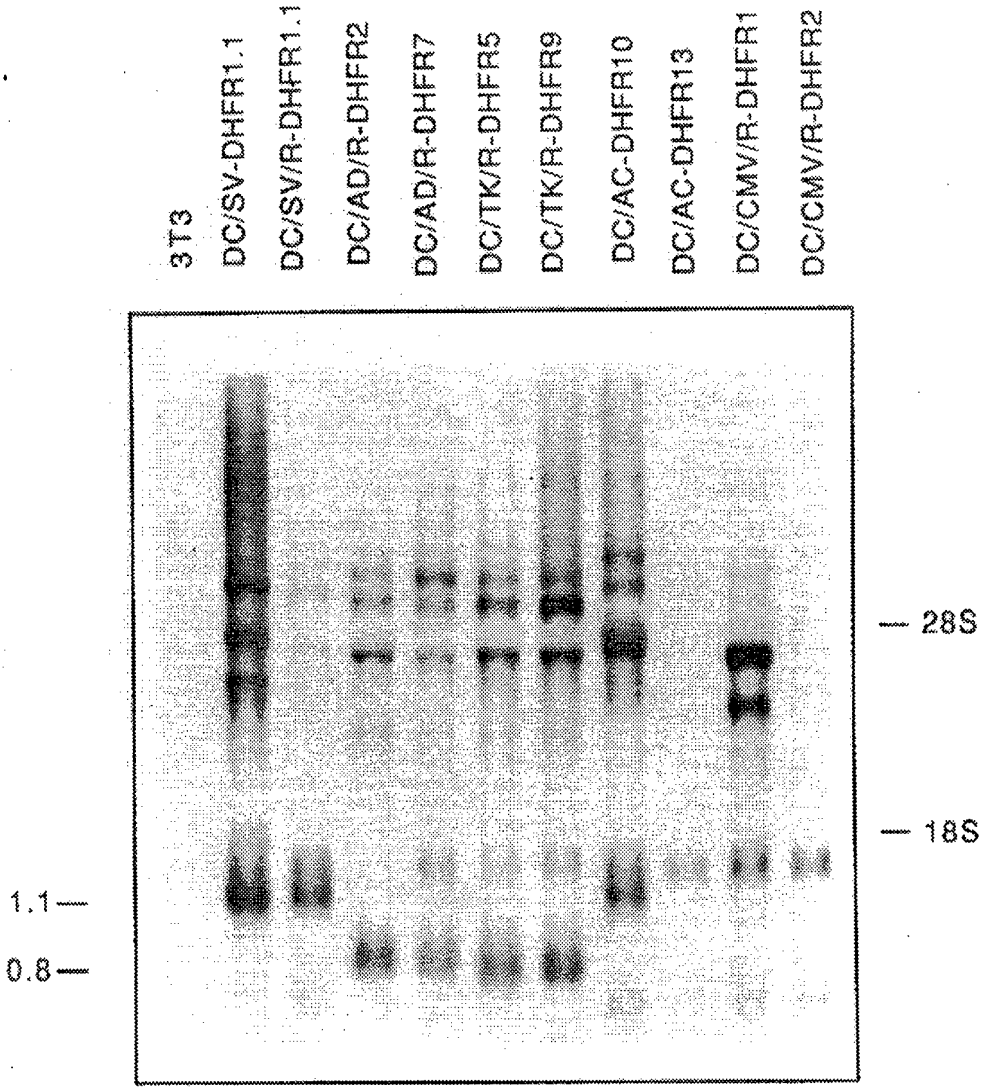


FIGURE 12B

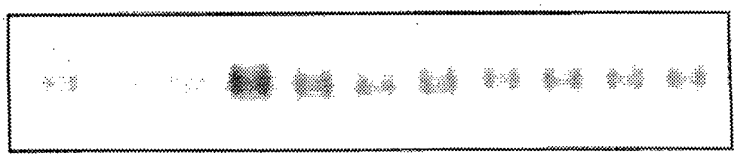


FIGURE 13A

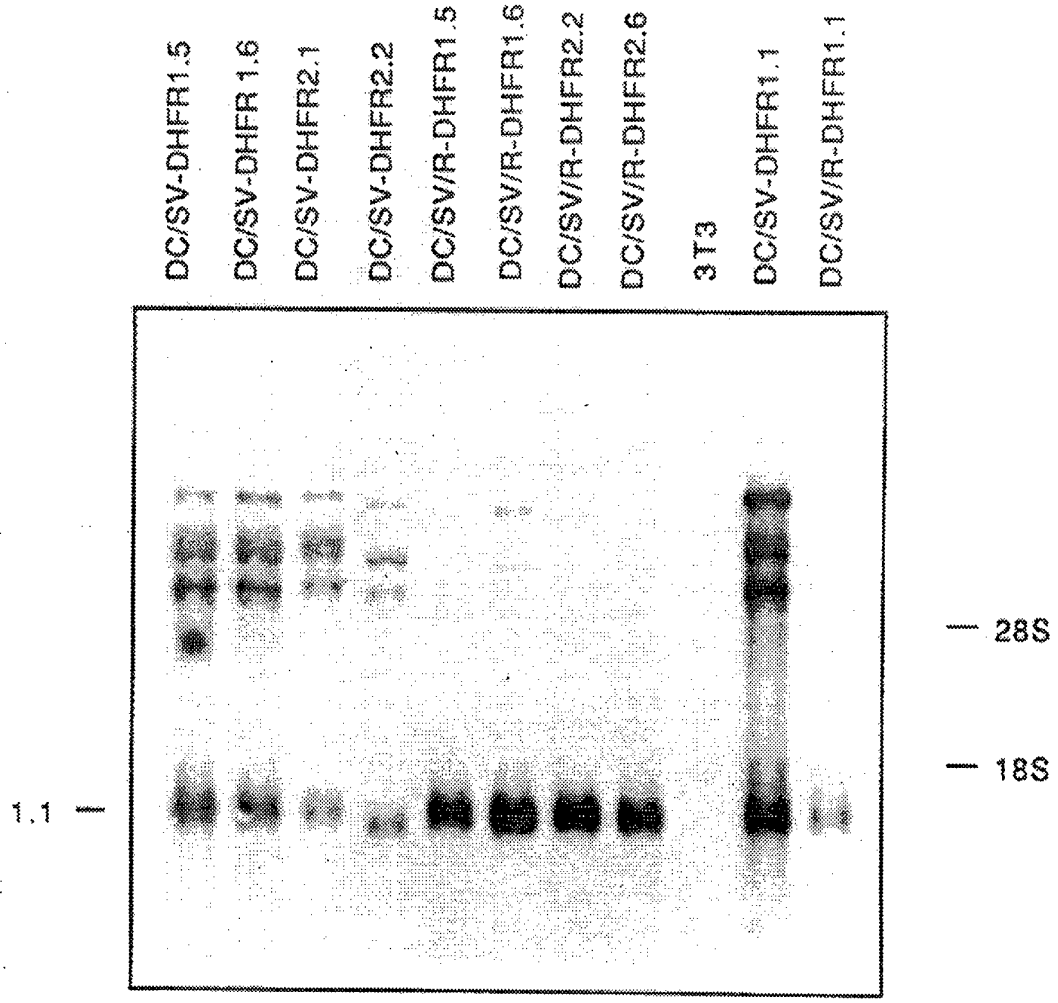


FIGURE 13B

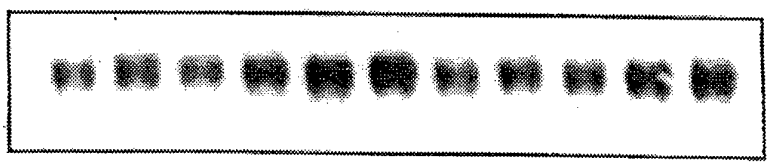


FIGURE 14A

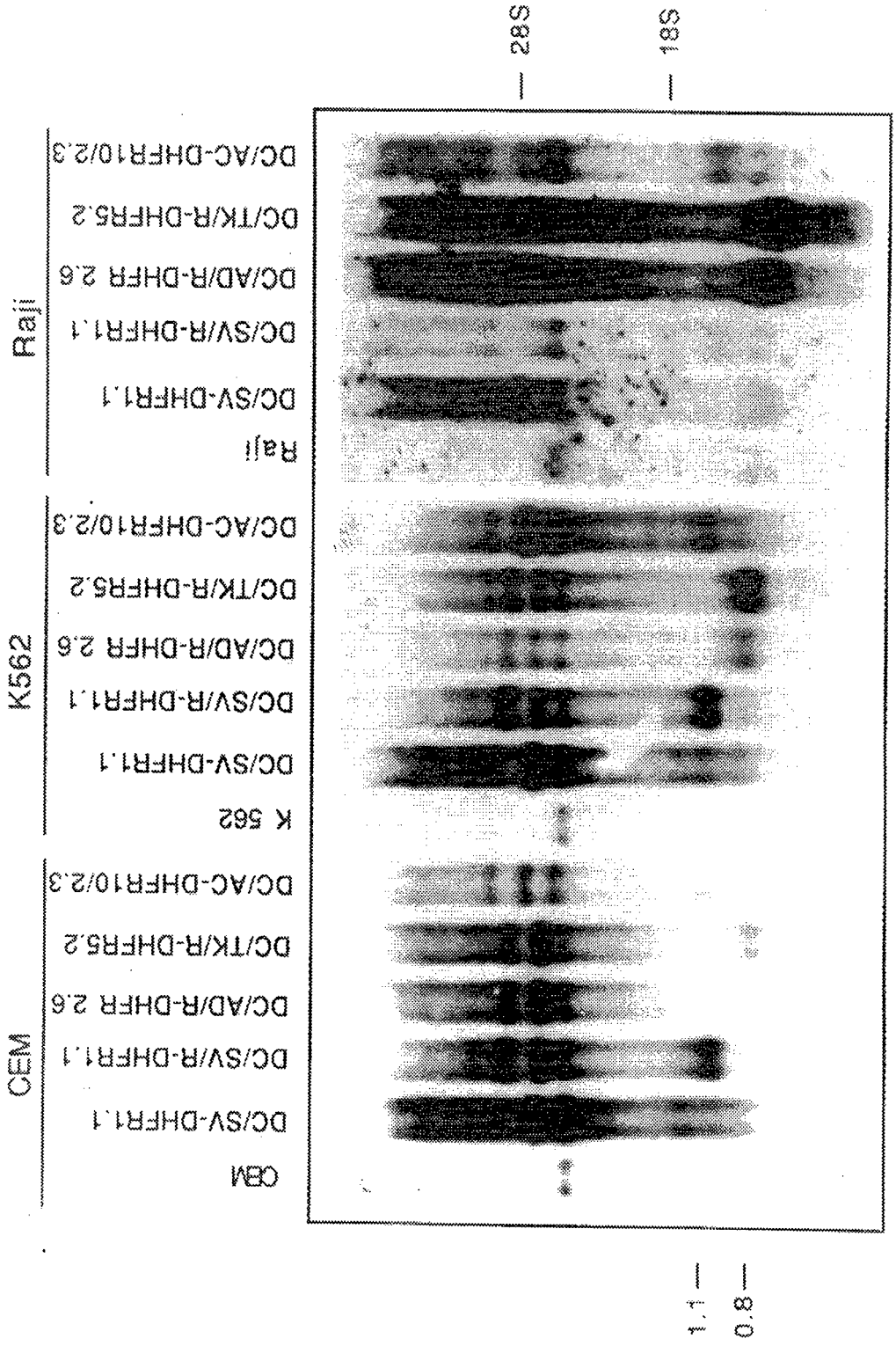

28S
18S
CEM
K562
Raji
CEM
DC/SV-DHFR1.1
DC/SV/R-DHFR1.1
DC/AD/R-DHFR 2.6
DC/TK/R-DHFR5.2
DC/AC-DHFR10/2.3
K 562
DC/SV-DHFR1.1
DC/SV/R-DHFR1.1
DC/AD/R-DHFR 2.6
DC/TK/R-DHFR5.2
DC/AC-DHFR10/2.3
Raji
DC/SV-DHFR1.1
DC/SV/R-DHFR1.1
DC/AD/R-DHFR 2.6
DC/TK/R-DHFR5.2
DC/AC-DHFR10/2.3
1.1
0.8

