

Construction and Use of a Dominant, Selectable Marker: a Harvey Sarcoma Virus-Dihydrofolate Reductase Chimera

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The transcriptional promoter of the Harvey sarcoma virus long terminal repeat has been used to construct a biologically active dihydrofolate reductase chimera. The construction placed the long terminal repeat at the 5' end of a dihydrofolate reductase cDNA. This chimera mediated methotrexate resistance when introduced into wild-type NIH3T3 mouse cells by transfection. The chimeric sequences were expressed in the form of polyadenylated RNA and dihydrofolate reductase protein and were amplified when the methotrexate-resistant transfectants were selected to grow in increasing methotrexate concentrations. This chimera was dominant acting and able to confer a methotrexate-resistant phenotype on wild-type NIH3T3 cells. It has been used in cotransfection experiments with DNA from human tumor cells to obtain foci of methotrexate-resistant transformed NIH3T3 cells resulting from uptake of exogenous DNA. The transfected methotrexate-resistant cells carried double minute chromosomes that appeared to contain DNA acquired during transfection.

The development of gene transfer techniques has made possible the introduction of a variety of DNA sequences into appropriate eucaryotic recipients (16, 17, 28, 32, 46, 47). This transfer need not depend upon the expression of the gene of interest in the recipient cells. Rather, one may ensure its presence by introducing such a nonselectable DNA sequence together with a selectable marker gene into suitable recipient cells (49, 50). The few recipient cells that take up transfected DNAs can be shown to take up large amounts of DNA, including both the selectable gene and the cotransfected, nonselectable sequence of interest (31). These concomitantly acquired sequences become fused in the recipient cell into a large array which is preserved for many cell generations. This makes possible the retention of the unselected DNA sequence by virtue of its linkage to the selectable gene whose presence is ensured by the selection protocol (49, 50).

A frequently used selectable marker in such cotransfections is the herpesvirus thymidine kinase (TK) gene (32, 46, 49, 50). The continued presence of this marker in TK⁻ cells can be readily selected by growth in HAT medium (27). For example, cotransfection of the herpesvirus TK gene and cloned copies of a beta-globin gene have generated cell populations which have acquired both the selected TK gene and the unselected globin sequence (49, 50). The association

between these transfected genes persists within the transfectants. This apparent physical linkage between the cotransfected selectable and unselectable genes in the recipient cell ensures that those cells selected to retain the TK gene will, with great likelihood, retain copies of the linked, cotransfected globin gene.

The utility of the TK cotransfection procedure is limited by the range of recipient cells in which the presence of this gene can be scored. Only mutant cell lines that are TK⁻ provide a suitable recipient population in which a TK⁺ transfectant can be scored. This limitation can be overcome by use of a selectable gene which acts dominantly with respect to the wild-type genotype of normal cells. An example of such a dominant selectable marker is the dihydrofolate reductase gene (DHFR), whose presence in high copy number confers resistance to high levels of the folate antagonist methotrexate (MTX) (10, 38, 48). Wild-type cells possess only a diploid complement of DHFR genes and are killed by low concentrations of MTX. Mouse cells may acquire resistance to high levels of MTX by amplifying the copy number of their resident DHFR genes (38). MTX resistance makes possible the selection of cells which have amplified their complement of DHFR genes.

In this communication, we describe the construction and use of a functional DHFR clone which is dominant and amplifiable in wild-type

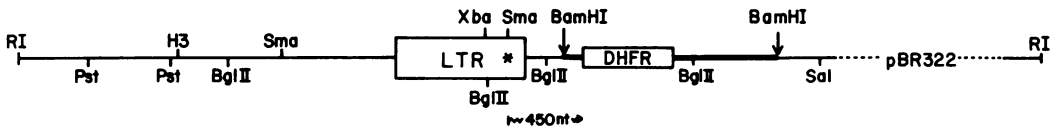


FIG. 1. Restriction map of the pLTRdhfr26 construct. The left *EcoRI*-*BamHI* fragment (3.3 kb) bearing the LTR was derived from a proviral clone of HaSV (clone H-1). The large open box represents the LTR sequences, and the asterisk indicates the approximate mRNA cap site. The central *BamHI* fragment (1.3 kb) is the pDHFR26 cDNA sequence (9) subcloned into this construct after addition of *BamHI* linkers as described in the text. The open box (DHFR) denotes the amino acid coding region, and the thick black lines indicate the 5' and 3' untranslated portions of the DHFR mRNA. The rightmost *BamHI* to *EcoRI* fragment is pBR322 derived. The *EcoRI*-cleaved, linear version of the construct as shown here was used for transfection experiments. The restriction endonuclease sites are abbreviated on the map as follows: RI, *EcoRI*; Pst, *PstI*; H3, *HindIII*; Sma, *SmaI*; Xba, *XbaI*; Sal, *SalI*. The distance from the cap site to the beginning of the DHFR coding sequence is indicated as approximately 450 nucleotides.

NIH3T3 mouse cells. This marker has been used successfully in cotransfection with cellular transforming genes. Recipient cells transfected with this marker carry double minute chromosomes (DMs) which appear to contain the acquired DNA sequences.

MATERIALS AND METHODS

Molecular DNA clones and pLTRdhfr26 construction. DHFR cDNA clones (pDHFR7, pDHFR12, and pDHFR26) were obtained from R. Schimke (9). Harvey murine sarcoma virus (HaSV) proviral clone H-1 (12) was a gift from E. Scolnick and D. Lowy. The pLTRdhfr26 construct was made by juxtaposing a DHFR cDNA sequence next to a long terminal repeat (LTR)-containing fragment of HaSV. The H-1 clone is a permuted proviral clone (in the vector λ gtWES.1B) with a single LTR lying approximately in the middle of the insert sequence. An *EcoRI*-*BamHI* (New England Biolabs) fragment of the H-1 clone, bearing the LTR, was resolved by agarose gel electrophoresis and isolated by electroelution. This fragment was subcloned by ligating it into an *EcoRI*-*BamHI*-digested plasmid, pBR322. The DHFR cDNA fragments were isolated as above after *PstI* digestion. The 3' overhanging ends of these fragments were removed with the Klenow fragment of DNA polymerase I (Boehringer Mannheim), and *BamHI* linkers (Collaborative Research) were ligated to the blunt-end fragments. These fragments were then subcloned into the *BamHI* site of the LTR-bearing fragment. The resulting construct was propagated in *Escherichia coli* strain C600. The orientation of the DHFR cDNA sequence was established by digesting the *BglII* sites (Fig. 1).

