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Co-Expression and Amplification of Dihydrofolate Reductase cDNA and the *Escherichia coli* XGPRT Gene in Chinese Hamster Ovary Cells

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Summary: We have transformed Chinese hamster ovary cells with a plasmid containing mouse dihydrofolate reductase (DHFR) cDNA and the *Escherichia coli* xanthine-guanine phosphoribosyl transferase (XGPRT) gene under the control of the mouse mammary tumor virus and SV40 early promoters, respectively. Selection for the expression of XGPRT using the dominant selection scheme described by Mulligan and Berg yields clones that simultaneously express DHFR. Growth of these cells in progressively increasing concentrations of methotrexate, results in selection of cells that overproduce DHFR and its messenger RNA 250–500 fold. Analyses of the plasmid DNA sequences in these cells reveal that the increased production of DHFR is due in part to gene amplification (~50-fold) and in part to a selective overproduction of DHFR RNA. Last, the methotrexate-resistant cells contain 50 times more XGPRT RNA and DNA than the initial transformant; this demonstrates the potential for using gene amplification as a means for overproducing the products of genes linked to DHFR cDNA in plasmid vectors.
Key Words: Eukaryotic plasmid vectors—Dihydrofolate reductase DNA—Gene amplification—Recombinant DNA.

The advent of procedures for introducing well-characterized genes into mammalian tissue culture cells has provided novel approaches to studying gene expression and regulation (1–5). We and others have recently succeeded in introducing plasmid vectors containing a mouse dihydrofolate reductase (DHFR) cDNA into hamster cells that are deficient in this enzyme (6,7). Under the direction of a promoter derived from either SV40 or mouse mammary tumor virus (MMTV) DNA, sufficient DHFR is produced to complement the defect in these hamster cells. Similarly, the *Escherichia coli* gene for xanthine-guanine phosphoribosyl transferase (XGPRT) can also be expressed in mammalian cells from plasmid vectors containing a viral promoter (8). In this manuscript we describe the expression of DHFR cDNA and XGPRT in hamster cells transformed with a plasmid vector carrying both of these markers.

Cell lines derived by growth in progressively higher concentrations of the 4-amino analog of

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folic acid, methotrexate (MTX), have been shown to contain increased levels of DHFR associated with an increase in the number of DHFR genes (9,10). In this and other cases of gene amplification, the DNA sequences that become amplified appear to include the coding (exon) and noncoding (intron) regions of the gene as well as large extents of flanking chromosomal DNA (10,11). Here we show that hamster cells transformed with the plasmid vector containing mouse DHFR cDNA become resistant to increasing concentrations of MTX both by amplifying a region of DNA carrying integrated plasmid(s) and by selectively increasing the production of DHFR RNA. Furthermore, the *E. coli* XGPRT gene sequences that were present on the transforming plasmid have also been amplified, resulting in overproduction of XGPRT RNA.

MATERIALS AND METHODS

Cells

Chinese hamster ovary (CHO) cells deficient in dihydrofolate reductase (DHFR) were propagated as described by Urlaub and Chasin (12). Growth in nonselective conditions was in Ham's F12 medium supplemented with 4% each of newborn and fetal calf serum (Irvine Scientific). Expression of the *E. coli* XGPRT gene was monitored by growth in this same medium containing 25 μg/ml of mycophenolic acid (gift from the Eli Lilly Co.) and 250 μg/ml of xanthine (Sigma). Expression of DHFR was assessed by growth in Dulbecco's modified Eagle's medium supplemented with serum (as above) and 35 μg/ml of proline.

DNA-Mediated Transformation of Cells

The plasmid pMDSG has been described previously (6); its important features are summarized in the legend to Fig. 1. Transformation of the DHFR-deficient CHO cells with pMDSG was performed by the procedure of Graham and Van der Eb (13) as modified by Parker and Stark (14), except that carrier DNA was omitted. In short, 10^6 cells were exposed to a calcium-phosphate precipitate containing 10–20 μg of pMDSG and treated with 20% (vol/vol) glycerol for 5 min in PBS 4 h later. After growth for 3 days in nonselective medium, the cells were passaged (1:10) and placed into XGPRT selective medium. Colonies of transformed cells appeared approximately 10 days later and were isolated with cloning cylinders after 15–20 days. All procedures were carried out with Stanford University and NIH approval, according to NIH guidelines on recombinant DNA.