FIGURE 14B

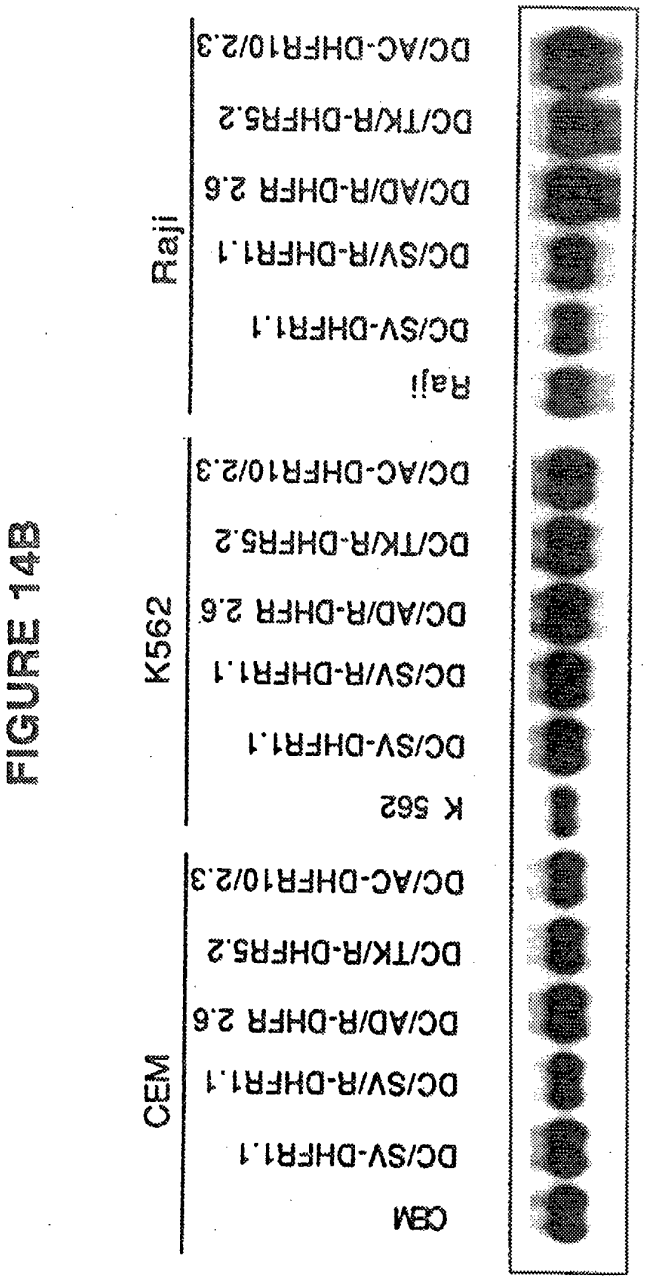

CEM
DC/SV-DHFR1.1
DC/SV/R-DHFR1.1
DC/AD/R-DHFR 2.6
DC/TK/R-DHFR5.2
DC/AC-DHFR10/2.3
K562
K 562
DC/SV-DHFR1.1
DC/SV/R-DHFR1.1
DC/AD/R-DHFR 2.6
DC/TK/R-DHFR5.2
DC/AC-DHFR10/2.3
Raji
Raji
DC/SV-DHFR1.1
DC/SV/R-DHFR1.1
DC/AD/R-DHFR 2.6
DC/TK/R-DHFR5.2
DC/AC-DHFR10/2.3

FIGURE 15A

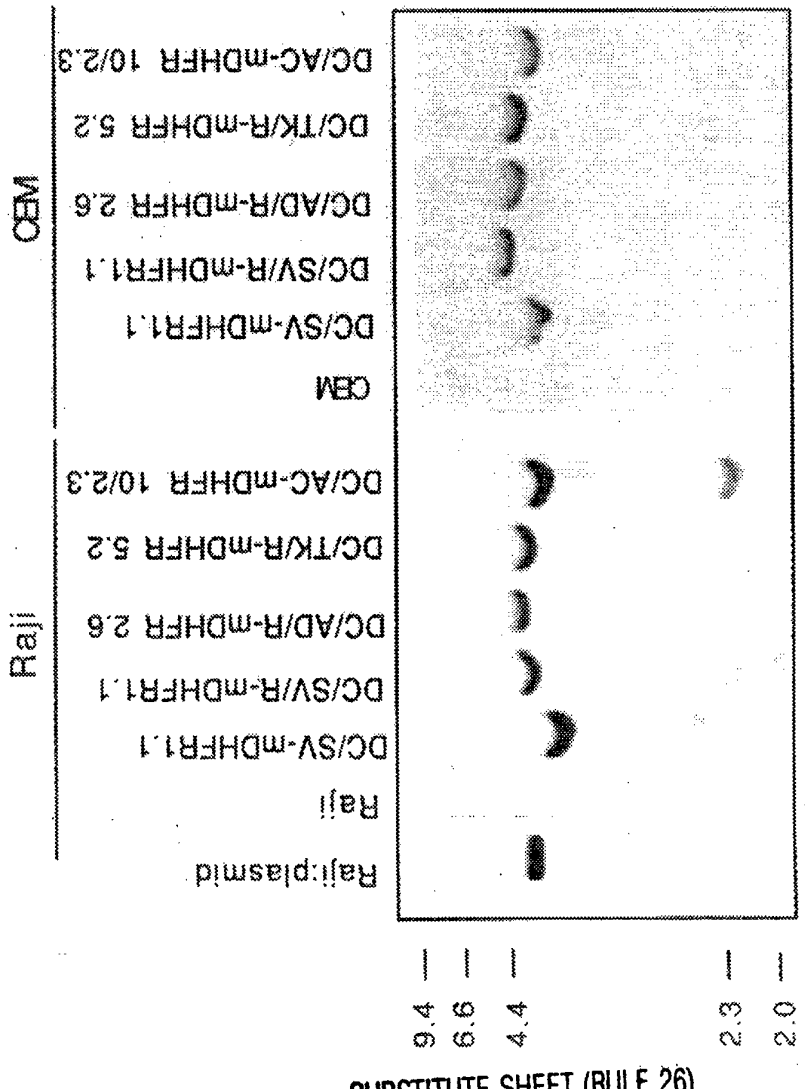


FIGURE 15B

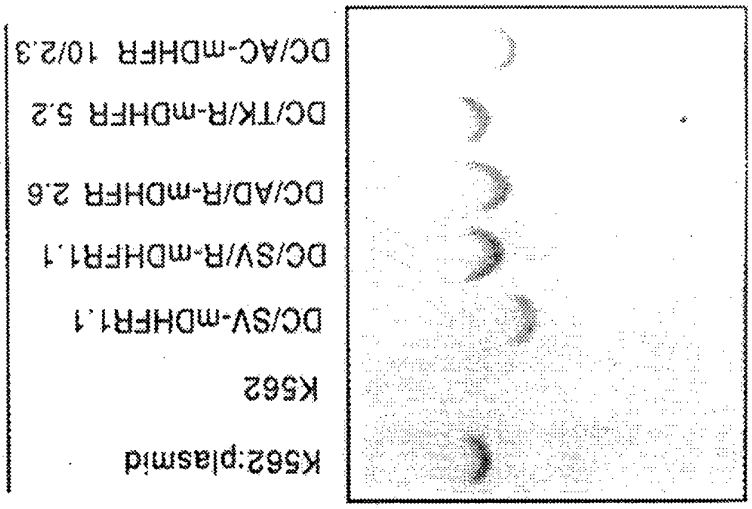


FIGURE 16

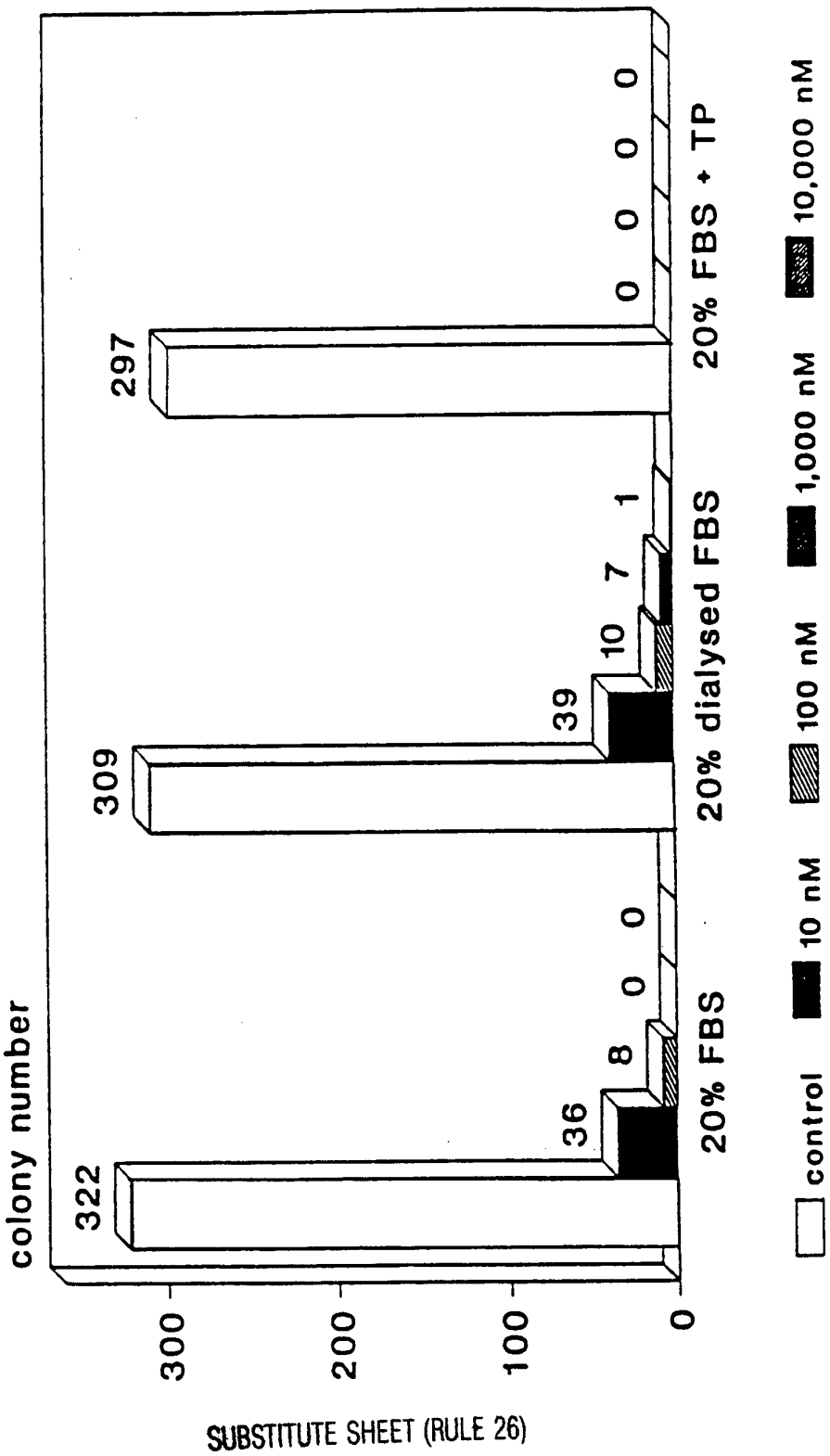

colony number
300
200
100
0
322
36
8
0
0
309
39
10
7
1
297
0
0
0
0
20% FBS
20% dialysed FBS
20% FBS + TP
control
10 nM
100 nM
1,000 nM
10,000 nM