DNA transfection and MTX selection. The cloned pLTRdhfr26 construct was transfected into NIH3T3 cells by the calcium phosphate precipitation technique (18). NIH3T3 cell carrier DNA was coprecipitated and transfected with 0.5 to 1 μ g of the construct DNA. The details of this procedure have been described previously (2; C. Shih and R. A. Weinberg, Cell, in press). Approximately 20 h after transfection the cells were trypsinized and split 1:6. Twenty-four hours after the transfected cells were split, they were refed with medium containing 0.3 μ M MTX (Sigma Chemical Co.) and 10% dialyzed calf serum. The medium was replaced once a week, and 10 to 14 days later the surviving colonies were scored by crystal violet staining. After the plates were washed twice with phos-

phate-buffered saline, the colonies were stained for 2 min by applying 1 ml of 1% crystal violet in 20% EtOH and then removing the stain and washing with water. Some of the colonies were picked from unstained plates with the aid of cloning cylinders and grown into cell cultures.

Amplification of the input pLTRdhfr26 sequences was achieved by growing cells derived from MTX-resistant colonies in increasing concentrations of the drug. Typically, the MTX concentration was increased by twofold jumps (e.g., 0.3 to 0.6 to 1.2 μ M, etc.). The cells were maintained at each new MTX level for about 2 weeks and were split at least twice at a given MTX concentration before proceeding to the next level. Three such cell lines (called 26-1, 26-8, and 26-19) were selected to grow in 80 μ M MTX after about 3 months of this protocol.

Southern transfer analysis of MTX-resistant cell DNA. DNA of the transfected MTX-resistant (MTX^r) cell lines was prepared as previously described (Shih and Weinberg, in press). After restriction endonuclease digestion, 10 μ g of the DNA of each cell line was fractionated by electrophoresis through a 1% agarose gel in 40 mM Tris (pH 7.9)-50 mM sodium acetate-1 mM EDTA. After electrophoresis, the DNA was transferred to nitrocellulose by the method of Southern (40), and the resulting blots were hybridized with a DHFR cDNA probe (9). The DHFR cDNA probe was ³²P-labeled by nick translation (34), using 100 μ Ci each of ³²P-labeled dATP and dCTP (New England Nuclear). The labeled probe was hybridized with the nitrocellulose blots for 18 to 24 h in the presence of 10% dextran sulfate (45) at 40°C. After hybridization, the blots were washed in 2 \times SSC (= 0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.4)-0.1% sodium dodecyl sulfate (SDS) at 68°C for several hours.

Preparation and analysis of polyadenylated RNA. Polyadenylic acid-containing RNA was prepared by the procedure of Varmus et al. (44). Briefly, MTX-resistant cells were lysed in 0.5% SDS-0.1 M NaCl-0.01 M Tris-hydrochloride (pH 7.5)-0.002 M EDTA-200 μ g of proteinase K (Boehringer Mannheim) per ml. The lysate was incubated at 37°C for 30 min and then sonicated. After sonication, the lysate was adjusted to 0.5 M NaCl, oligodeoxythymidylic acid-cellulose (Collaborative Research) was added (1 mg per 10-15-cm plates of cells), and the mixture was stirred for several hours at room temperature. The mixture was then poured into a column and washed, and the RNA was eluted in the same buffer without NaCl.

A 5- μ g portion of polyadenylated RNA was electrophoresed in 1.4% agarose gels containing methyl mercuric hydroxide and transferred to diazobenzoyloxymethyl paper by the procedure of Alwine et al. (1). The immobilized RNA sequences were hybridized with 32 P-labeled DHFR cDNA (9) or pBR322 probes, using the same protocol used for Southern hybridizations except that the presoak solution was supplemented with 0.2% sodium azide and 1% glycine. To reuse the diazobenzoyloxymethyl blot, the probe was removed by two 15-min washes in formamide with 0.1% SDS at 68°C. The washed blot was exposed to X-ray film to ensure complete removal of the first probe; then it was presoaked again and hybridized with a second probe.

Preparation and analysis of labeled cellular protein extracts. Cells in the logarithmic phase of growth were rinsed with phosphate-buffered saline and incubated in Dulbecco minimal essential medium with 25 μ Ci of [35 S]methionine (New England Nuclear) per ml and 5% dialyzed fetal calf serum for 1 h at 37°C. Cells were then rinsed with cold phosphate-buffered saline, lysed in RIPA buffer (150 mM NaCl, 20 mM Tris [pH 7.4], 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 5 mM EDTA, and 0.05% sodium azide) with 1 mM phenylmethylsulfonyl fluoride, and stored at -20°C. 35 S incorporation was monitored by trichloroacetic acid precipitation. A total of 10^5 cpm from each sample were applied to Laemmli SDS gels (24) containing 15% acrylamide-0.86% *N*'-methylene bisacrylamide. The gels were fixed in 10% methanol-10% acetic acid, treated with Enhance (New England Nuclear), and dried. Dried gels were exposed to preflashed Kodak XR-5 film (6).

The above extracts were analyzed by immunoprecipitation, utilizing *Staphylococcus aureus* as the immunoabsorbant (23), precisely as described previously (R. J. Kaufman and P. A. Sharp, *J. Mol. Biol.*, in press). Rabbit anti-mouse DHFR was provided by B. Dolnick (University of Wisconsin, Madison) and J. Bertino (Yale University, New Haven, Conn.).

DM preparation and visualization. DMs were fractionated from preparations of metaphase chromosomes by filtration (19) as described by Schimke et al. (37). Metaphase cells were collected after 12-h treatment with colcemid (0.06 μ g/ml) by mitotic shake-off (approximately 10^8 cells having >50% mitotic index). Total metaphase chromosomes were prepared by neutral pH extraction as described by Blumenthal et al. (5) with slight modifications (37). Cells were washed twice at 37°C with buffer A [150 mM Tris-hydrochloride (pH 7.4), 60 mM NaCl, 15 mM KCl, 2 mM EDTA, 0.5 mM ethylene glycol-bis (β -aminoethyl ether)-*N,N*-tetracetic acid, 14 mM β -mercaptoethanol, 2 mM spermine, 0.5 mM spermidine] containing 0.06 μ g of colcemid per ml. Cells were then suspended at 5×10^6 /ml in buffer A with 0.5 M hexylene glycol and 0.1% digitonin (37°C) and passed through a 23-gauge syringe needle three times. Cell lysis and release of metaphase chromosomes were monitored after each cycle (5). The preparation was layered over 20% (wt/vol) sucrose in buffer A and centrifuged at 1,000 rpm for 5 min. The supernatant above the sucrose was saved. The pellet was washed by resuspension in 20 ml of buffer A and centrifuged again over 20% sucrose in buffer A. The two supernatants were pooled and centrifuged at 1,000 rpm for 10 min. This final supernatant was passed through a Nuclepore hydrophobic 2- μ m-pore size fil-