Selection of Methotrexate-Resistant Cells and Growth Studies

The clone pMDSG.9 was propagated in DHFR$^-$ selective medium (see above) and exposed to progressively increasing concentrations of methotrexate (Lederle) as described in the text; cells resistant to 10^{-6} *M* methotrexate are designated MTXR6. Sensitivity to the drug was analyzed by first growing cells for 3–4 generations in the absence of methotrexate, followed by addition of varying concentrations of the drug to 60 mm dishes containing 10^5 cells. Cells were fed after 2 days and counted in a hemocytometer after 4 or 5 days. The percentage of growth inhibition was determined by comparing the number of cells present in the methotrexate-treated cultures to that in control cultures without drug.

Quantitation of DHFR

Extracts of cells (1–2 × 10^7) were prepared by three cycles of freeze-thawing in liquid nitrogen and a 37°C waterbath in a buffer containing 50 m*M* potassium phosphate, pH 7.4, followed by centrifugation in a microfuge for 15 min. The concentration of protein in each extract was determined by the procedure of Bradford (15) with bovine γ-globulin as the standard. Varying concentrations of protein (100–300 μg from pMDSG.9 and 0.5–1.5 μg for

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MTXR6) were incubated with [^{3}H]methotrexate (Amersham, 200 mCi/mmol) for 10 min at 25°C, as previously described (6). Total protein was kept constant by addition of bovine serum albumin. The incubation mixture was passed over an 8 ml Sephadex G-50 column equilibrated in 10 m*M* potassium phosphate, pH 6/0.15 *M* KCl and the counts in the excluded volume were determined in a scintillation spectrometer. Control experiments showed no binding of [^{3}H]methotrexate to extracts from DHFR-deficient CHO cells.

DNA Extraction and Hybridization

Plasmid DNAs were isolated from *E. coli* by the procedure described by Hirt for isolation of polyoma DNA (16) and centrifuged to equilibrium in cesium chloride/ethidium bromide gradients. High molecular weight chromosomal DNA for use in restriction endonuclease analyses was prepared by treatment of tissue culture cells ($1-2 \times 10^8$) with sodium dodecyl sulfate (0.5%) and Pronase (500 μg/ml) for 1 h at 37°C. Protein was removed by gentle extraction with phenol/chloroform (1:1 vol/vol) and the DNA was dialyzed exhaustively against 5 m*M* Tris HCl, pH 7.4/0.1 m*M* EDTA. Restriction endonucleases were purchased from Bethesda Research Labs and used according to BRL catalog descriptions. DNAs were cleaved with restriction endonucleases, subjected to electrophoresis through agarose gels, transferred to nitrocellulose membranes (Schleicher and Schuell) as described by Southern (17), and hybridized with ^{32}P-nick-translated pMDSG DNA or *E. coli* XGPRT DNA ($0.5-1 \times 10^8$ cpm/μg).

Analysis of mRNA Levels

The relative amounts of DHFR and XGPRT RNAs present in pMDSG.9 and MTXR6 cells were estimated by hybridization of total cytoplasmic RNAs to an excess of end-labeled probes. The DNA probes were prepared essentially as described (6). The plasmids pMTV dhfr (6) and pSVM gpt (see Fig. 7) were cut at a unique *Bgl*II site (at the end of the DHFR sequence and in the XGPRT sequence, respectively), treated with alkaline phosphatase, and labeled with [^{32}P]ATP using T4 polynucleotide kinase. After digestion with *Eco*RI, the DNA fragments were separated by agarose gel electrophoresis and the appropriate fragments were recovered by dissolving the gel slice in 7 *M* sodium perchlorate and adsorbing the DNA to glass powder (18). DNA was eluted from the glass in 10 m*M* Tris HCl pH 7.4/0.1 m*M* EDTA and used for hybridizations to cytoplasmic RNAs in 80% formamide at 50°C for 12–16 h, according to the method of Berk and Sharp (19). Hybrids were treated with S1 nuclease (Boehringer-Mannheim) and run on nondenaturing agarose (1.4%) or polyacrylamide (6%) gels.

RESULTS

Analysis of CHO DHFR$^-$-Cells Transformed with pMDSG

A plasmid (pMDSG) containing mouse DHFR cDNA fused to the mouse mammary tumor virus (MMTV) promoter and the *E. coli* XGPRT gene fused to the SV40 early promoter (Fig. 1) was used to transform CHO cells that are deficient in dihydrofolate reductase (DHFR). We have previously demonstrated that using the DNA-mediated transformation procedure of Graham and Van der Eb (13), cells expressing either the *E. coli* XGPRT gene or the mouse DHFR cDNA can be selected at a frequency of $1-2 \times 10^{-4}$/cell (6). In the following experiments we have analyzed three clones of CHO transformants that were selected for functional expression of the XGPRT sequences by growth in mycophenolic acid and xanthine (8). Interestingly, all three of these clones were able to grow in medium lacking glycine and thymidine (Fig. 2), suggesting that they were also expressing the DHFR cDNA sequences.