FIGURE 17

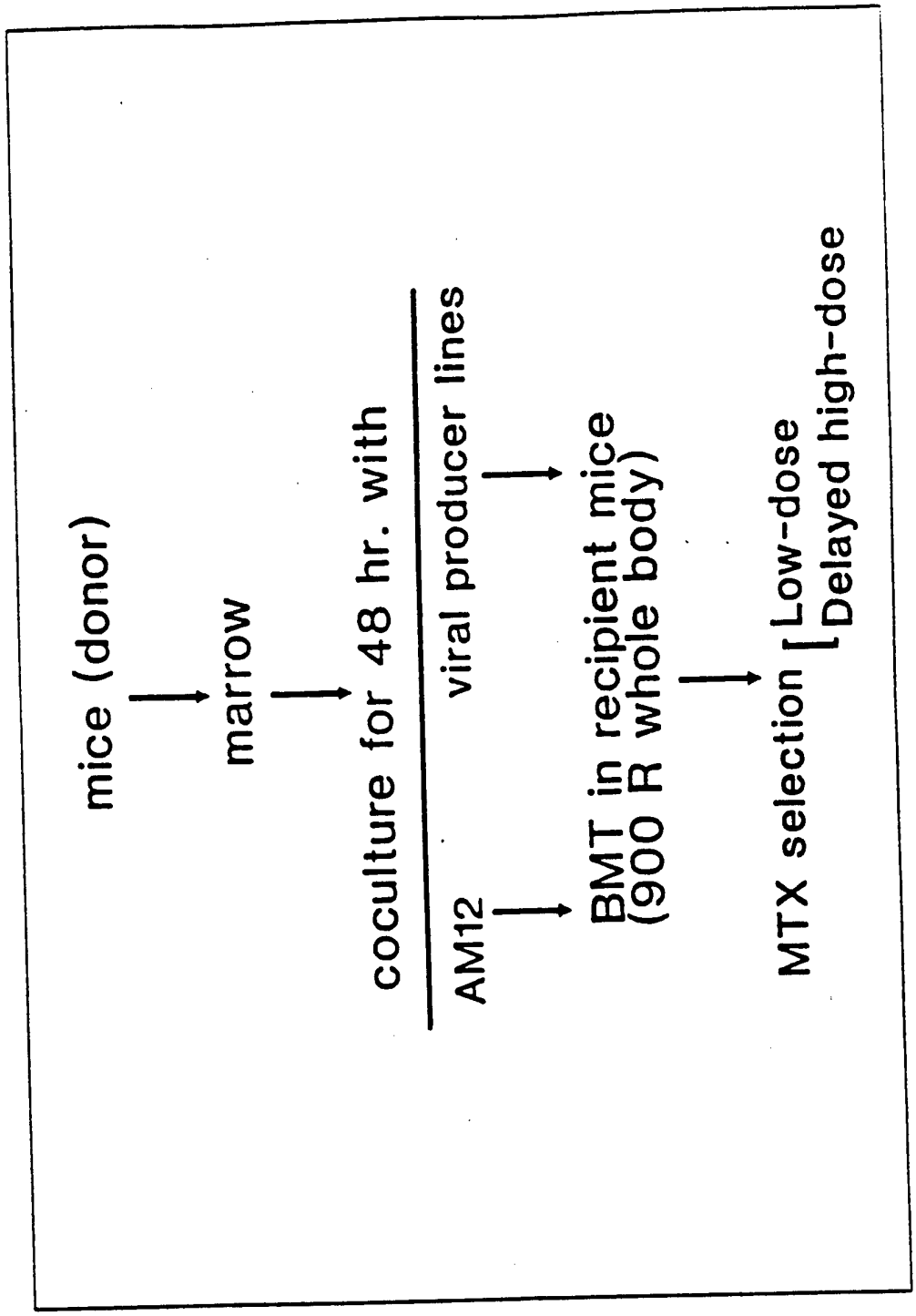

mice (donor)
marrow
coculture for 48 hr. with
AM12
viral producer lines
BMT in recipient mice
(900 R whole body)
MTX selection
Low-dose
Delayed high-dose