ter (142-mm diameter) with gentle vacuum and extensive washing with buffer A containing hexylene glycol. To monitor the fractionation by fluorescence microscopy, samples of the starting supernatant and 1 μ m of filtrate were layered over 20% sucrose in buffer A, and the chromosomes were centrifuged onto 12-mm glass cover slips for 20 min at 7,000 rpm in a Sorvall HB4 rotor (41). The cover slips were fixed in methanol-acetic acid (3:1) and stained with ethidium bromide (1 μ g/ml in 10 mM Tris [pH 7.4], 1 mM EDTA). These preparations were photographed with a fluorescence microscope. The remaining filtrate was centrifuged at 24,000 rpm at 4°C for 1 h in a Beckman SW27 rotor, and DNA was extracted from the resulting pellet.

For examination of the karyotype of the transfectants, metaphase spreads were prepared from cells exposed to 0.34 μ g of colcemid per ml. Two hours later, the cells were suspended by trypsinization, swollen with 0.075 M KCl, and fixed twice in 3:1 methanol-acetic acid. Slides of fixed cells were made by air-drying, and the slides were stained with 33258 Hoechst (0.5 μ g/ml). Randomly selected metaphases were photographed on Tri-X film, using a Leitz Orthoplan microscope and incident illumination (25). DMs were scored from overexposed photographic prints of metaphase cells.

RESULTS

An LTR-DHFR cDNA construct mediates MTX resistance. The DHFR markers used in previous cotransfection procedures were uncloned cellular genes (10, 48). Because of the large size of the cellular DHFR gene, ~42 kilobases (kb) (30), biologically active molecular clones of the genomic sequence have been difficult to obtain. The absence of such molecular clones reduces the utility of this cotransfection since one may need to vary the molar ratio of the DHFR gene to a second unselected gene. Such flexibility is not possible when the DHFR gene exists as an uncloned sequence amid a 10^5 -fold excess of other cellular DNA sequences. We therefore constructed a biologically active molecular clone of DHFR by fusion of a cDNA copy of the DHFR mRNA with an appropriate transcriptional promoter.

We chose the transcriptional promoter of the murine retrovirus HaSV. At least two advantages accrued from use of a retrovirus promoter. First, the promoter appears to be highly active since an infected cell carrying a small number of proviruses will display many thousands of copies of the viral RNA transcript (13). Second, the viral genomic RNA of HSV would appear to be transported to the cytoplasm without being previously spliced (43). The viral sequences responsible for this transport are likely close to the 5' end (15).

A group of three different cDNA clones of mouse DHFR mRNA was obtained from R. Schimke. These three clones (pDHFR7, -12, and -26), whose detailed structure is described elsewhere (9), differed primarily in their extent of 5'

untranslated sequence. Clone pDHFR26 also lacks a significant portion of the 3' untranslated sequence. These three clones were fused individually to a fragment of HaSV comprising the left 3,330 bases of a circulary permuted proviral clone obtained from D. Lowy and E. Scolnick (12). Such constructions (Fig. 1) placed the 5' end of the coding sequence about 450 base pairs downstream from the transcriptional promoter site of the HaSV LTR (43). The three resulting chimeras were tested for biological activity by transfection onto monolayers of NIH3T3 mouse fibroblasts after being linearized by treatment with endonuclease *Eco*RI (Fig. 1). After transfection, MTX was applied to the recipient monolayers at a concentration of 0.3 μ M. This drug concentration will kill virtually all of the untransfected NIH3T3 cells (data not shown). Clone pLTRdhfr26, which derived from DHFR cDNA clone pDHFR26 (9), was found to induce approximately 1,000 MTX-resistant colonies per μ g of transfected DNA. The other two chimeric genes derived from pDHFR7 and pDHFR12 had minimal biological activity and their characterization was not further pursued.

Several MTX^r colonies were picked, and their DNAs were analyzed for the presence of the transfected construct by the Southern transfer hybridization method (40). These cells (termed the 26 series) all acquired multiple copies of the transfected gene (Fig. 2). The common band seen at about 1.3 kb is an internal fragment of DHFR cDNA origin and was released from the construct by *Bam*HI digestion (Fig. 1). The higher-molecular-weight bands of different sizes are detected because of homology to pBR322 sequences in the probe and indicate that each construct is present in a different context of surrounding DNA sequences. The intensity of these bands indicated that each of these acquired genes became amplified severalfold after transfection. The resident DHFR gene of the recipient NIH3T3 cells does not appear to be amplified. These resident genes exhibit a banding pattern (30) distinct from that of the transfected pLTRdhfr26 clone, and their single-copy level is barely detected in these transfectants. The pattern of the resident genes is provided by analysis of the DNA of the 3T3-R500 line, which carries ca. 100 to 200 copies of the mouse DHFR gene. This apparent lack of alteration of the resident DHFR genes suggests strongly that the MTX^r phenotype of these cell lines derives from the newly acquired, exogenous DHFR sequences.

Two of these MTX^r clones were subsequently grown for about 2 months in MTX-free medium. The previously acquired pLTRdhfr26 sequences were lost (Fig. 3, lanes d and h). When placed back into medium containing 0.3 μ M MTX,