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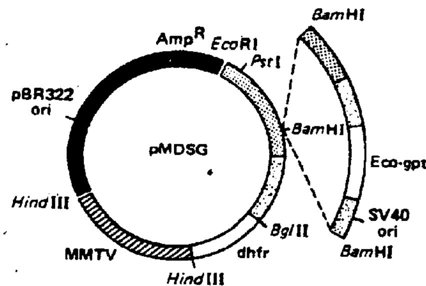

FIG. 1. Structure of the plasmid pMDSG. This molecule was constructed by inserting the *Bam*HI fragment containing XGPRT (Eco-gpt) into the plasmid pMTV dhfr as previously described (6). The solid black segment is a 2.3 kb fragment of pBR 322 extending from the *Eco*RI site to the *Pvu*II site that contains the β-lactamase gene (ampicillin resistance) and the origin of replication. The lightly and heavily stippled regions represent fragments derived from SV40 DNA that provide the SV40 early promoter (linked to Eco-gpt) and RNA processing signals (a splice and a site for polyadenylation). The hatched region represents the MMTV LTR which encompasses the promoter for viral RNA synthesis. The open regions represent either mouse DHFR cDNA (6,7) or *E. coli* XGPRT DNA (25). Transcription from both the MMTV promoter, and the SV40 early promoter is in the counterclockwise direction, as are the coding sequences for DHFR and XGPRT. The sizes of the fragments are not drawn to scale.

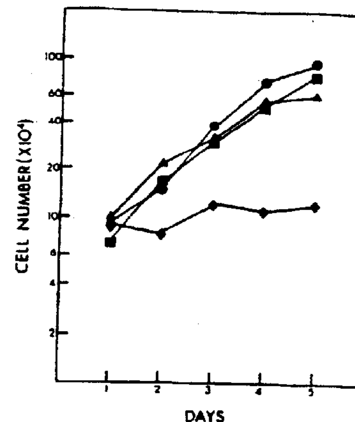

FIG. 2. Growth of pMDSG-transformed clones of DHFR-deficient CHO cells in DHFR selective medium. Individual clones selected for expression of XGPRT after DNA infection with pMDSG were plated at approximately 10^5 cells/60 mm dish in DHFR selective medium (see Methods). Cells were fed every 2 days and counted in a hemocytometer: (▲—▲) pMDSG.3; (■—■) pMDSG.9; (●—●) pMDSG.12; (◆—◆) parental CHO DHFR⁻ cells.

To determine whether these clones arose from independent events, samples of whole cell DNA were cleaved with either *Eco*RI or *Hind*III (enzymes that cleave the plasmid at one or three sites, respectively), separated on 1% agarose gels, transferred to nitrocellulose filters and hybridized with ^{32}P-nick-translated pMDSG (17). It is apparent that the pattern of the hybridizing bands is different in each of the three clones (Fig. 3). One of these, pMDSG.9, contains a large number of plasmid-derived sequences, whereas pMDSG.12 and pMDSG.3 contain few. Although there appears to be a band with a mobility that corresponds to that of linear plasmid itself in the DNA from all three clones, we have not ascertained whether this represents tandem integrations of the plasmid, freely replicating plasmid, or a serendipitous event. The transformed phenotype, however, is stable even in the absence of selective pressure (Ringold, unpublished observation). Thus, it is probable that the bulk of the plasmid DNAs in these transformants is associated with chromosomal DNA and that these three clones are indeed the products of independent transformation events. We have not characterized the integrated structures of these plasmids in any further detail.

Increased Expression of DHFR in Cells That Are Resistant to Methotrexate

One of the three clones described above (pMDSG.9) was chosen at random for initial attempts to select MTX-resistant cells. The growth of this cell line is inhibited at MTX concentrations of 10^{-9} to 10^{-8} *M* (Fig. 4). Approximately 10^6 cells were plated in 10^{-8} *M* MTX, and after 2 weeks, 10–12 colonies appeared on the dish. A single colony was picked, grown to mass culture, and subsequently subjected to growth in 10^{-7} *M* MTX. Again, a few colonies survived at this concentration. The resistant cells were grown to mass culture without cloning and then placed in 10^{-6} *M* MTX.