FIGURE 18A

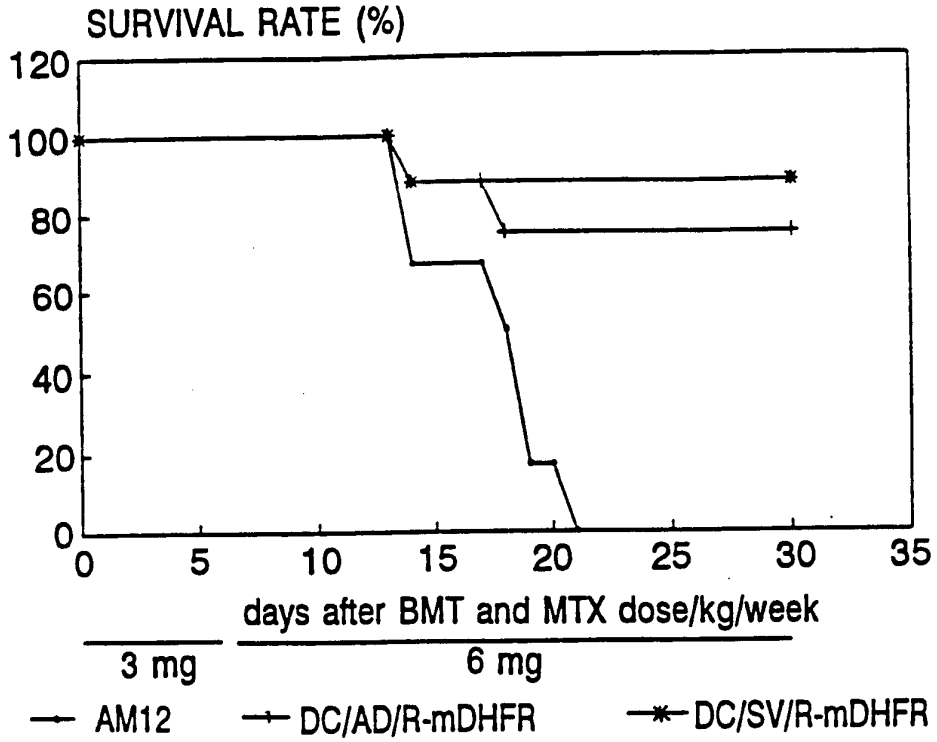


FIGURE 18B

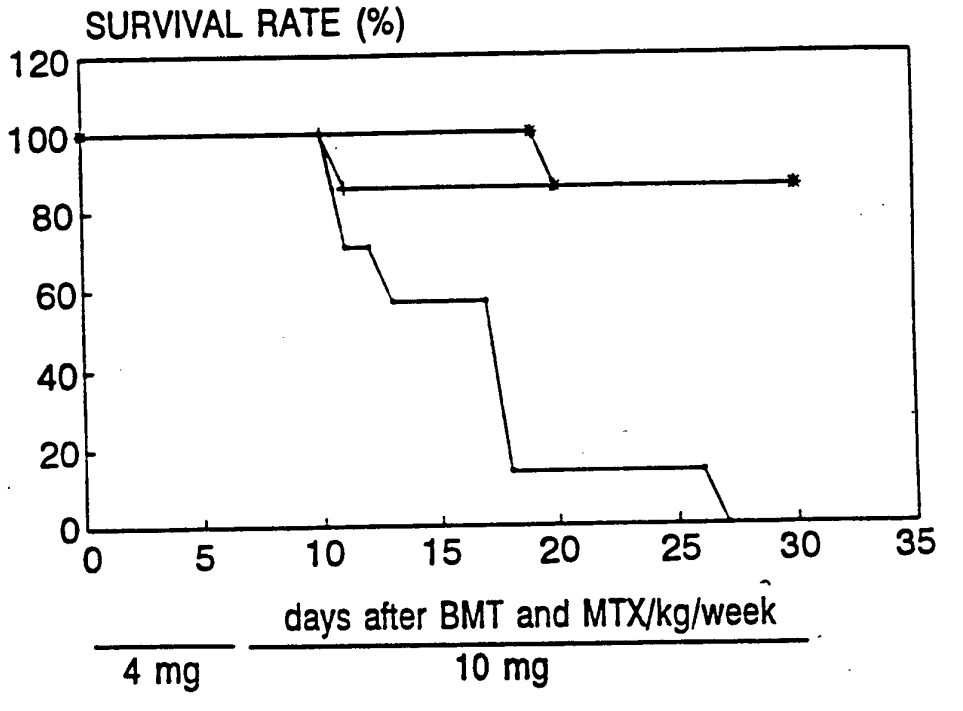


FIGURE 19A

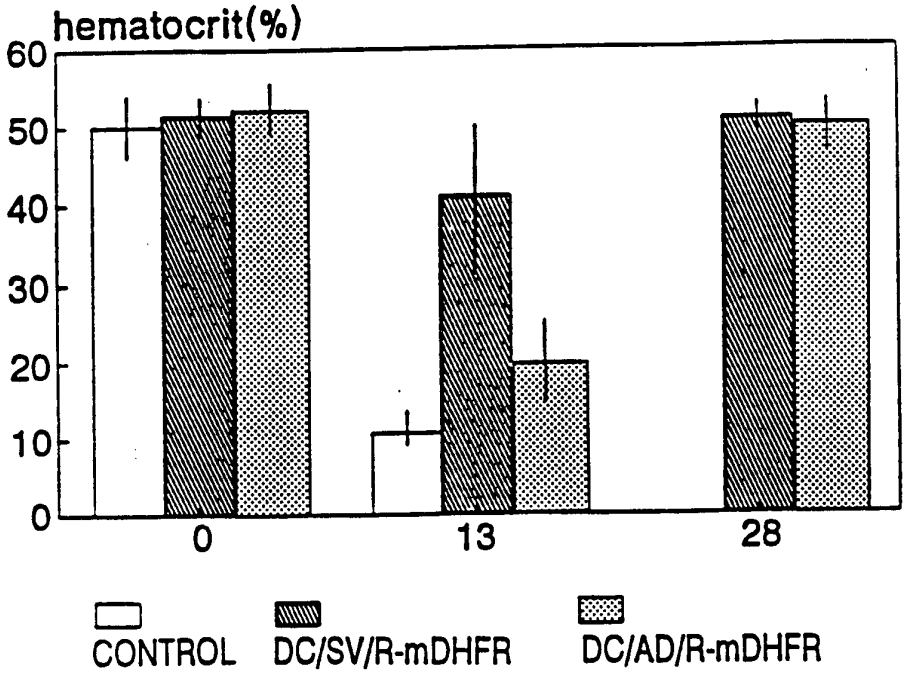


FIGURE 19B

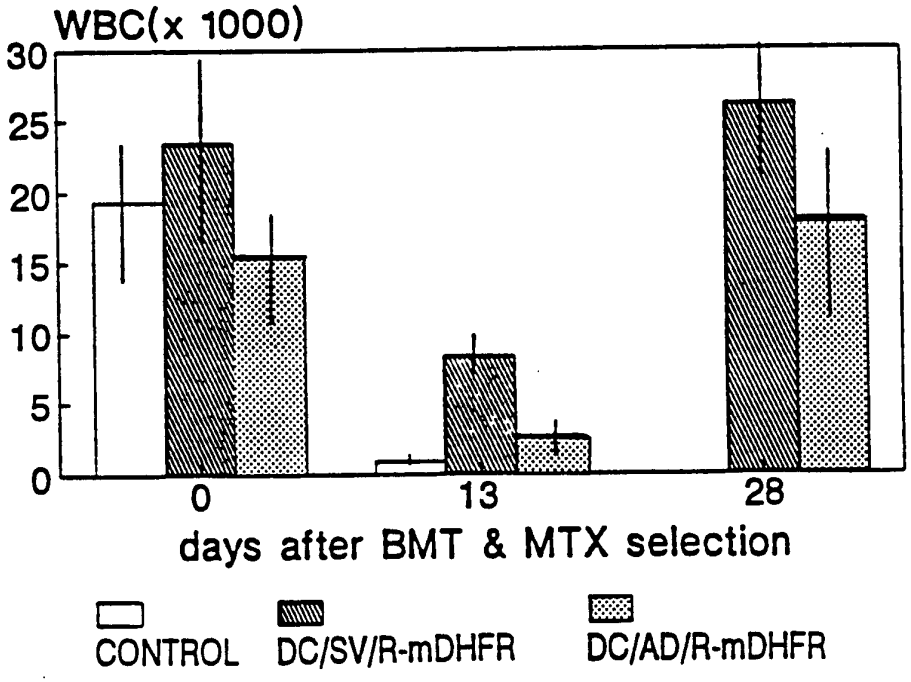


FIGURE 20A

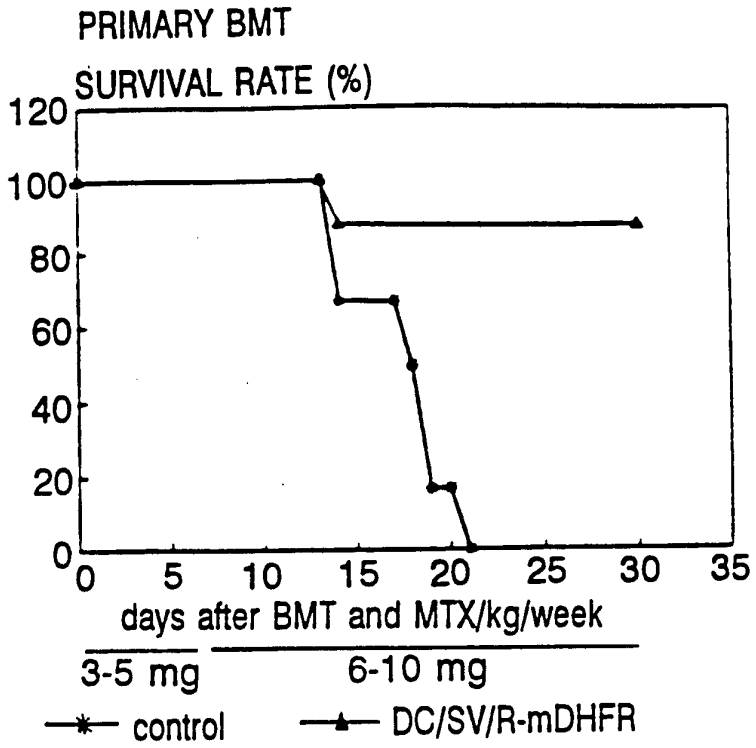


FIGURE 20B

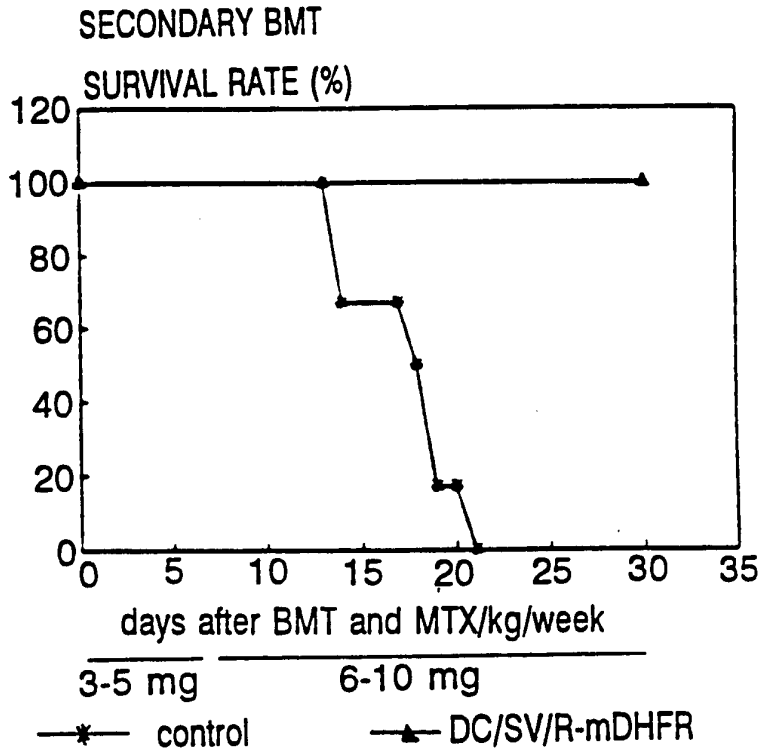


FIGURE 20C

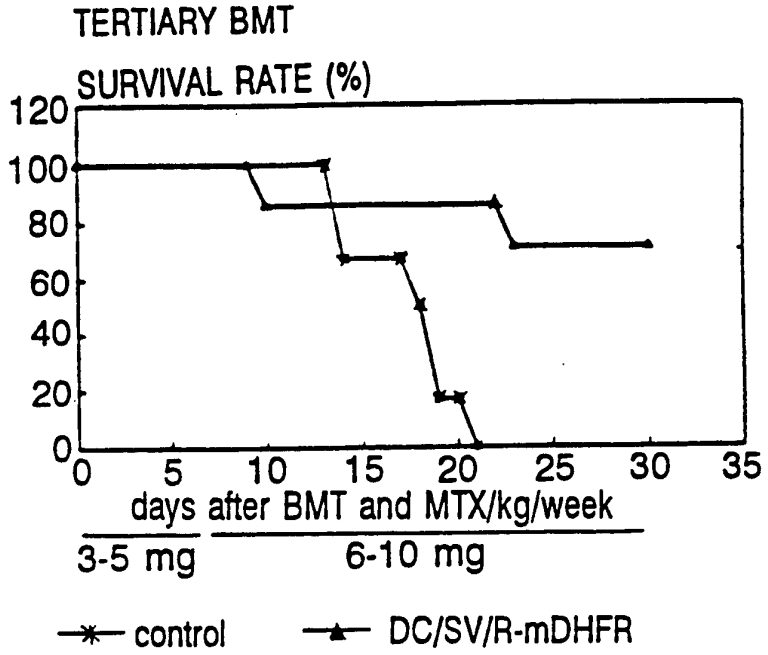

TERTIARY BMT
SURVIVAL RATE (%)
120
100
80
60
40
20
0
0 5 10 15 20 25 30 35
days after BMT and MTX/kg/week
3-5 mg
6-10 mg
control
DC/SV/R-mDHFR