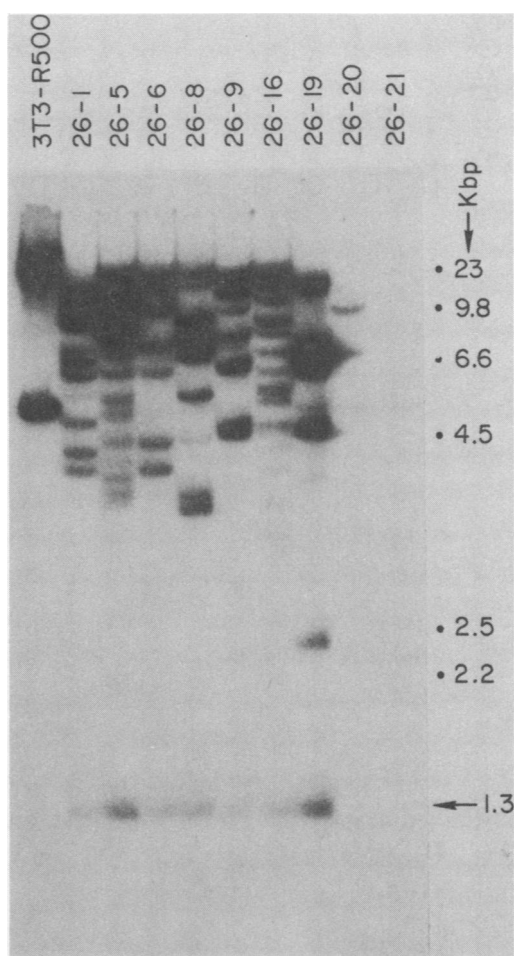


FIG. 2. Detection of pLTRdhfr26 sequences in MTX-resistant transfectants. The DNA of each transfectant was digested with endonuclease *Bam*HI, followed by agarose gel electrophoresis and Southern transfer as described in the text. The resulting blot was hybridized with a ³²P-labeled DHFR cDNA (pDHFR26) probe (9). The 3T3R500 lane contains 5 μ g of DNA from 3T3R500 cells, a line derived by Schimke and co-workers containing many amplified copies of the endogenous mouse DHFR gene. The other lanes contain 10 μ g of DNA from a series (termed the 26 series) of cell colonies surviving transfection with pLTRdhfr26 and MTX selection. The positions of migration of molecular weight markers are indicated on the right in thousands of base pairs. The arrow indicates the DHFR cDNA sequences released from the construct by *Bam*HI digestion (Fig. 1). Reprobing this blot with ³²P-labeled pBR322 results in hybridization to all bands in the transfectants except the 1.3-kb cDNA band.

these cells were unable to grow. This parallels the findings of others that unstably amplified DHFR genes are usually retained in multiple copies only in the continued presence of selective pressure and are eliminated once such pres-

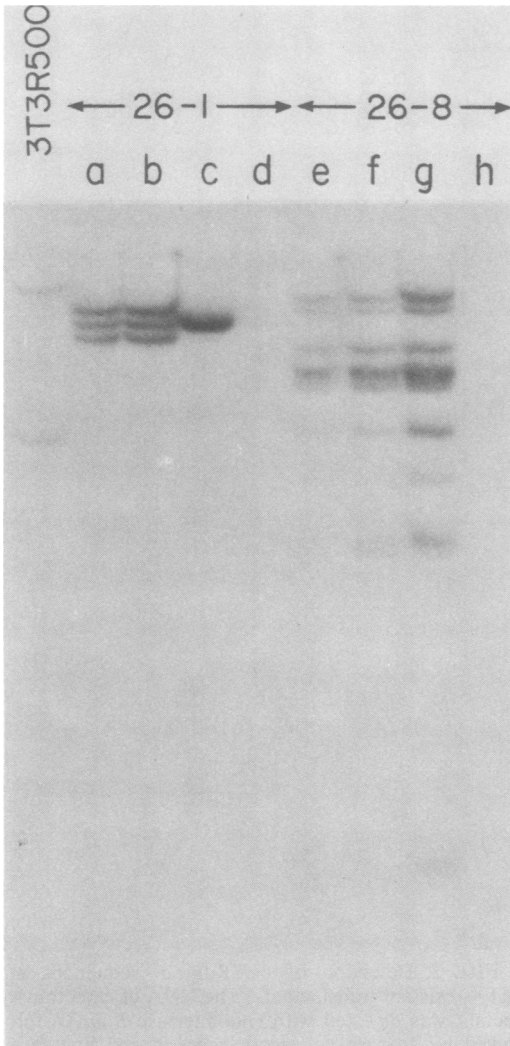


FIG. 3. Amplification and loss of transfected pLTRdhfr26 sequences in response to MTX selection pressure. Some of the MTX-resistant transfectants analyzed in Fig. 2 were selected to grow in increasing concentrations of MTX as described in the test or were grown without MTX. At various stages during this selection, DNA was extracted from the cells surviving at that MTX level and analyzed as in the legend to Fig. 2 by hybridization to a DHFR cDNA probe. The 3T3R500 lane contains DNA from a cell line with many amplified copies of the endogenous DHFR gene. Lanes a to d contain DNA from 26-1 cells grown in 0.3, 2.4, 20 μ M MTX, and no MTX, respectively. Lanes e to h contain DNA from 26-8 cells grown in 0.3, 2.4, 20 μ M MTX, and no MTX, respectively.

sure is removed (21, 22). The retention of these cloned genes in the presence of MTX and their loss upon drug removal further support the contention that their presence mediates the MTX^r phenotype.

Southern blot analysis revealed that the

DNAs of 1 of 20 of the MTX^r colonies that arose during these transfections and which we examined gave no evidence of acquired pLTRdhfr26 sequences (Fig. 2, lane 26-21). Instead, DNA of this clone only gave indication of the resident cellular DHFR gene in the form of a faintly detected DNA fragment of 5 kb. The development of the MTX^r phenotype in this cell line appears to depend on an alteration other than acquisition of the chimeric gene. For example, an altered drug transport step would confer this resistance (14).

Control experiments have confirmed that the pLTRdhfr26 fragments observed in Fig. 2 have an internal structure identical to that of the transfected clone. For example, the acquired gene retains the *Xba*I and *Sal*I endonuclease sites (Fig. 1) present in the cloned chimera, indicating that the linkage between the DHFR cDNA sequences and the LTR remains intact (Fig. 4, 2.5-kb band).

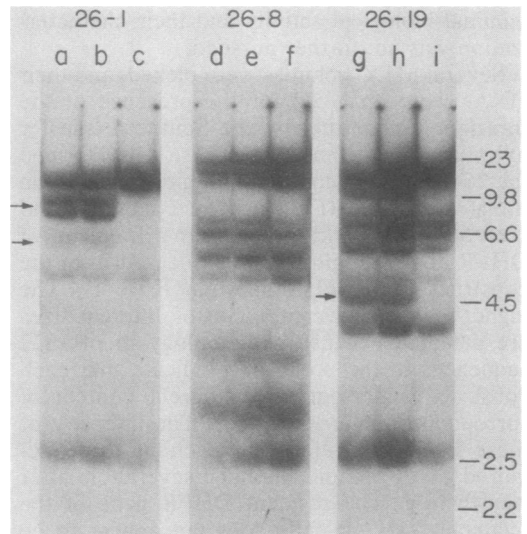


FIG. 4. Rearrangement of pLTRdhfr26 sequences during growth in increasing MTX concentrations. MTX-resistant transfectants were selected to grow in increasing concentrations of MTX. At various stages during this selection, DNA was extracted from the cells, digested with *Xba*I and *Sal*I, and analyzed as in the legend to Fig. 2 by hybridization to the ³²P-labeled pDHFR26 cDNA probe. The analysis was performed on cell lines 26-1, 26-8, and 26-19. Lanes a, d, and g contain DNA from these cells grown in 0.3 μ M MTX; lanes b, e, and h contain DNA from these cells grown in 2.4 μ M MTX; and lanes c, f, and i contain DNA from cells grown in 20 μ M MTX. Arrows indicate bands which were lost during the selection to grow in higher MTX concentrations. The band seen at 2.5 kb in all lanes is an internal DHFR cDNA-bearing fragment released after digestion with *Xba*I and *Sal*I (Fig. 1). The molecular weight markers, in kilobase pairs, are indicated on the right.