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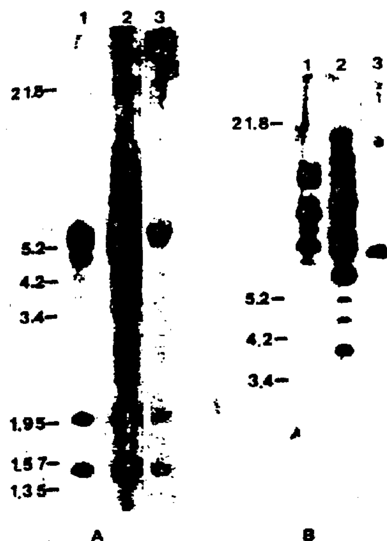

FIG. 3. pMDSG sequences in DNAs extracted from XGPRT transformants. Approximately 10 μg of high molecular weight DNA cleaved with either *Hin*dIII (A) or *Eco*RI (B) were subjected to electrophoresis in 1% agarose and transferred to nitrocellulose filters. Filters were hybridized with ³²P-nick translated pMDSG. Size markers (in kb) are from a digest of phage λ DNA with *Eco*RI and *Hin*dIII. Lane 1: pMDSG.12; lane 2: pMDSG.9; lane 3: pMDSG.3.

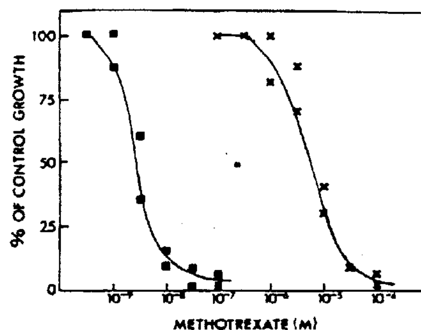

FIG. 4. Methotrexate sensitivity of pMDSG.9 and MTXR6 cells. Cells (~10⁵) were plated in 60 mm dishes; after 24 h, medium containing the indicated concentrations of methotrexate was added. Cells were fed after 2 days and counted in a hemocytometer 5 days after addition of methotrexate. The data are plotted relative to the number of cells in dishes containing no methotrexate; this was done for both cell lines since we have observed that the MTXR6 cells grow more slowly than do the pMDSG.9 cells. (■—■), pMDSG.9; (X—X), MTXR6.

Cells surviving in this concentration of the drug (designated MTXR6) were used for all of the following analyses.

In order to ascertain the relative resistance of MTXR6 and pMDSG.9 cells to MTX, growth inhibition studies with the drug were performed. The results shown in Fig. 4 indicate that the highly resistant cells are capable of growing in concentrations of MTX that are approximately 2000-fold higher than the parental cells. We have assessed the stability of the drug-resistant phenotype by growing MTXR6 cells in the absence of MTX for approximately 55 generations; after this time, the cells remain resistant to the same levels of MTX as the cells kept in the drug continuously (data not shown).

Since there is not necessarily a 1:1 correlation between the absolute amount of DHFR and the level of MTX resistance (20), we measured the content of DHFR in extracts of the sensitive and resistant cell lines. Using [³H]MTX as a ligand to quantitate DHFR molecules, we estimate that the MTXR6 cells contain approximately 400 times as much DHFR as do pMDSG.9 cells (Table 1). The reasons for the discrepancy between the increased levels of DHFR and methotrexate resistance in these and others cells are not clear.

Overproduction of MMTV-DHFR RNA and Amplification of Plasmid Sequences

We have estimated the relative amounts of DHFR RNA in pMDSG.9 and MTXR6 cells by the procedure of Berk and Sharp (19). Using this approach we have also been able to determine the approximate 5′ end of the DHFR transcripts. The results shown in Fig. 5 indicate that there is 200–500 times as much DHFR RNA in the MTXR6 cells as in pMDSG.9, in agreement with our estimates of the increase in enzyme production in these cells. The major transcript in these cells (corresponding to the

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TABLE 1. *Overproduction of DHFR in methotrexate-resistant cells*

Cell line	cpm [^{3}H]MTX bound/μg protein^a	
	Expt 1	Expt 2
pMDSG.9	1.9	3.0
MTXR6	980	960

^{a} Binding of [^{3}H]methotrexate was measured by exclusion chromatography as described in Materials and Methods.

band at 1.1 kb) appears to initiate approximately 250–300 nucleotides upstream of the DHFR insert (see Fig. 1). Since this is the approximate region of the 5′ end of MMTV RNA (21), our results are consistent with the notion that the DHFR RNA is produced by utilization of the MMTV promoter. A minor band is also present in the MTXR6 RNA at about 1.6 kb. We do not know whether this represents an authentic site of initiation of RNA synthesis, a spliced messsenger RNA, or an anomaly of this analysis. In pMDSG.9 this band is not apparent; however, since there is so little DHFR RNA in these cells, we may not be able to detect such a minor species. Further characterization of this larger RNA as well as more refined mapping of the 5′ end(s) of the DHFR RNA is warranted. Lastly, there are two additional bands present in the products of the hybridization to pMDSG.9 RNA which migrate at approximately 3.0 and 3.5 kb. These bands, which are also present in long exposures of MTXR6 and CHO DHFR$^-$ RNA (data not shown), appear to be artifacts of the S1 treatment.