FIGURE 21A

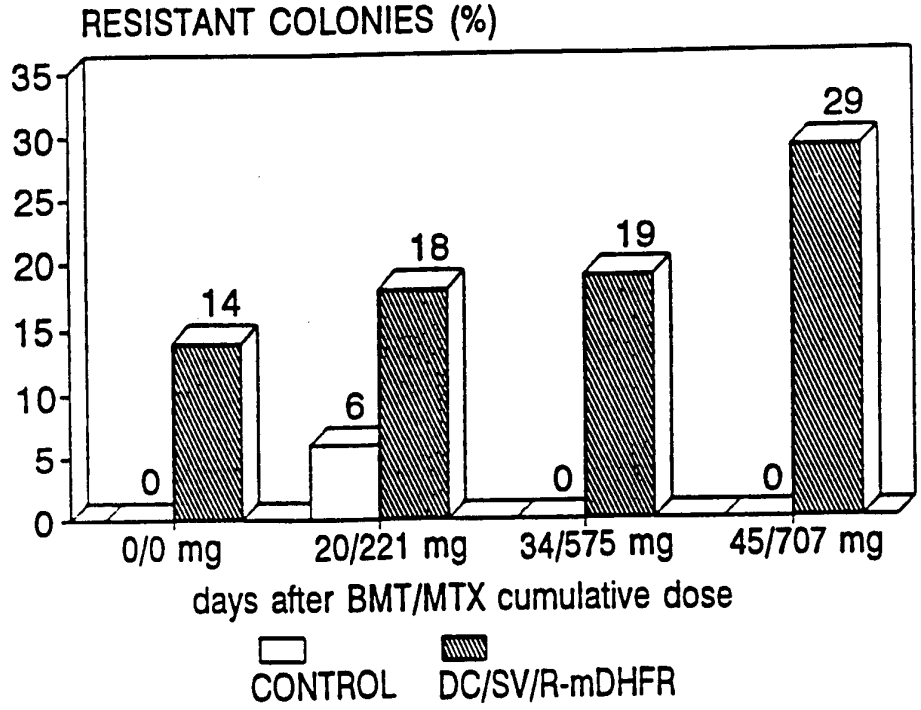


FIGURE 21B

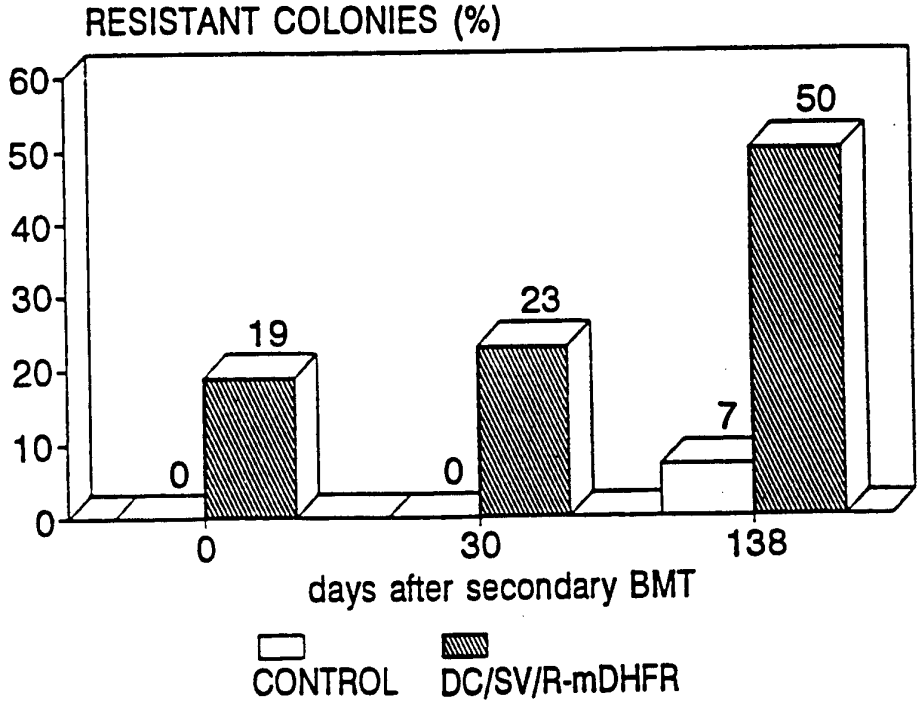


FIGURE 22A

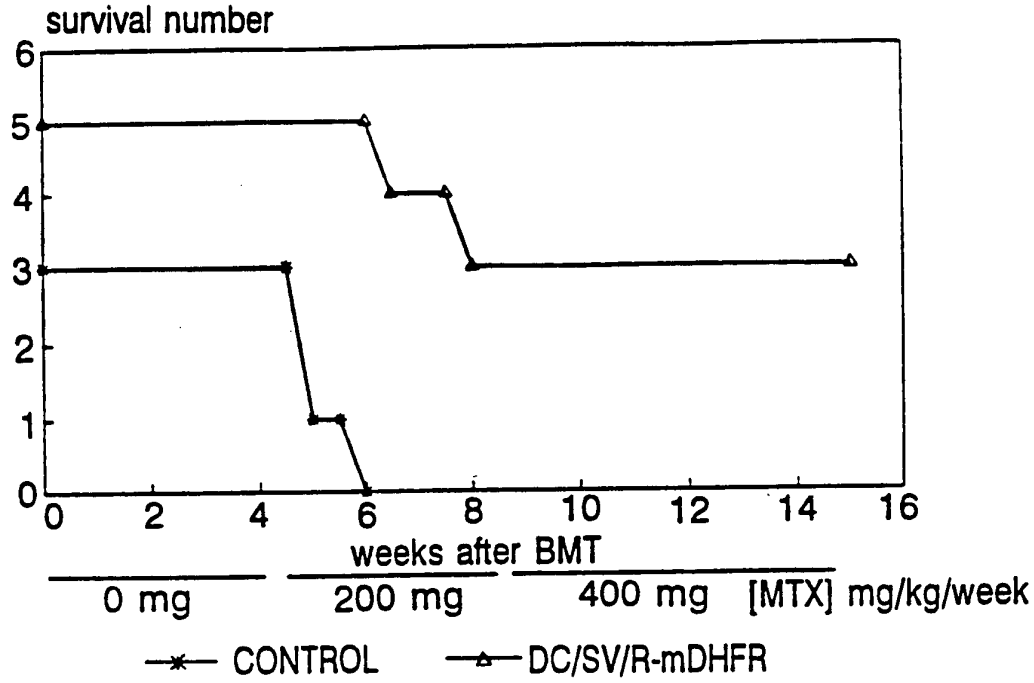


FIGURE 22B

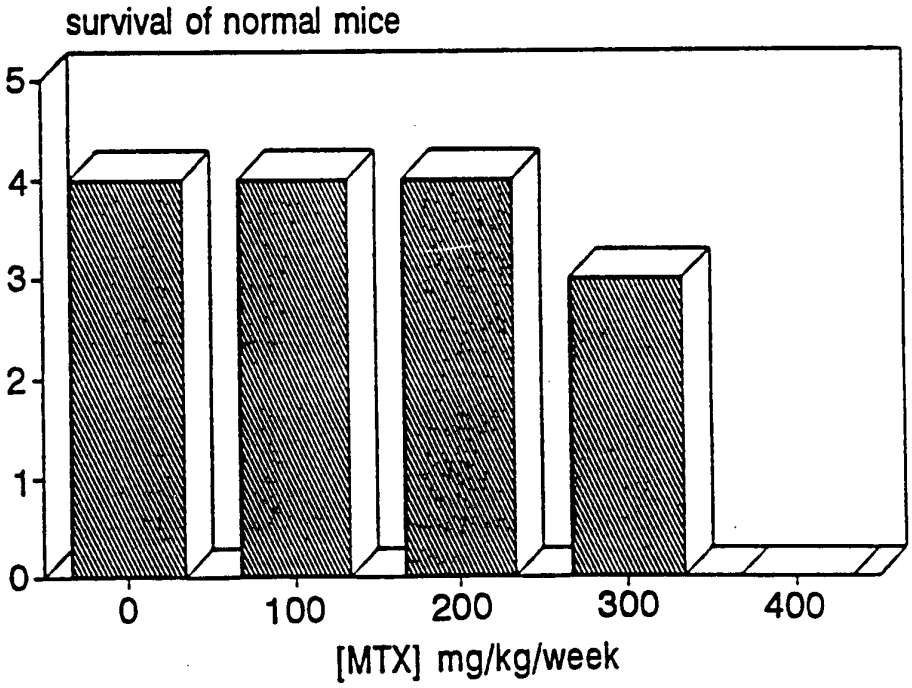


FIGURE 23A

1. Normal mouse spleen
2. Normal mouse bone marrow
3. DC/SV/R-mDHFR spleen
4. DC/AD/R-mDHFR spleen
5. DC/SV/R-mDHFR liver
6. No template
7. Marker
8. Blank

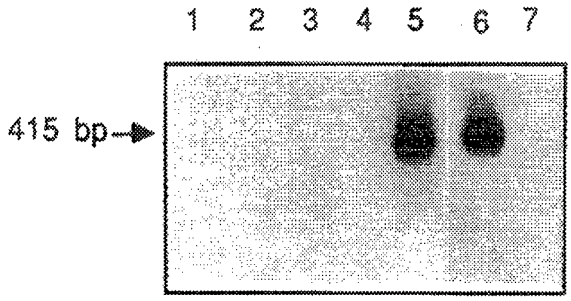
FIGURE 23B

1. DC/SV/R-mDHFR spleen
2. DC/SV/R-mDHFR liver
3. No template
4. AM12 spleen
5. AM12 liver
6. Marker

FIGURE 23C

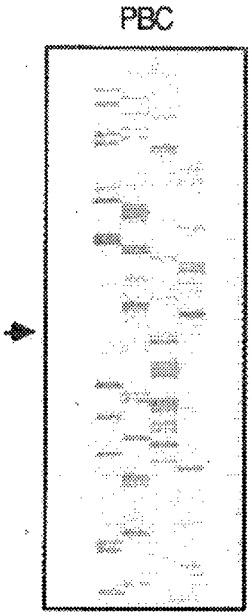
1. No template
2. AM12 spleen
3. AM12 liver
4. DC/SV/R-mDHFR/3T3
5. Marker

FIGURE 24



1. Marker
2. Normal mouse
3. AM12
4. Blank
5. DC/SV/R-mDHFR (5 weeks after primary BMT)
6. DC/SV/R-mDHFR (4 months after secondary BMT)
7. No template