Expression of pLTRdhfr26 sequences in transfected cells. The conclusion that the MTX-resistant phenotype of these transfected cell lines is being mediated by the acquired pLTRdhfr26 construct is further supported by experiments that examine the expression of the cloned sequences in these cells. Transfected cells selected to grow in 80 μ M MTX (see below) produce a protein which comigrates with the DHFR protein found in cells containing many amplified copies of the resident mouse DHFR gene (Fig. 5A, lane 1). This protein is so abundant in the transfectants that it is readily detectable upon electrophoresis of radiolabeled whole-cell lysate (Fig. 5A, lanes 3, 4, and 5). Furthermore, immunoprecipitation of the lysates with anti-DHFR serum yields a protein whose electrophoretic mobility is indistinguishable from that of the normal mouse enzyme (Fig. 5B). Since the

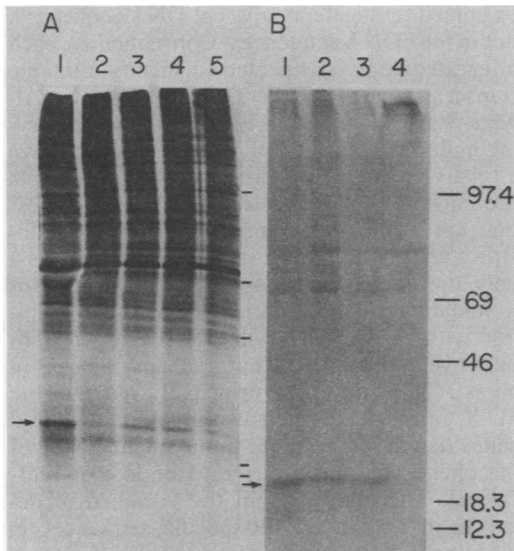


FIG. 5. SDS-polyacrylamide gel electrophoresis of labeled proteins in MTX-resistant transfectants. Growing cells were pulse-labeled for 1 h with [35 S]methionine, whole-cell extracts were prepared, and immunoprecipitations were performed as described in the text. (A) Results of SDS-polyacrylamide gel electrophoresis of total protein from MTX-resistant 3T3R500 cells (lane 1), NIH3T3 cells (lane 2), and three transfectants selected for resistance to 80 μ M MTX: 26-1 (lane 3), 26-8 (lane 4), and 26-19 (lane 5). (B) Results of immunoprecipitation of the same extracts, using monospecific rabbit anti-mouse DHFR serum. Lanes 1 through 4 are immunoprecipitations from 26-1, 26-8, 26-19, and NIH3T3 cells, respectively. (A) and (B) are from different gels. The distance of migration of molecular weight standards for each gel is shown by horizontal lines on the right of each panel and the sizes, in thousands of daltons, are indicated on the right of (B). The arrows indicate the DHFR protein.

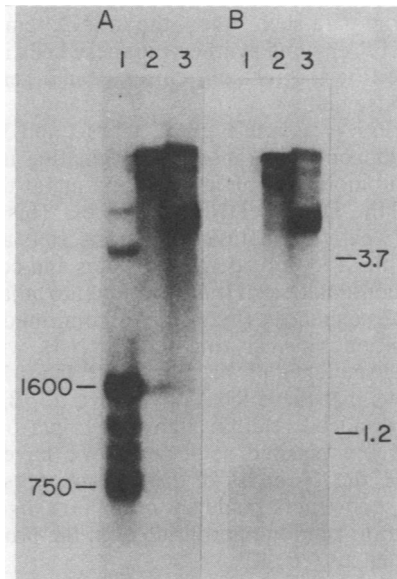


FIG. 6. Analysis of DHFR-specific RNAs. Polyadenylated RNA was prepared from MTX-resistant cells, fractionated by agarose gel electrophoresis in the presence of methyl mercuric hydroxide, and transferred to diazobenzoyloxymethyl (DBM) paper as described in the text. The transfectants analyzed here had been selected to grow in 80 μ M MTX. (A) RNAs from 3T3R500 cells (lane 1) and two pLTRdhfr26 transfectants, 26-1 (lane 2) and 26-19 (lane 3), hybridized with a DHFR cDNA probe (pDHFR26). (B) Same DBM blot after removing the cDNA probe and rehybridizing with a pBR322 probe; lane 1 is 3T3R500 RNA, lane 2 is 26-1 RNA, and lane 3 is 26-19 RNA. The sizes designated in bases on the left of (A) are published values for those RNAs (39). The size markers on the right of (B), in thousands of bases, are from DNA markers run on the gel.

resident DHFR gene does not appear to be amplified in these cells, we presume that the great bulk of this enzyme is encoded by the transfected pLTRdhfr26 construct.

Further support of these conclusions comes from analyses shown in Fig. 6 in which the polyadenylated RNAs of two transfected cell lines are analyzed by the Alwine-Stark hybridization procedure (1) for sequences homologous to the cloned DHFR gene. In Fig. 6A (lanes 1, 2, and 3), the RNAs are probed for the presence of sequences homologous to a DHFR cDNA. The four transcripts produced in a cell line with amplified copies of the resident DHFR gene are seen in lane 1 (39). They range from 750 to 1,600 nucleotides in length. Two higher-molecular-weight precursors are also seen in this preparation. The RNAs from pLTRdhfr26 transfected cell lines (lanes 2 and 3) contain a different pattern of transcripts. In both cases, the predominant species are >4,000 nucleotides long and only trace amounts of the major endogenous

transcripts are seen. Thus, the mRNA template for DHFR enzyme synthesis in these cells is not specified by the resident chromosomal gene of the mouse cell.

The RNAs seen in Fig. 6A, lanes 2 and 3, are much longer than transcripts originating in the HSV promoter region and terminating at the 3' end of the DHFR cDNA sequences. This suggested weak termination of the transcripts at the cDNA-derived polyadenylation sites and consequent continuation of transcription into adjacent pBR322 sequences (Fig. 1). We confirmed this supposition by analyzing these RNAs with a pBR322 sequence probe. Figure 6B (lanes 2 and 3) shows that these RNAs hybridize to pBR322 and are indeed read-through transcripts of the DHFR and plasmid sequences. We have not mapped the 5' ends of these transcripts but similar constructs made by other investigators begin transcription appropriately in the promoter sequences (26, 42).