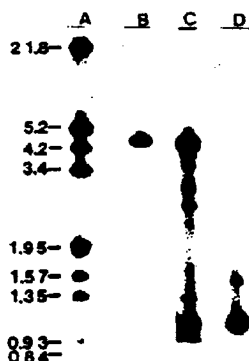

FIG. 5. DHFR RNAs in pMDSG.9 and MTXR6 cells. Cytoplasmic RNAs were isolated from cells as described (19) and hybridized with 50,000 cpm of [^{32}P]DNA labeled at the *Bgl*II site present at the end of the DHFR coding region of pMTV dhfr (see Fig. 1 and ref. 6); the probe extended to the *Eco*RI site. Hybridization was at 50°C in 80% formamide for ~ 16 h. DNA-RNA hybrids were treated with S1 nuclease at 50°C, precipitated with EtOH, and analyzed by electrophoresis on a 1.4% agarose gel. Lane A: ^{32}P-labeled phage λ DNA cut with *Eco*RI and *Hind*III; lane B: end-labeled probe alone; lane C: RNA from pMDSG.9 (~150 μg); 12-day exposure; lane D: RNA from MTXR6 (~30 μg); 4-h exposure. We estimate from densitometer tracings of this and several other exposures that there is 250–500 times more DHFR RNA in MTXR6 than in pMDSG.9 cells. The dark band present in lane C at about 4.4 kb represents re-annealed probe; this could also be seen in longer exposures of lane D.

We have quantitated the amount of pMDSG DNA in the parental and highly resistant cells by the procedure of Southern (17). After cleavage of the DNA with *Eco*RI, an enzyme that cuts the plasmid at a single site, DNA from pMDSG.9 cells yields a complex pattern of bands that hybridize with ^{32}P-nick-translated pMDSG (Figs. 2 and 6A, lanes 5–7). In DNA from the MTXR6 cells, the identical pattern of bands is observed; however, approximately 50 times less DNA is required to obtain a pattern with equal autoradiographic intensity (Fig. 6A, lanes 1–4). We surmise that these MTX-resistant cells have amplified a region of chromosomal DNA containing multiple inserts of pMDSG DNA to a level approximately 50 times that in the parental cells. Thus, the level of gene amplification in the MTXR6 cell does not account completely for the increased amounts of enzyme or DHFR RNA.

XGPRT RNA Is Overproduced in MTXR6 Cells

Since pMDSG.9 cells were selected by their ability to express the XGPRT gene, it was of interest to see whether the selection for increased expression of DHFR had resulted in a coincident increase in XGPRT expression in the MTXR6 cells. Figure 6B shows that the XGPRT sequences, detected by hybridization with labeled XGPRT DNA rather than whole

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FIG. 6. Analysis and quantitation of pMDSG and XGPRT DNA in methotrexate sensitive and resistant cells. High molecular weight DNAs from pMDSG.9 and MTXR6 cells were digested with *Eco*RI, run on an 0.8% agarose gel, and transferred to nitrocellulose filters. DNA samples were as follows: Lanes 1–4: MTXR6 DNA; 0.5, 0.2, 0.1, and 0.05 μg, respectively. Lanes 5–7: pMDSG.9 DNA; 30, 10, and 3 μg, respectively. The DNA filters were hybridized with either ^{32}P-nick-translated pMDSG (panel A) or ^{32}P-nick-translated XGPRT DNA (panel B) isolated by *Bam*HI and *Hind*III digestion of plasmid pL10 (25). pL10 DNA contains the XGPRT sequence cloned in pBR 322.

plasmid, are also amplified about 50-fold in the MTXR6 cells. This result might have been anticipated since the DHFR and XGPRT coding sequences were both present on the plasmid used for transformation. In experiments similar to these, Wigler et al. (26) have demonstrated that pBR 322 sequences become amplified in mouse cells co-transformed with a mutant DHFR gene and pBR 322 after exposure to increasing concentrations of methotrexate.