FIGURE 25A



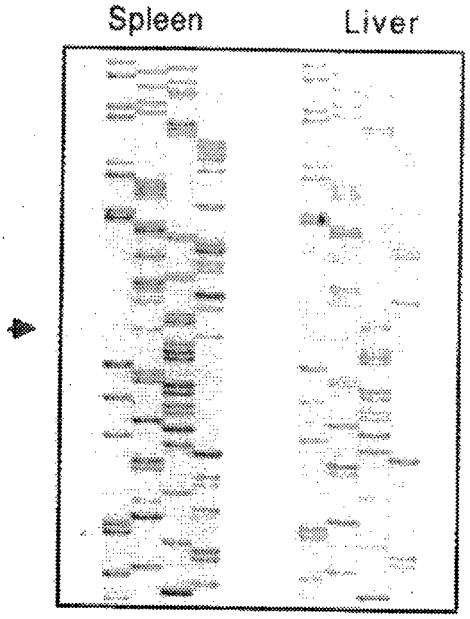
Leu

Wild type (non-coding): GCCTCTG GAT GGGA

Mutant (non-coding): GCCTCTG GCT GGGA

Arg

FIGURE 25B



Leu

Wild type (non-coding): GCCTCTG GAT GGGA

Mutant (non-coding): GCCTCTG GCT GGGA

Arg

FIGURE 26A

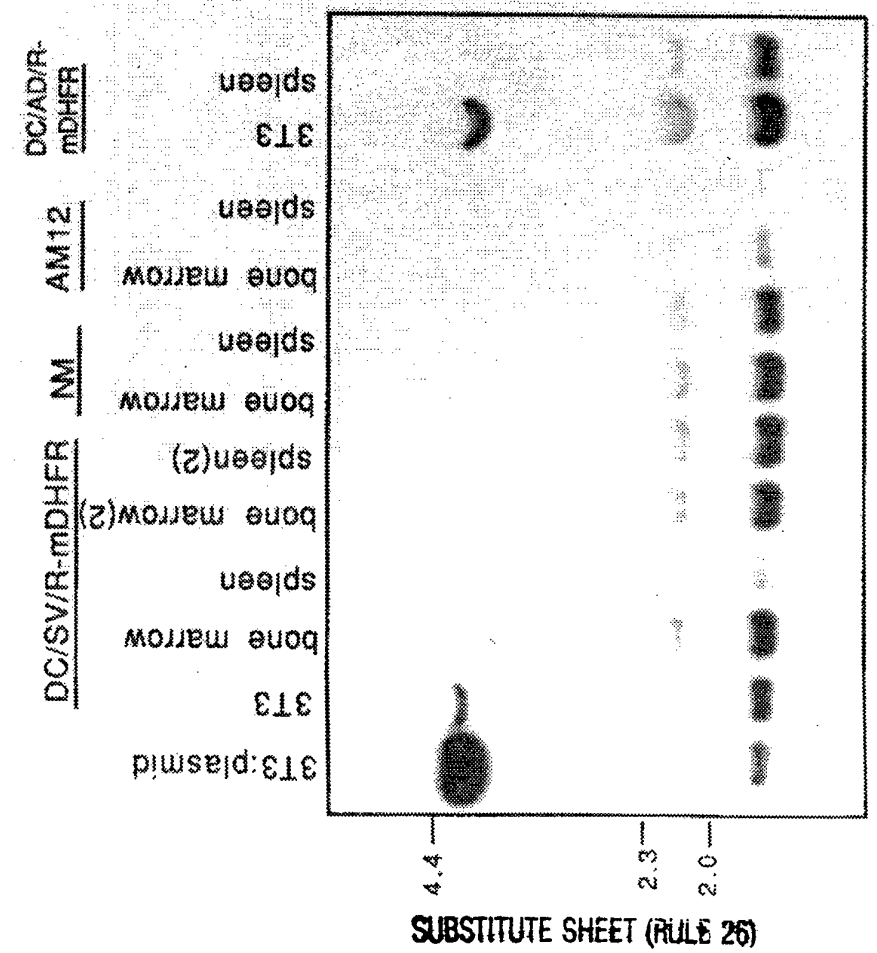


FIGURE 26B

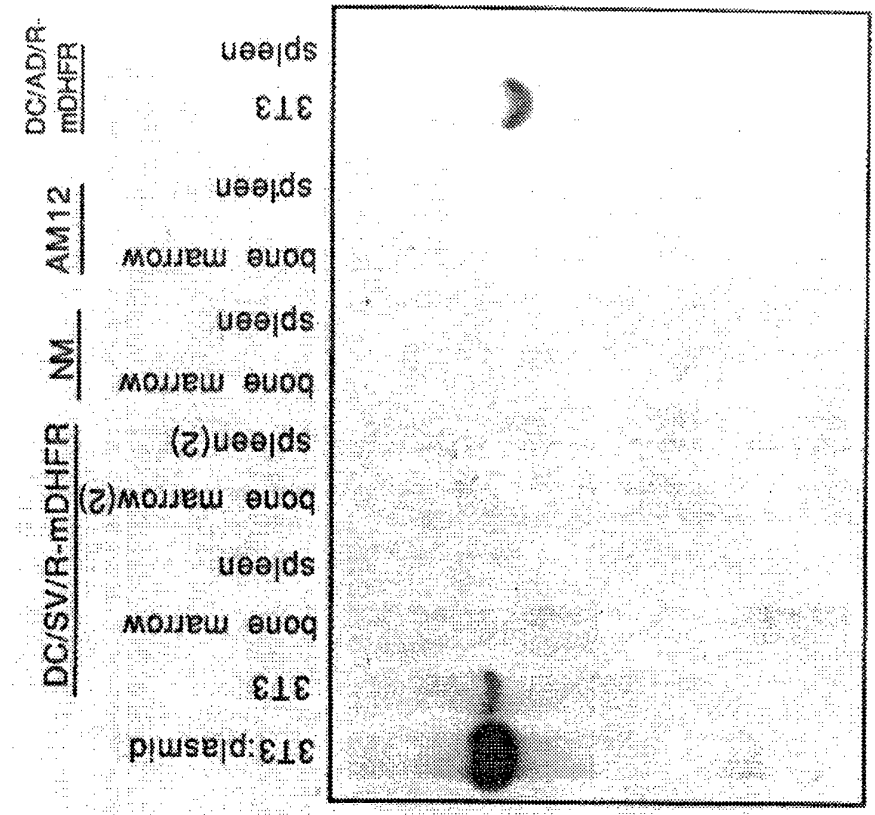


FIGURE 27

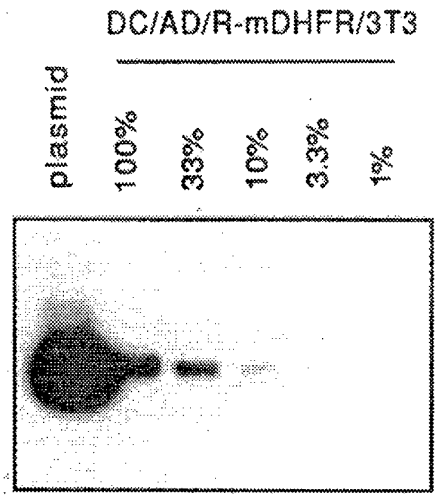


FIGURE 28A

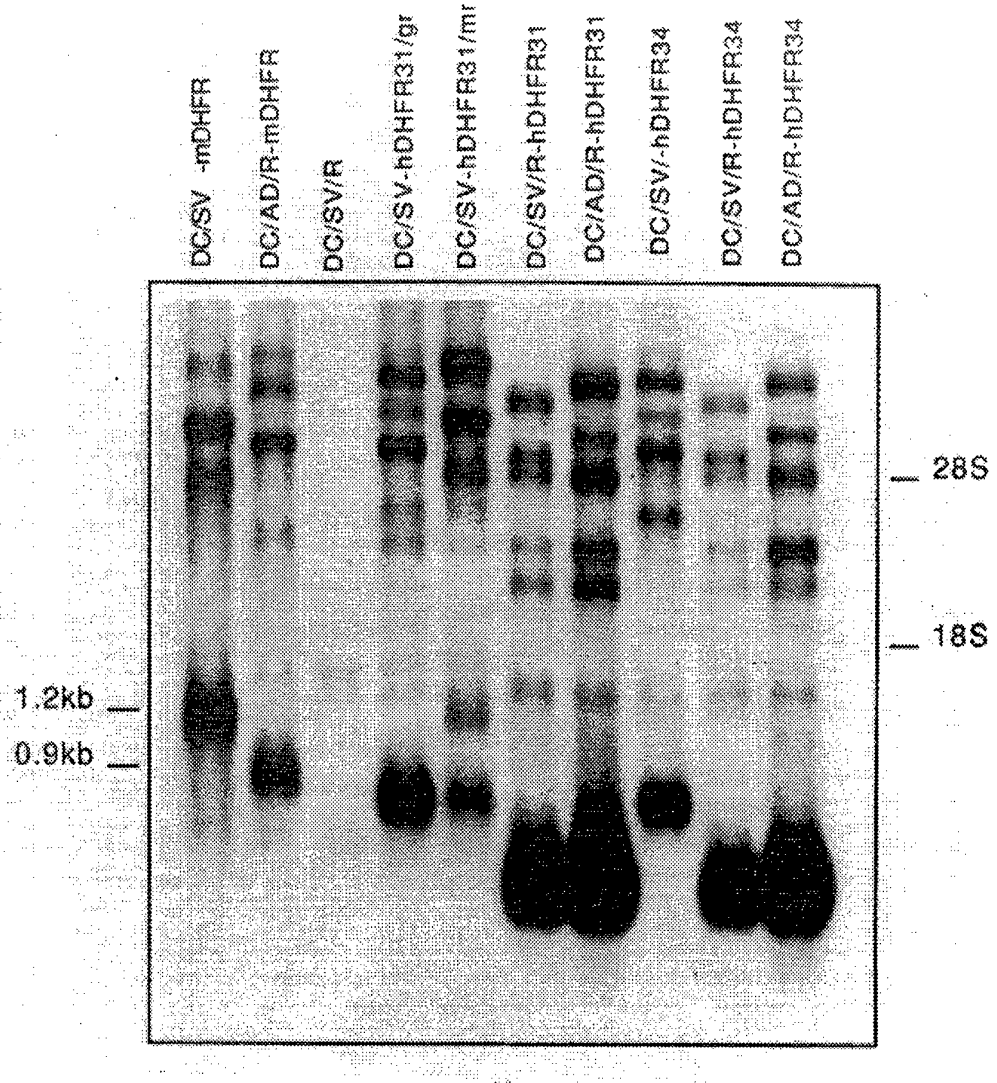

DC/SV -mDHFR
DC/AD/R-mDHFR
DC/SV/R
DC/SV-hDHFR31/gr
DC/SV-hDHFR31/mr
DC/SV/R-hDHFR31
DC/AD/R-hDHFR31
DC/SV/-hDHFR34
DC/SV/R-hDHFR34
DC/AD/R-hDHFR34
28S
18S
1.2kb
0.9kb

FIGURE 28B

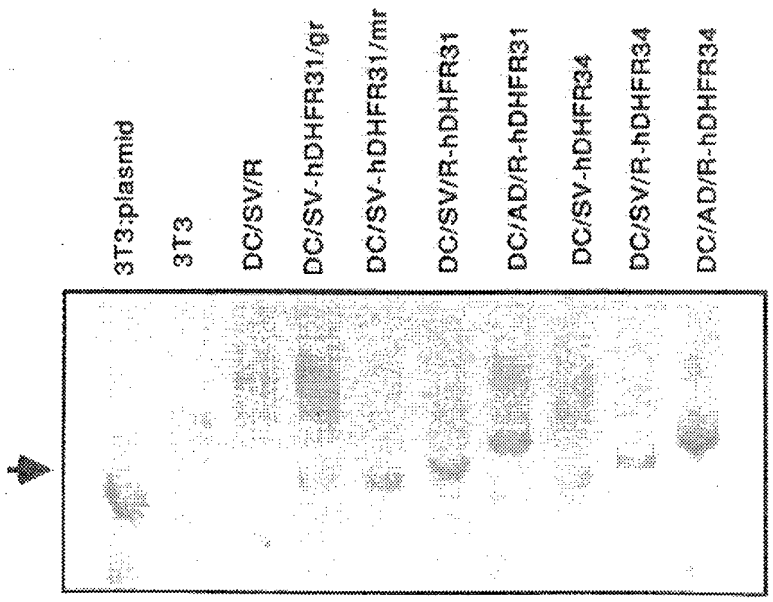

3T3:plasmid
3T3
DC/SV/R
DC/SV-hDHFR31/gr
DC/SV-hDHFR31/mr
DC/SV/R-hDHFR31
DC/AD/R-hDHFR31
DC/SV-hDHFR34
DC/SV/R-hDHFR34
DC/AD/R-hDHFR34

FIGURE 29B

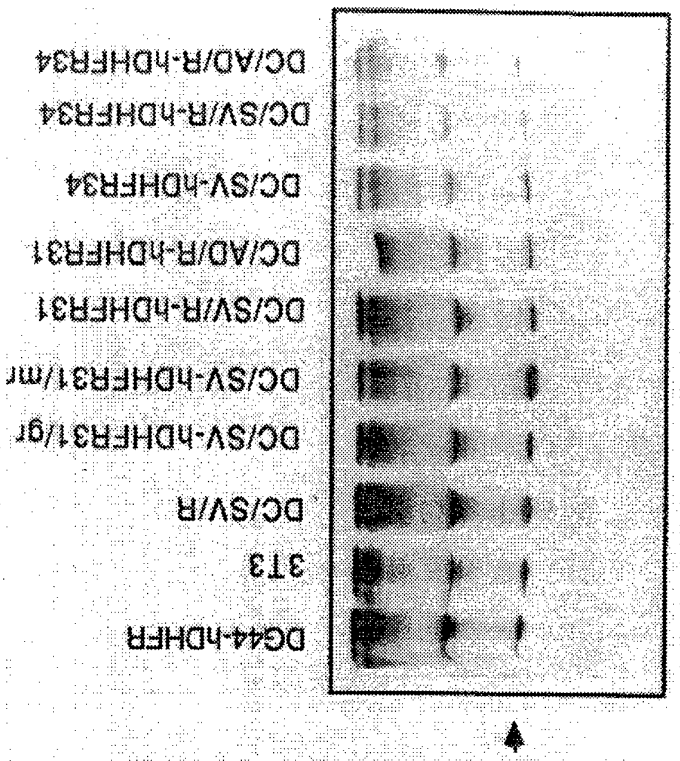


FIGURE 29A

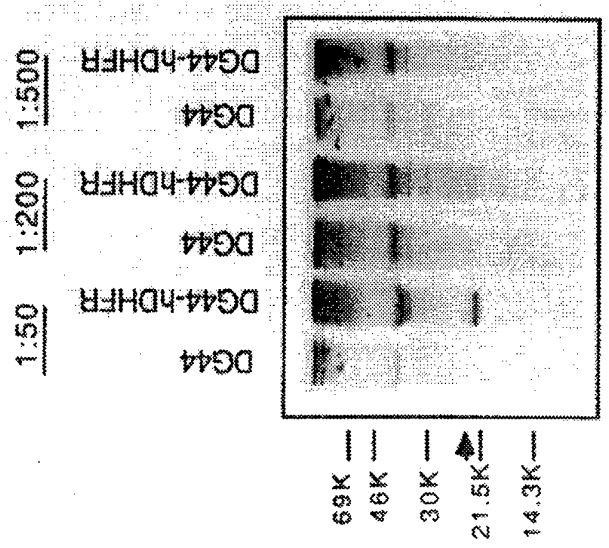


FIGURE 30A

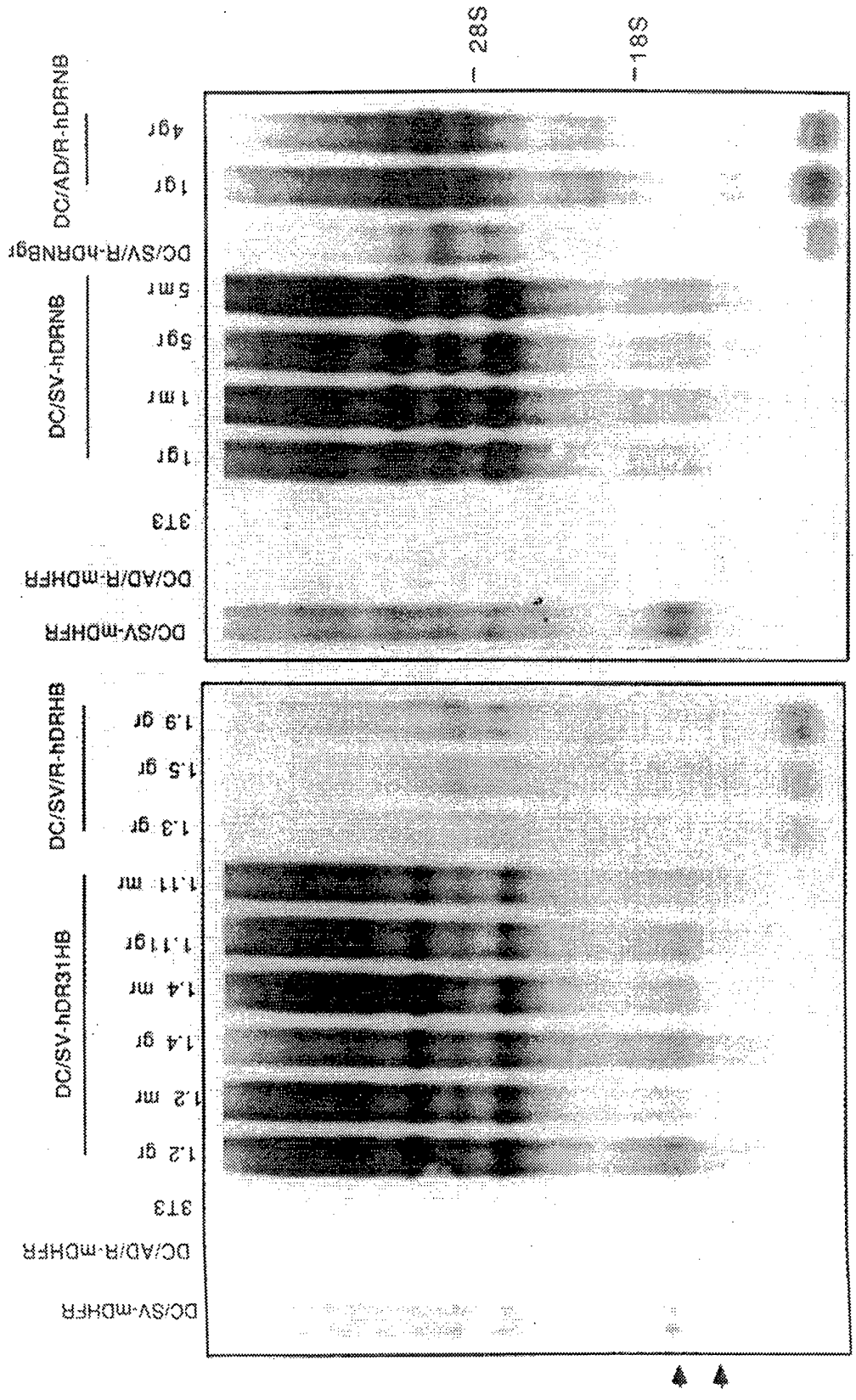

28S
18S
DC/AD/R-hDRNB
4gr
1gr
DC/SV/R-hDRNBgr
DC/SV-hDRNB
5mr
5gr
1mr
1gr
3T3
DC/AD/R-mDHFR
DC/SV-mDHFR
DC/SV/R-hDRHB
1.9 gr
1.5 gr
1.3 gr
DC/SV-hDR31HB
1.11 mr
1.11gr
1.4 mr
1.4 gr
1.2 mr
1.2 gr
3T3
DC/AD/R-mDHFR
DC/SV-mDHFR