These results rule out the possibility that the MTX resistance of these transfected cells stems from an unamplified, but transcriptionally hyperactive resident DHFR gene. Rather, it is clear that the transcripts responsible for the increased DHFR enzyme level derive from the exogenous pLTRdhfr26 sequences introduced via transfection.

Amplification of the pLTRdhfr26 sequences. The previous experiments of others have shown that gradual stepwise increases in the MTX concentration in the medium results in the selective outgrowth of cells which have a compensatory increase in the copy number of resident cellular DHFR genes (8, 38). This increase in gene copy number occurs because the product of the gene, the DHFR enzyme, is stoichiometrically inhibited by MTX. Only those cells that have amplified their DHFR gene copy number are able to synthesize enough enzyme to resist MTX killing.

We wished to know whether the transfected pLTRdhfr26 construct was amplifiable like the cellular gene. Three of the transfected cell lines were selected for growth in increasing MTX concentration. These cell lines had originally been selected in 0.3 μ M MTX. The MTX concentration was increased by stepwise doublings up to 2.4 μ M with no dramatic effect on the cell growth. Presumably, the initially acquired dosage of pLTRdhfr26 genes resulted in an enzyme level adequate to overcome these higher MTX concentrations. At concentrations above 2.4 μ M there was significant cell killing but subsequent outgrowth of resistant cell populations. These cells were eventually adapted to grow in 80 μ M MTX after 3 months of stepwise selection.

Southern blot analysis of the DNAs of the selected cell lines give indications of sequence

amplification and rearrangement. Figures 3 and 4 show DNAs prepared from transfectants at three stages (0.3, 2.4, and 20 μ M MTX) during amplification, hybridized with a DHFR cDNA probe. Clone 26-8 (Fig. 3 and 4), when grown under increased selective pressure, amplified its entire complement of transfected genes three- to fivefold. A second clone, termed 26-1 (Fig. 2, 3, and 4), has lost all of its acquired pLTRdhfr26 genes save one, which is now present in about five times the original copy number. A third clone, 26-19 (Fig. 4), has lost some of its acquired genes and amplified others. This indicates that the array of initially acquired pLTRdhfr26 sequences is not absolutely stable in the cell.

The data above (Fig. 2, 3, and 4) indicate that these MTX-resistant transfectants harbor multiple copies of the pLTRdhfr26 construct integrated at different sites within the recipient cell. As discussed above, the pLTRdhfr26 transcripts examined terminate in adjacent DNA sequences, not in the cDNA sequences. Consequently, each integrated copy is expected to specify a unique transcript size. However, Fig. 6 shows by Alwine-Stark analysis that the transfectants carrying multiple integrated pLTRdhfr26 copies have a relatively simple pattern of DHFR transcripts (i.e., a single major species). These data suggest that not all of the acquired copies of the construct are giving rise to stable mRNA. Perhaps only the copy which has acquired an efficient termination-polyadenylation signal by integration with carrier DNA will give rise to substantial levels of RNA (Kaufman and Sharp, in press). This copy may principally mediate the MTX resistance, whereas the other acquired genes may not contribute to the maintenance of the phenotype and are free to be lost or rearranged. This may explain the paradoxical decrease in certain DHFR-specific sequences in those cell lines undergoing extensive rearrangements in response to increased MTX pressure (e.g., Fig. 4).

Cotransfection of pLTRdhfr26 with the DNA of a human promyelocytic leukemia cell line. The use of the DHFR gene as a reagent has several advantages in studying cellular genes whose passage via transfection is normally difficult to achieve. An example of such a gene is provided by the transforming gene of the human promyelocytic leukemia cell line HL60 (11).

We reasoned that transfection of the HL60 oncogene with the DHFR gene offered two related advantages. First, the MTX selection would only allow outgrowth of transfectants and thus suppress the growth of spontaneously transformed cells whose presence in a monolayer might obscure small numbers of genuinely transfected, transformed cells. Second, any

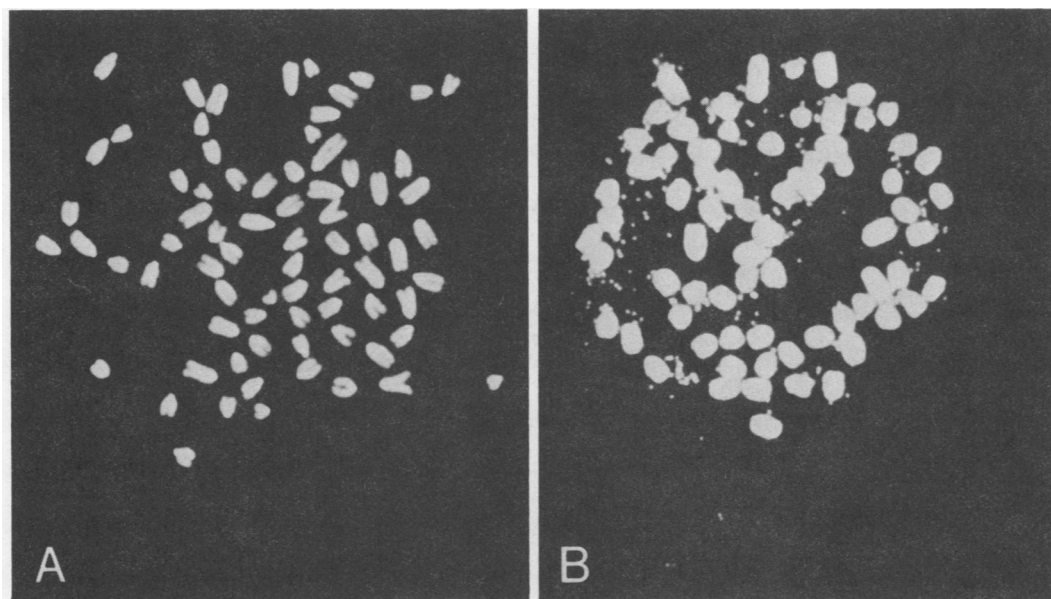


FIG. 7. DMs in NIH3T3 cells transfected with pLTRdhfr26 and human carrier DNA. The metaphase spreads were prepared from transfected cells grown in the absence (A) or presence (B) of MTX (20 μ M). Photographs were obtained under fluorescence microscopy after staining with 33258 Hoechst. No DMs are present in (A); approximately 100 are present in (B). The average DM content in cells cultured under the same conditions as those in (A) and (B) were 1.9 ± 0.8 and 14.2 ± 3.3 , respectively.

transformed cells found among the surviving colonies have arisen, with great likelihood, via uptake of exogenous DNA.