We have quantitated the relative amounts of XGPRT RNA in pMDSG.9 and MTXR6 by hybridization with a probe labeled at the *Bgl*II site of the XGPRT gene in the plasmid pSVM gpt (Fig. 7A). Since an XGPRT RNA initiating at the SV40 promoter of pMDSG would only have about 135 nucleotides in common with this probe, the protected fragment should be approximately this length. As seen in Fig. 7B, this is in fact the case. Of particular interest, however, is that the amount of XGPRT RNA in MTXR6 cells is 40–70-fold greater than that in pMDSG.9 cells. Thus, unlike DHFR RNA, the relative increase in the level of the XGPRT RNA corresponds very well to the extent of gene amplification. The data on levels of gene amplification and expression of both XGPRT and DHFR are summarized in Table 2.

DISCUSSION

Transformation of CHO Cells with pMDSG

We have demonstrated that CHO cells infected with the vector pMDSG are capable of expressing DHFR cDNA and *E. coli* XGPRT simultaneously. In the experiments reported here, cells were selected for their ability to express the XGPRT sequences. Subsequent experiments have been performed demonstrating that CHO cells selected for expression of DHFR cDNA may also express XGPRT (Ringold, unpublished observations). As suggested by Mulligan et al. (22) and demonstrated here, plasmid vectors such as pMDSG may be of general utility for introducing selectable or nonselectable markers into mammalian cells with high efficiency. In this regard, the *E. coli* XGPRT gene serves as a wide spectrum dominant marker; we have thus far succeeded in transforming mouse 3T6, CHO, and rat hepatoma cells with the selective conditions described by Mulligan and Berg (8).

Mechanisms of Methotrexate Resistance

Transformation of the DHFR-deficient CHO cells with pMDSG has provided a unique set-

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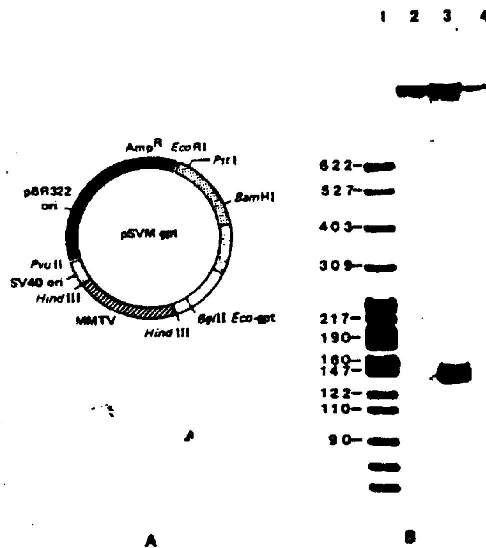

FIG. 7. (A) Structure of the plasmid pSVM gpt. Its salient features are as described for pMDSG in the legend to Fig. 1. The *Bgl*II site within the Eco gpt (XGPRT) sequence was labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase; the probe used in panel B extended to the *Eco*RI site. The *Bgl*II site is approximately 125 nucleotides from the closest *Hind*III site at the proximal end of the MMTV DNA. Therefore, a hybrid between this probe and an XGPRT RNA initiated from the SV40 promoter within pMDSG would only be 125 nucleotides in length. (B) XGPRT RNA in pMDSG.9 and MTXR6 cells. The same RNAs described in Fig. 5 were hybridized with end-labeled XGPRT probe (25,000 cpm). Hybrids generated by S1 nuclease treatment were analyzed on a 6% acrylamide gel. Lane 1: ^{32}P-labeled *Hpa*II digest of pBR 322 (sizes are in base pairs); lane 2: RNA from pMDSG.9 (~150 μg); lane 3: RNA from MTXR6 (~30 μg); lane 4: RNA from parental DHFR⁻ CHO cells (~150 μg). The autoradiogram depicted in lanes 2–4 was exposed for 12 h. Using densitometer tracings of this and other exposures, and taking into account the different inputs of RNA in hybridizations, we estimate that there is 40–60 times more XGPRT RNA in MTXR6 than in pMDSG.9 cells. The band present at a position above the 622 bp marker in lanes 2–4 corresponds to re-annealed probe.

ting in which to study the mechanisms by which cells become resistant to methotrexate. As first observed by Schimke and his colleagues (20), cells selected in progressively higher concentrations of methotrexate overproduce DHFR as a consequence of gene amplification. In the experiments reported here, we have demonstrated that an artificial gene containing the DHFR coding region fused to the MMTV promoter and RNA processing signals derived from SV40 can be amplified in a similar fashion. One may infer from this result that the large intervening sequences and flanking chromosomal sequences in and around the natural gene are not absolutely required for the amplification process.

It is clear that many alterations in a cell could give rise to the methotrexate-resistant phenotype. In the case of MTXR6 cells, it appears that resistance is due to a combination of gene amplification and a selective increase in DHFR RNA production. The amount of DHFR produced in these cells is approximately 400 times greater than in the pMDSG.9 cells.