FIGURE 30B

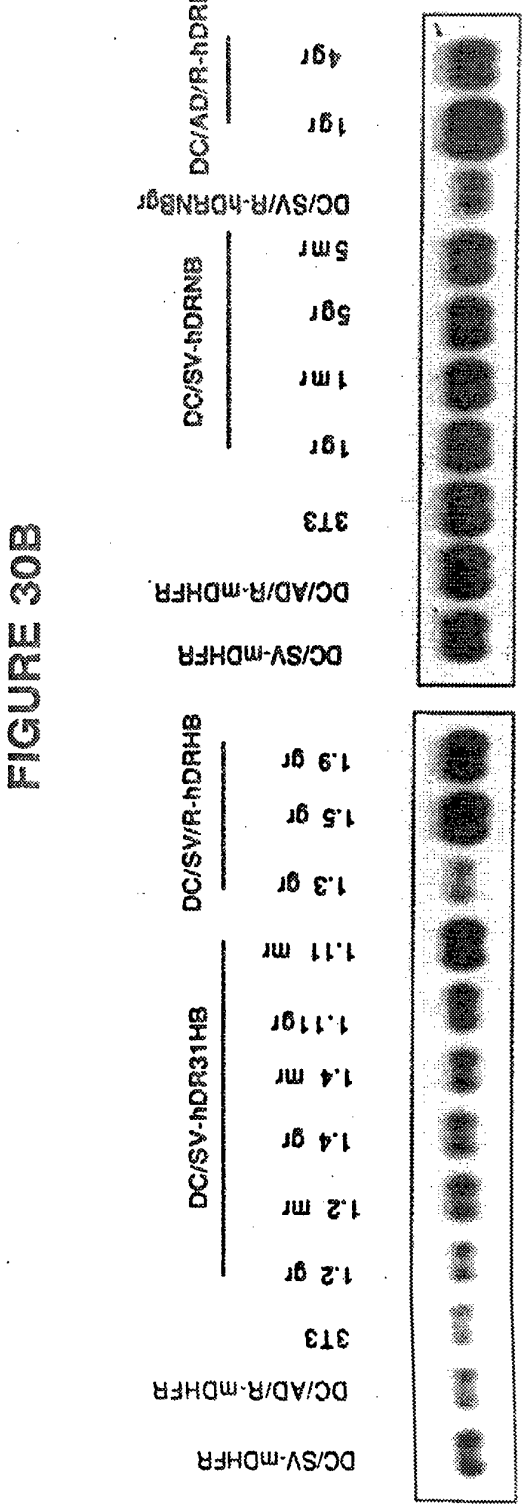

DC/SV-mDHFR
DC/AD/R-mDHFR
3T3
DC/SV-hDR31HB
1.2 gr
1.2 mr
1.4 gr
1.4 mr
1.11gr
1.11 mr
DC/SV/R-hDRHB
1.3 gr
1.5 gr
1.9 gr
DC/SV-mDHFR
DC/AD/R-mDHFR
3T3
DC/SV-hDRNB
1gr
1mr
5gr
5mr
DC/SV/R-hDRNBgr
DC/AD/R-hDRNB
1gr
4gr

FIGURE 31A

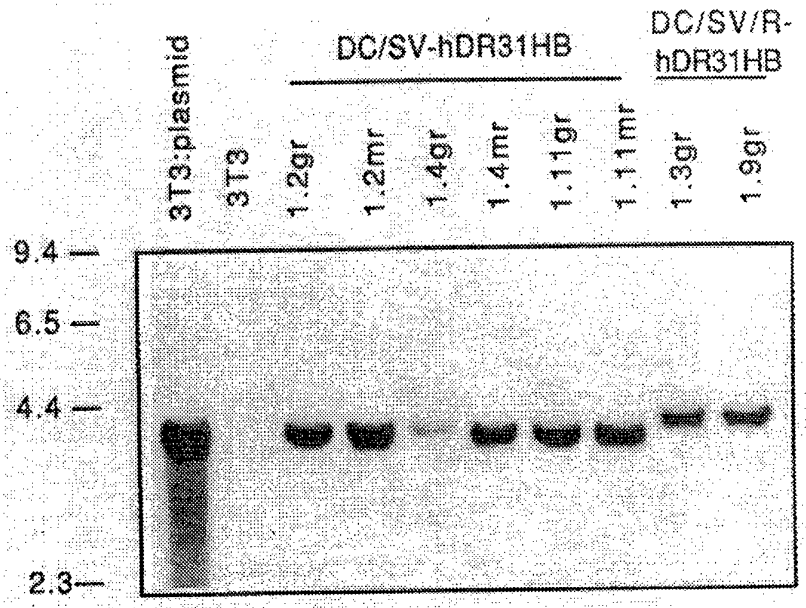


FIGURE 31B

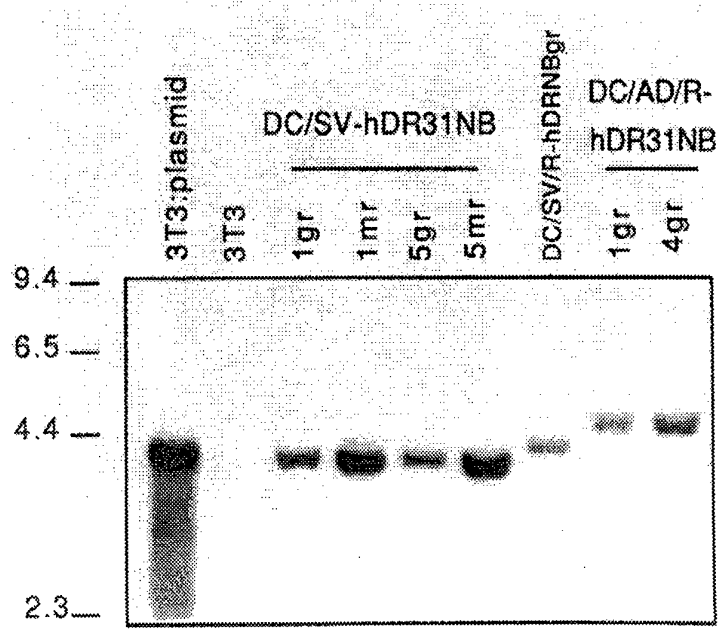


FIGURE 32A

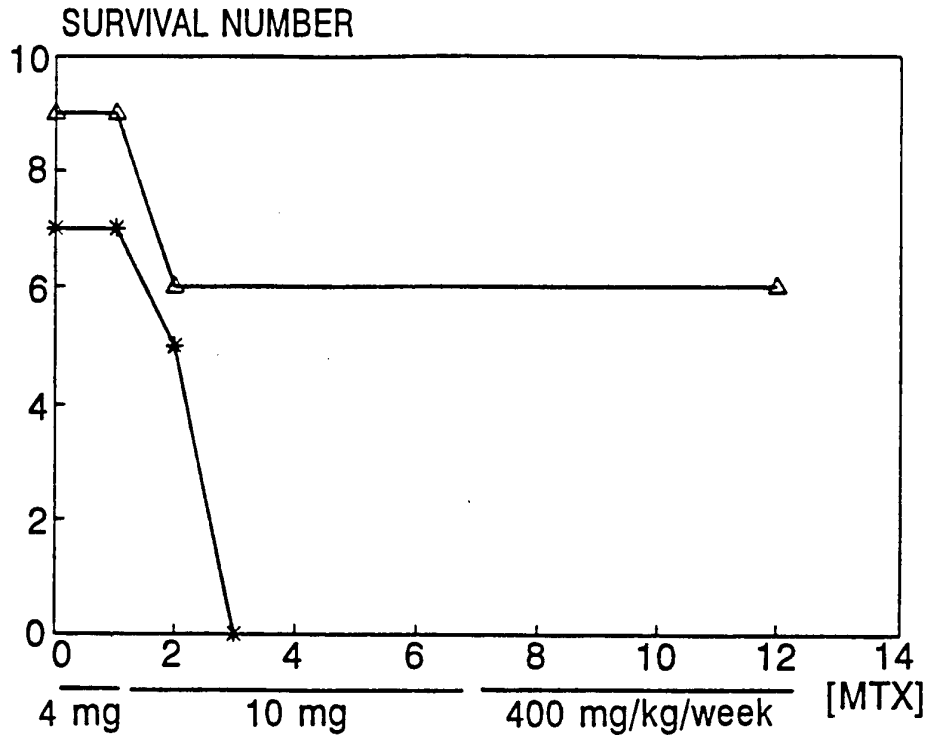


FIGURE 32B

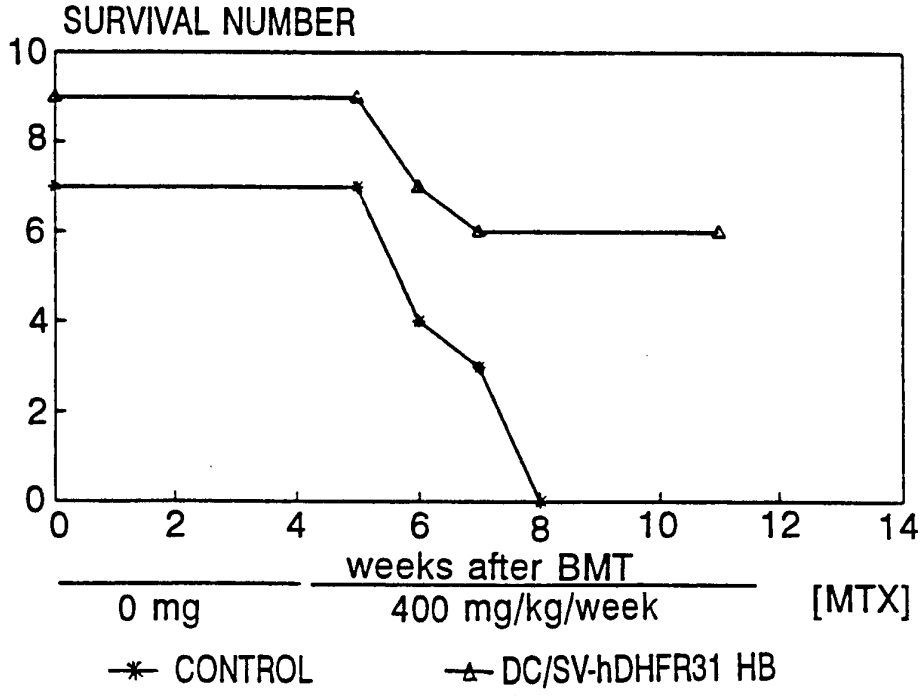
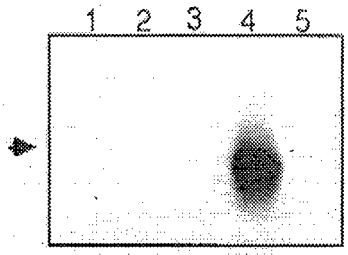