HL60 cell DNA was cotransfected with 1 μ g of DNA of the pLTRdhfr26 clone. Of the approximately 680 MTX^r colonies which arose, one contained refractile cells which appeared morphologically transformed. These cells were picked and grown up, and their DNA was seen to carry both acquired pLTRdhfr26 and human repetitive DNA sequences. Other experiments, using a cloned sequence probe specific for the HL60 oncogene, showed that these cells had also concomitantly acquired the transforming gene.

Presence of DMs in pLTRdhfr26 transformed cells. DMs have been found in a variety of tumor cells and in some unstably MTX^r cells (3, 4, 7, 20, 33). They have not been reported to arise in cells after transfection with selectable markers. We examined the karyotype of three cell lines established from MTX^r colonies as a consequence of uptake of the pLTRdhfr26 construct. DMs were found in all metaphase spreads in moderate to large numbers. A representative metaphase plate of such cells is seen in Fig. 7B, which shows a preparation from cells cotransfected with pLTRdhfr26 and human (HL60) carrier DNA. DMs also arise in cells cotransfected with NIH3T3 carrier DNA (data not shown).

DMs are known to appear in cells which have

become MTX^r by amplification of their resident DHFR genes. The MTX^r phenotype of these DM-containing cells is often unstable (7, 20). It was of interest to determine whether the DMs arising after pLTRdhfr26 transfection, like the DMs of endogenous origin, are lost from cells after relief of the MTX selective pressure. Some of the MTX^r cell lines derived above were incubated in medium lacking MTX for 2 to 3 weeks, after which their DM complement was examined. The cells now appeared to contain few if any DMs (Fig. 7A). These transfection-induced DMs therefore behave like those found in unstably resistant cells (7, 20). Their presence depends upon continued application of selective pressure.

We believe it is likely that the DMs in these cells are composed largely of the exogenous DNA transfected into the cell. Data presented above support this notion. There is an apparent correlation between the behavior of the DMs and the pLTRdhfr26 sequences: both occur in the cell after transfection and respond in parallel to increase or withdrawal of MTX. These observations suggest that DMs are in part composed of DNA introduced into the recipient cells during transfection. To support this, we attempted to directly analyze the sequence content of the DMs. Perhaps the most direct proof of the presence of pLTRdhfr26 sequences in the DMs would come from isolation and subsequent anal-

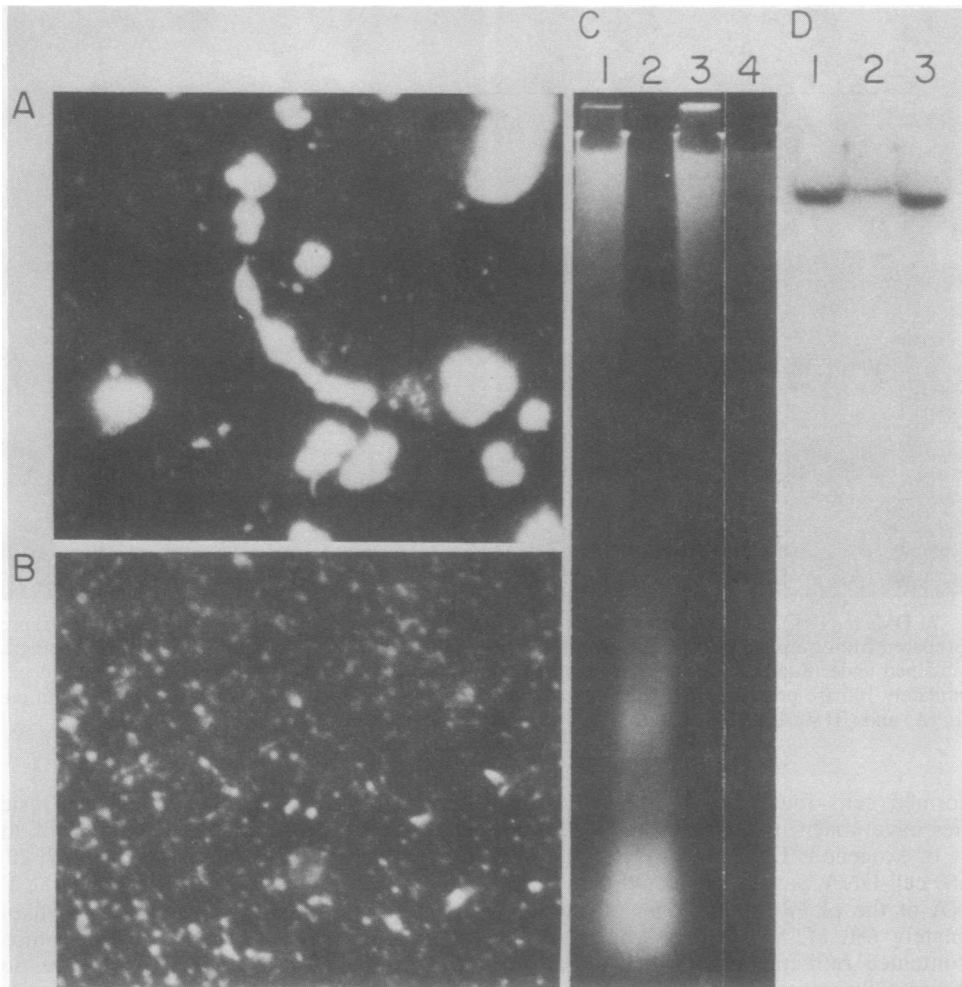


FIG. 8. DM enrichment and analysis. Metaphase chromosomes were prepared from 26-1 transfectants growing in 20 μ M MTX (Fig. 3, lane c), and an enriched DM fraction was obtained by Nuclepore filtration (see text). (A) Fluorescent micrograph of an ethidium bromide-stained sample of the material retained by the Nuclepore filter and (B) of the filtrate. DNA obtained from the enrichment protocol fractions (A and B) was analyzed by agarose gel electrophoresis and Southern transfer as in legends to Fig. 2 and 3. (C) Ethidium bromide-stained agarose gel: lane 1 contains 10 μ g of *Bam*HI-digested total cell DNA from 26-1 cells; lane 2 contains a sample (<0.5 μ g) of *Bam*HI digested DNA from the Nuclepore filtrate (B); lane 3 contains 10 μ g of *Bam*HI-digested DNA from nuclei prepared from colcemid-treated cells before Nuclepore filtration; lane 4 is a reference lane from the same gel that contains 0.5 μ g of *Bam*HI-digested cellular DNA which serves as a standard for calibrating ethidium bromide fluorescence intensity. The Nuclepore filtrate (lane 2) was not RNase treated before electrophoresis and a significant amount of RNA can be seen in this preparation. (D) Autoradiogram obtained after the gel in (C) was transferred and the resulting blot was hybridized with the 32 P-labeled pDHFR26 cDNA probe. The lanes in (D) are the same as in (C).