TABLE 2. *Relative amounts of DHFR and XGPRT in methotrexate sensitive and resistant cells*

	Protein[a]		RNA[b]		DNA[c]	
	pMDSG.9	MTXR6	pMDSG.9	MTXR6	pMDSG.9	MTXR6
DHFR	1	300–500	1	250–500	1	40–60
XGPRT	N.T.[d]	N.T.	1	40–70	1	40–60

[a] DHFR was measured by [^{3}H]MTX binding (see Table 1).
[b] RNA levels were estimated from the analyses shown in Figs. 5 and 7.
[c] DNA levels were estimated from the Southern blots shown in Fig. 6.
[d] N.T., not tested.

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whereas there is a similar 250–500-fold increase in the amount of cytoplasmic DHFR RNA, there is only about a 50-fold increase in the gene copy number. Thus, there may be alterations that lead to increased production of DHFR RNA from the MMTV promoter (promoter-up mutations) or there could be changes in the processing and/or stability of the DHFR RNA in the MTXR6 cells that lead to increased accumulation of the messenger RNA. Since it is likely that the MTXR6 cells are not a clonal isolate, multiple phenotypes among the cells may contribute to the high level of methotrexate resistance. This, however, might also be true in a clonal isolate harboring multiple copies of the gene since one could envision the presence of mutant and wild type genes within a single cell.

Integration and Amplification of Plasmid Sequences

The transformants we have analyzed vary substantially from one another in their content of pMDSG DNA. The Southern blots shown in Fig. 3, revealed that individual transformants contain as few as 1–2 copies or as many as 20 or more copies of pMDSG-derived DNA. Although the restriction endonuclease analysis does not constitute absolute proof, it seems likely that most if not all of the vector DNA is integrated into chromosomal sequences. We have made preliminary attempts to identify freely replicating plasmid in these cells by the procedure of Hirt (16) without success.

In the transformant pMDSG.9, the plasmid-derived sequences are present in a very complex array, as determined by Southern blotting analysis. Strikingly, the pattern observed in the Southern blots of cellular DNA from the MTX-resistant cells is identical to that from pMDSG.9 (Fig. 6). It seems highly unlikely that DNA integrated into various locations in the cellular genome would be amplified coordinately. Rather, we believe that the multiple insertions of pMDSG have occurred within a small region of a chromosome during the initial DNA infection and that amplification of these integrated plasmid sequences results from expansion of a chromosomal region encompassing all or most of the plasmid DNAs.

This interpretation is consistent with the results of others who have recently studied the acquisition of DNA in transformants selected by expression of the herpes virus thymidine kinase (TK) gene (4,23). Their experiments indicate that stable transformants contain only integrated as opposed to freely replicating TK DNA. Furthermore, in co-transformation experiments using TK and the rat growth hormone gene, Robins et al. (24) have demonstrated that individual transformants may harbor from 1 to 100 copies of the growth hormone DNA. Particularly intriguing is the observation that in cells containing many copies, the bulk of the growth hormone DNA appears to be integrated into a single chromosomal region; these "hot spots" for integration of exogenous DNA seem to be associated with chromosomal break-points (24). Our results indicating that the multiple pMDSG sequences are amplified coordinately are consonant with these observations.

Special Features of pMDSG Transformed Cells

The results of previous experiments suggest that the MMTV promoter is weak in comparison to the SV40 early or the cellular DHFR promoter (6). This is reflected by the ability to inhibit growth of the pMDSG transformants with very low (10^{-8} *M*) concentrations of MTX; for comparison, the growth of wild type CHO cells is inhibited at approximately 10^{-7} *M* MTX. In order to isolate wild type CHO cells that overproduce DHFR several hundred fold, it is necessary to select for cells that grow in concentrations of methotrexate between 10^{-4} and 10^{-3} *M* (10). In the case of the MTXR6 cells described here, these levels of enzyme overproduction (not absolute levels of DHFR) are achieved at much lower concentrations of the drug. It may therefore be possible to isolate pMDSG transformed cells that overproduce DHFR many thousand fold by progressively

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increasing the concentration of MTX to 10^{-4} or 10^{-3} *M*.