FIGURE 33



1. Marker
2. AM 12
3. Blank
4. DC/SV-hDHFR31
5. No template

FIGURE 34

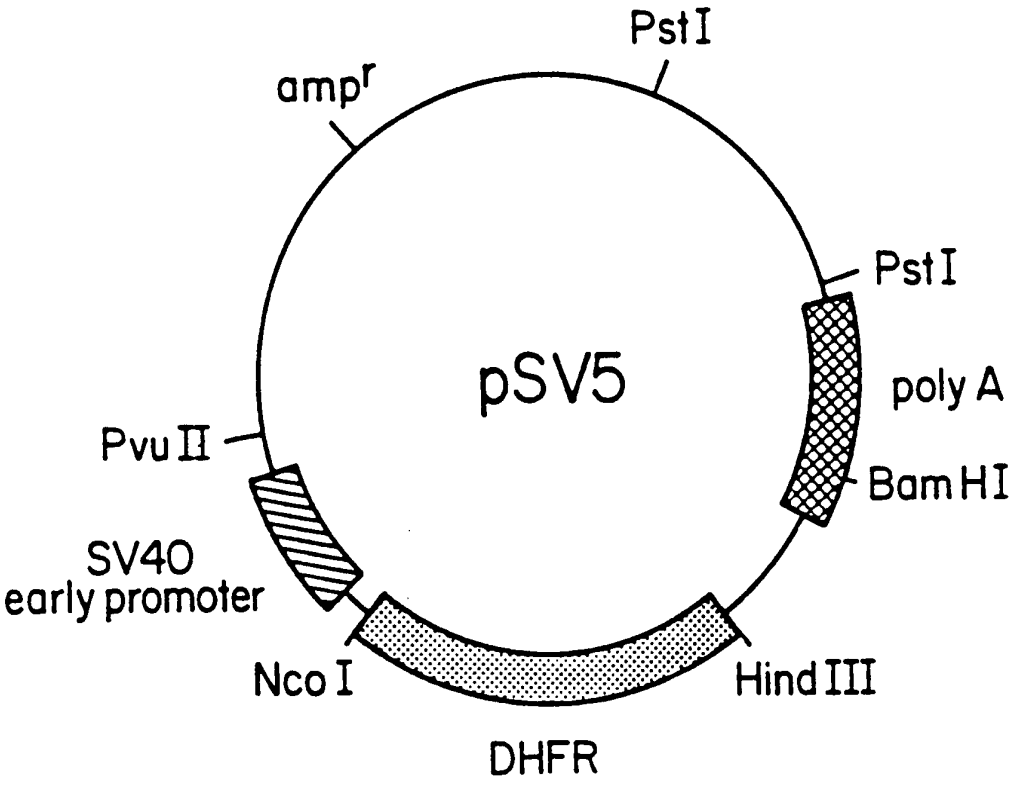

PstI
ampr
PstI
pSV5
poly A
Pvu II
BamHI
SV40
early promoter
Nco I
Hind III
DHFR

FIGURE 35A

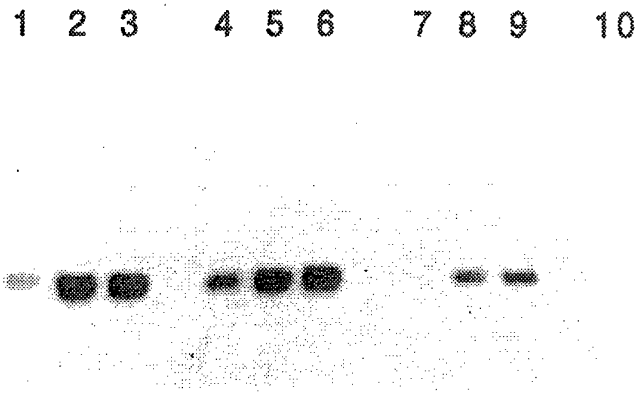


FIGURE 35B

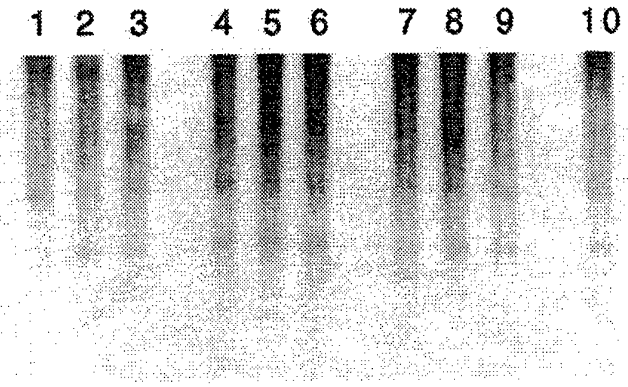


FIGURE 36A

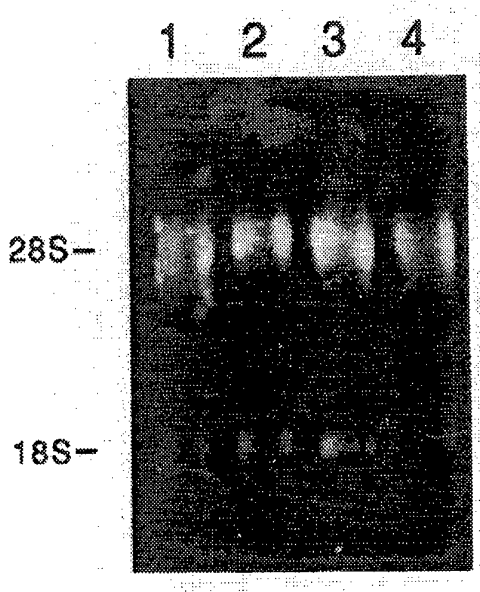


FIGURE 36B

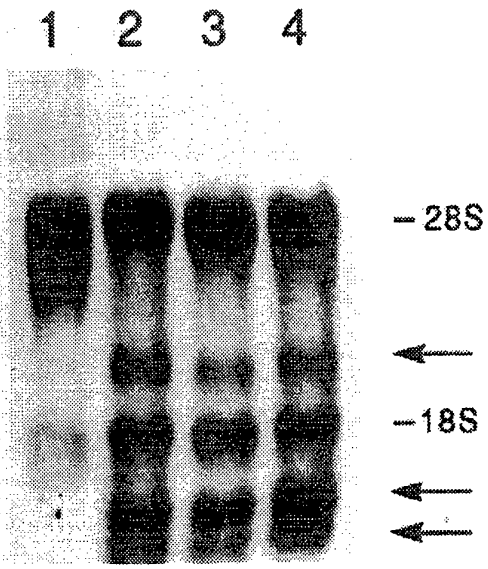
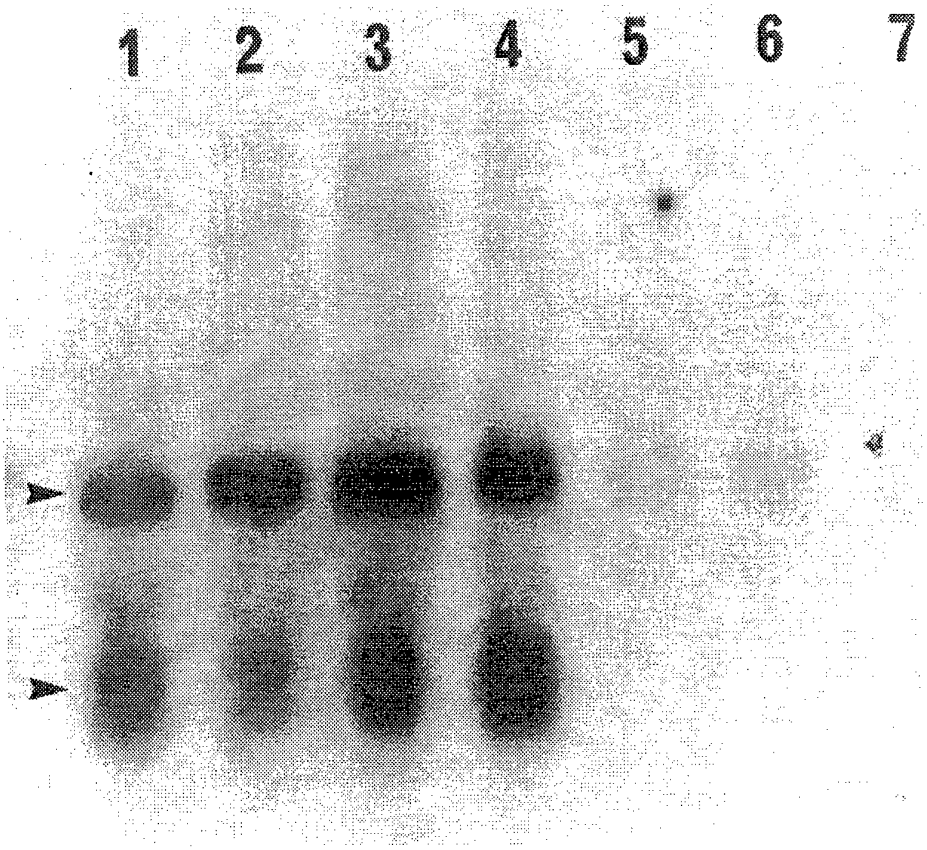


FIGURE 37

Northern Analysis of Trp 15 cDNA Transfected
and Untransfected Chinese Hamster Ovary Cells

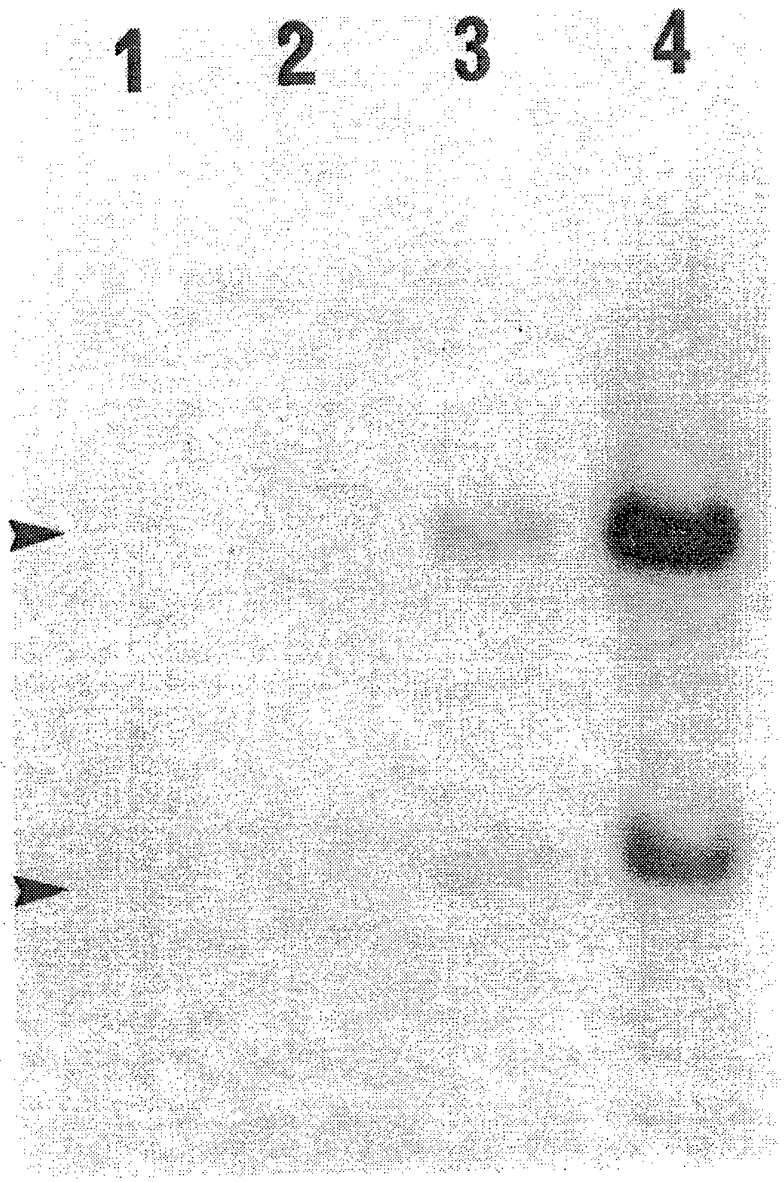


Lanes 1-4 Four individual clones of Trp 15 cDNA transfected CHO cells

Lanes 5-7 Three individual clones of untransfected wild type CHO cells

FIGURE 38

Southern Analysis of NcoI/HindIII Digested Genomic DNA Isolated From Trp 15 Transfected and Untransfected Chinese Hamster Ovary Cells



Lane 1 Untransfected CHO cells
Lane 2 Trp 15 transfected CHO cells selected in G418
Lane 3 Trp 15 transfected CHO cells selected for 4 weeks in 6μM MTX
Lane 4 Trp 15 transfected CHO cells selected for 4 weeks in 6μM and 4 weeks in 15μM MTX

INTERNATIONAL SEARCH REPORT

International application No.: PCT/US94/04129

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00, 15/11, 15/12, 15/85, 15/86; A61K 48/00
US CL :435/320.1, 69.1, 240.2, 172.1; 536/23.1, 23.2, 23.5; 424/93A
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 69.1, 240.2, 172.1; 536/23.1, 23.2, 23.5; 424/93A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Medline, Medicine, Biotech
Serach Terms: retrovirus, vector, gene therapy, dihydrofolate reductase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Human Gene Therapy, Volume 1, issued 1990, David A. Williams, "Expression of Introduced Genetic Sequences in Hematopoietic Cells Following Retroviral-Mediated Gene Transfer" pages 229-239, see pages 235-236.	1-20
Y	The Journal of Biological Chemistry, Volume 264, Number 34, issued 05 December 1989, Schweitzer et al., "Probing the Role of Two Hydrophobic Active Site Residues in the Human Dihydrofolate Reductase by Site-directed Mutagenesis", pages 20786-20795, see the Abstract and page 20789.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search: 26 MAY 1994

Date of mailing of the international search report: JUN 24 1994

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