ysis of their associated DNA. Currently available DM purification techniques only offer relative enrichment of the DMs away from the other chromosomes (20). We undertook such an enrichment by filtration through Nuclepore membranes. The results of this enrichment can be seen in Fig. 8. Figure 8A is an example of the material retained by the filter and Fig. 8B is one of the filtrate. DNA was extracted from these

preparations and analyzed by Southern transfer. The analysis shown in Fig. 8 is of the 26-1 cell line adapted to grow in 20 μ M MTX (see Fig. 3, lane c). Less than 0.5 μ g of the DM filtrate DNA (Fig. 8C, lane 2) gave an autoradiographic signal (Fig. 8D) after hybridization with the DHFR cDNA probe one-third as strong as that obtained with 10 μ g of total cell DNA (lanes 1 and 3). By this analysis the DM preparation was

enriched four- to sixfold for pLTRdhfr26 sequences compared with the total cellular DNA. A second DM enrichment performed on transfectants obtained with pLTRdhfr26 and human carrier DNA showed a three- to fourfold enrichment of human repetitive DNA sequences in the DM preparation. These data indicate that much of the acquired DNA resides in the DMs. More effective purification procedures will be required before this conclusion can be made more precisely.

DISCUSSION

The technique of cotransfection, described extensively by others (10, 31, 48-50), allows the simultaneous introduction of multiple sequences into a recipient cell. Because these cotransfected sequences become linked into an array within the recipient cell (31), the cotransfected sequences may be retained if selective pressure is applied to encourage the retention of a single marker of the array.

Such an experimental strategy is more useful if it can be applied to a variety of recipient cells. This consideration motivated us to develop a functional molecular clone of a DHFR cDNA. Other chimeric clones carrying DHFR cDNA sequences have recently been reported which are able to confer the MTX^r phenotype on DHFR⁻ recipient cells (26, 42; Kaufman and Sharp, in press). In contrast, the present pLTRdhfr26 clone is able to convert wild-type cells as well to this phenotype. Consequently, multiple acquired copies of this clone should allow many types of cells to survive concentrations of MTX which would kill their untransfected counterparts.

Others have shown that an uncloned genomic DHFR gene, when cotransfected with an unselected cloned sequence, may confer an MTX^r phenotype on the recipient cell (10, 48). Application of increased concentrations of MTX resulted in amplification of the donor DHFR gene along with copies of the cotransfected cloned sequence. Such a strategy is of limited usefulness however, since the uncloned DHFR gene cannot be transfected with a second, unselected, single-copy gene with any assurance that a recipient cell will acquire both genes. This is because a recipient cell may stably acquire only about 0.1% of a donor cell complement of DNA (31). As a consequence, very few recipients (<1:16⁶) will simultaneously acquire two unlinked single-copy donor sequences.

This difficulty can be circumvented if the unselected, uncloned single-copy donor gene is cotransfected with many copies of a selectable marker sequence such as the pLTRdhfr26 clone constructed here. Among the 500 to 1,000 colonies which survive MTX after a single transfection,

one or two should carry as well the unselected gene of interest. These few colonies can only be identified if the unselected gene of interest induces some additional phenotype.

The viability of this approach is indicated by the experiments which used transformed cell (HL60) DNA cotransfected with the pLTRdhfr26 clone. Of the 680 MTX^r colonies which survived, 1 displayed the additional unselected phenotype of transformation. This led subsequently to the identification of a transforming sequence associated with the HL60 DNA (29). Once such a linked array of DHFR and unselected gene has been established in a recipient cell, one may select for variant subpopulations which have amplified their DHFR copy number and presumably coamplified the linked, unselected gene. This amplification is achieved in transfected cells because the donor sequences appear to exist in a genetically unstable state which is unusually susceptible to amplification or loss. The use of gene amplification to overproduce specific gene products has recently been demonstrated (35; Kaufman and Sharp, in press). Coamplification of selected and unselected genes depends as well on the stability of linkage between these two elements. This factor represents a serious limitation of the present approach. We find that the cotransfected array is unstable in some of our recipient cells, since one marker can be lost while another is retained.

An unexpected result of this work was our ability to visualize DMs within the transfectants. We suggest that these DMs derive largely from transfected donor DNA. This can only be proven by biochemical purification of these particles, which is currently difficult if not impossible to achieve. Others have reported that the donor DNA array becomes associated with a chromosomal locus in recipient cells (36). At least in the present experiments, we would suggest that much of this array can be perpetuated in an extrachromosomal form in the cell. It is possible that the MTX selection favors the outgrowth of cells which preserve their acquired sequences in these extrachromosomal particles.

The observed DMs do not contain amplified recipient host cell DHFR sequences. This is indicated most directly by the Southern blotting data (Fig. 2 and 3), which indicates virtually no amplification of resident chromosomal DHFR genes (30). Moreover, the numbers of DMs correlate with the copy number of pLTRdhfr26 sequences detectable by sequence hybridization. Both are seen to increase in the presence of higher DHFR concentrations and to be lost together in the absence of MTX selection. Untransfected NIH3T3 cells are capable of developing DMs carrying amplified cellular DHFR genes in response to MTX selection (20). This

alternate mode of developing MTX resistance likely involves excision of resident DHFR sequences from the cellular chromosome.

The carrier DNA sequences may have an important role in establishing the structure of the DMs resulting after transfection. In experiments not shown here, we have transfected the pLTRdhfr26 chimera with *E. coli* DNA serving as carrier. In these experiments the numbers of MTX^r colonies surviving was diminished by a factor of >100. Moreover, the surviving MTX^r colonies examined contained no DMs in their karyotype (data not shown). This indicates that the cotransfected carrier serves as more than an inert matrix for the establishment of these DMs. Rather, the carrier would seem to contain regions of sequence which are functionally important for the acquisition of the pLTRdhfr genes or their perpetuation in extrachromosomal particles. Such a hypothesis suggests that the sequence content of the DMs may to some extent be designed by choice of appropriate carrier and marker DNAs.

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