Using DHFR cDNA as the amplifiable marker has allowed us to simultaneously increase the expression of the *E. coli* XGPRT gene in pMDSG transformants. However, in contrast to the several hundred fold increase in DHFR expression, the production of XGPRT in MTXR6 cells represents only a 50-fold increase over the level in pMDSG.9. As suggested elsewhere, it is conceivable that overexpression of XGPRT is detrimental to the cell (25). Thus, the maximum tolerable levels of this enzyme may have been reached at the time the plasmid sequences were amplified 50-fold. It would be of interest to ascertain whether one could obtain higher levels of gene amplification in cells transformed with the plasmid, pMTV DHFR which lacks the SV40-XGPRT transcriptional unit. Along the same lines, it may be possible to amplify other DNA sequences inserted at either the *Bam*HI or *Eco*RI sites of these plasmids as a general approach to obtaining high level expression of desired gene products in these DHFR-deficient cells.

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REFERENCES

1. Goff SP, Berg P: Construction and expression of simian virus 40 recombinant genomes containing the *Escherichia coli* gene for thymidine kinase and a *Saccharomyces cerevisiae* gene for tyrosine transfer RNA. *J Mol Biol* 133:359–383, 1979
2. Mulligan RC, Howard BH, Berg P: Synthesis of rabbit β-globin in cultured monkey kidney cells following transfection with a SV40 β-globin recombinant genome. *Nature* 277:108–114, 1979
3. Hamer DH, Smith KD, Boyer SH, Leder P: SV40 recombinants carrying rabbit β-globin gene coding sequences. *Cell* 17:725–735, 1979
4. Wigler M, Silverstein S, Lee S-S, Pellicer A, Axel, R: Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* 11:223–232, 1977
5. Sarver N, Gruss P, Law M-F, Khoury G, Howley P: Bovine papilloma virus deoxyribonucleic acid: a novel eukaryotic cloning vector. *Mol Cell Biol* 1:486–496, 1981
6. Lee F, Mulligan R, Berg P, Ringold G: Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumor virus chimeric plasmids. *Nature* 294:228–232, 1981
7. Subramani S, Mulligan R, Berg P: Expression of the mouse dihydrofolate reductase cDNA in simian virus 40 vectors. *Mol Cell Biol* 1:854–864, 1981
8. Mulligan RC, Berg P: Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc Natl Acad Sci USA* 78:2072–2076, 1981
9. Alt FW, Kellems RE, Bertino JR, Schimke RT: Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J Biol Chem* 253:1357–1370, 1978
10. Nunberg JH, Kaufman RJ, Schimke RT, Urlaub G, Chasin LA: Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line. *Proc Natl Acad Sci USA* 75:5553–5556, 1978
11. Wahl GM, Padgett RA, Stark GR: Gene amplification causes overproduction of the first three enzymes of UMP synthesis in *N*-(phosphonoacetyl)-L-aspartate-resistant hamster cells. *J Biol Chem* 254:8679–8689, 1979
12. Urlaub G, Chasin LA: Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc Natl Acad Sci USA* 77:4216–4220, 1980
13. Graham R, Van der Eb A: A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456–467, 1973
14. Parker BA, Stark GR: Regulation of simian virus 40 transcription: Sensitive analysis of the RNA species present early in infection by virus or virus DNA. *J Virology* 31:360–369, 1979
15. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
16. Hirt B: Selective extraction of polyoma DNA from infected mouse cell cultures. *J Mol Biol* 26:365–369, 1967
17. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98:503–517, 1975
18. Vogelstein B, Gillespie D: Preparation and analytical purification of DNA from agarose. *Proc Natl Acad Sci USA* 76:615–619, 1979
19. Berk AJ, Sharp PA: Sizing and mapping of early ade-

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novirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* 12:721–732, 1977

20. Schimke RT, Kaufman RJ, Alt FW, Kellems RF: Gene amplification and drug resistance in cultured murine cells. *Science* 202:1051–1055, 1978
21. Donehower LA, Huang AL, Hager GL: Regulatory and coding potential of the mouse mammary tumor virus long terminal redundancy. *J Virology* 37:226–238, 1981
22. Mulligan RC, Southern PJ, Howard BH, Yaniv M, Geller AI, Berg P: Construction and potential uses for a family of mammalian transducing vectors. Submitted for publication
23. Perucho M, Hanahan D, Wigler M: Genetic and physical linkage of exogenous sequences in transformed cells. *Cell* 22:309–317, 1980
24. Robins DM, Ripley S, Henderson A, Axel R: Transforming DNA integrates into the host chromosome. *Cell* 23:29–39, 1981
25. Mulligan RC, Berg P: Expression of a bacterial gene in mammalian cells. *Science* 209:1423–1427, 1980
26. Wigler M, Perucho M, Kurtz D, Dana S, Pellicer A, Axel R, Silverstein S: Transformation of mammalian cells with an amplifiable dominant-acting gene. *Proc Natl Acad Sci USA* 77:3567–3570, 1980