

Transformation of mammalian cells with an amplifiable dominant-acting gene

(animal cell vectors/methotrexate resistance/gene amplification)

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Communicated by J. D. Watson, March 10, 1980

ABSTRACT We have transferred a mutant hamster gene coding for an altered dihydrofolate reductase to wild-type cultured mouse cells by using total genomic DNA from methotrexate-resistant Chinese hamster ovary A29 cells as donor. By demonstrating the presence of hamster gene sequences in transformants we have provided direct evidence for gene transfer. Transformants selected for increased resistance to methotrexate contain increased amounts of the newly transferred gene. We have used this mutant *dhfr* gene to introduce the *Escherichia coli* antibiotic resistance plasmid pBR322 into animal cells. Amplification of the *dhfr* sequences results in amplification of the pBR322 sequences as well. The use of this gene may allow the introduction and amplification of virtually any genetic element in various new cellular environments.

The ability to transfer purified genes into cultured cells provides a unique opportunity to study the function and physical state of exogenous genes in new cellular environments. The development of systems for DNA transfer in animal cells originated with the lytic transfection of cells by using purified viral DNA (1, 2) and progressed to the stable transfer of viral transforming functions to appropriate recipient cells (3). Subsequently, viral genes from the herpesviruses coding for the biochemically selectable marker thymidine kinase (TK) (4-6) were transferred to enzyme-deficient mutant cells. Restriction fragments of herpes simplex virus type 1 encoding TK were isolated (6) and subsequently cloned into bacterial plasmids (7). Through the use of this selectable marker, virtually any gene can now be introduced into recipient cells (8, 9); however, these cells must be *tk*⁻ mutants. Other potential selection systems are available, and several laboratories have recently demonstrated the DNA-mediated transfer of cellular genes coding for selectable markers such as TK (10), adenine phosphoribosyltransferase (11) and hypoxanthine phosphoribosyltransferase (12, 13).

Dominant mutant cellular genes coding for drug resistance in principle could serve as generalized biochemical vectors for wild-type cells. Cultured mammalian cells are exquisitely sensitive to the folate antagonist methotrexate (Mtx). Mtx-resistant cell lines have been identified in three categories: (i) cells with decreased uptake of this drug (14, 15); (ii) cells that produce inordinately high levels of dihydrofolate reductase (DHFR) (16, 17); and (iii) cells with structural mutations which lower the affinity of DHFR for Mtx (18). When they were examined, cells producing high levels of DHFR were found to contain increased copy numbers of the *dhfr* gene (gene amplification) (19). An interesting Mtx-resistant variant cell line (A29) has been identified that synthesizes increased amounts of a mutant DHFR with decreased affinity for Mtx (18). We have used genomic DNA from this cell line to transfer the

mutant *dhfr* gene to wild-type Mtx-sensitive cells. Exposure of Mtx-resistant transformed cells to increasing levels of Mtx selects for cells that have amplified the transferred gene.

MATERIALS AND METHODS

Cell Culture. Mouse Ltk⁻ aprt⁻ cells (11) and NIH 3T3 cells (20) (the latter generously provided by R. A. Weinberg) were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum and antibiotics (growth medium). Chinese hamster ovary (CHO) cells and A29 cells (18), Mtx-resistant CHO derivatives (generously provided by L. Siminovitch), were maintained in growth medium supplemented with 3 times the usual concentration of nonessential amino acids. A29 cells were grown in the presence of Mtx at 20 µg/ml.

Extraction, Restriction Endonuclease Digestion, and Ligation of DNA. High molecular weight DNA was extracted from cultured cells as described (9). DNA was analyzed by electrophoresis in 0.3% agarose gels with restriction fragments of herpes simplex virus DNA as markers. Only DNA whose molecular weight average was 35×10^6 or greater was used for transformation experiments. CsCl/ethidium bromide-purified form I DNA of the *Escherichia coli* plasmid pBR322 was isolated from cultures of *E. coli* strain HB101 (21). A29 DNA and pBR322 were mixed at 3:1 mass ratios, completely digested with restriction endonuclease *Sal* I (under conditions recommended by supplier, Bethesda Research Labs), extracted once with aqueous buffer-saturated phenol/chloroform/isoamyl alcohol 25:24:1 (vol/vol), and once with chloroform/isoamyl alcohol, 24:1, and precipitated with ethanol. DNA was resuspended and ligated with T4 DNA ligase (Bethesda Research Laboratories, Rockville, MD) at 100 µg of DNA and 3 units of ligase per ml at 4°C for 24 hr in the buffers recommended by the supplier. The ligation product was reextracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol.

Transformation and Selection. Ltk⁻ aprt⁻ cells and NIH 3T3 cells were transformed with genomic DNA by the calcium phosphate coprecipitation method (2) as described (11). All DNAs were sterilized by ethanol precipitation and resuspended in 1 mM Tris-HCl/1 mM EDTA, pH 7.9. For tk⁺ transformation, cells were exposed to hypoxanthine/aminopterin/thymidine selective medium as described (10). Transformants resistant to Mtx were selected in growth medium containing either 0.1 or 0.2 µg of Mtx per ml with the same feeding schedule as for tk selection. After 2-3 weeks, colonies were isolated from individual dishes with cloning cylinders to ensure that each transformant arose from an independent event. In transformation with ligated DNAs, no more than 1 µg of pBR322 DNA was added to 10⁶ cells per dish because higher

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Abbreviations: DHFR, dihydrofolate reductase; Mtx, methotrexate (amethopterin); TK, thymidine kinase; CHO, Chinese hamster ovary; kb, kilobase.

concentrations of pBR322 inhibited transformation. Ltk⁻ aprt⁻ DNA was added as carrier in these cases to a final DNA concentration of 20 μ g per dish.

Filter Hybridization. DNA from parental and transformed cells was isolated, digested with restriction endonucleases, electrophoresed in 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized as described (9). The probes for these experiments were ³²P-labeled nick translated pBR322 or pdhfr-21, a cloned cDNA copy of mouse DHFR mRNA (22) (kindly provided by R. J. Kaufman and R. T. Schimke).

RESULTS

Transfer of the Mutant Hamster Dihydrofolate Reductase Gene to Mouse Cells. High molecular weight cellular DNA was prepared from wild-type Mtx-sensitive CHO cells and from A29 cells, and the ability of these DNA preparations to transfer either the *dhfr* gene or the *tk* gene to tk⁻ mouse cells (Ltk⁻ aprt⁻) or NIH 3T3 cells was tested (Table 1). DNAs from both mutant A29 and wild-type CHO cells were competent in transferring the *tk* gene to Ltk⁻ aprt⁻ cells. Mtx-resistant Ltk⁻ aprt⁻ colonies were observed after treatment of cells with DNA from A29. No colonies were observed in cells treated with wild-type CHO DNA. Similarly, there were 40-fold more Mtx-resistant 3T3 colonies after treatment of cells with A29 DNA than after treatment with wild-type CHO DNA. These data suggest that treatment of Mtx-sensitive cells with A29 DNA resulted in the transfer and expression of a mutant *dhfr* gene, thus rendering these cells insensitive to increased levels of Mtx.

In order to test this hypothesis directly, we demonstrated the presence of the hamster *dhfr* gene in DNA from transformants by using the filter hybridization method of Southern (23). A mouse *dhfr* cDNA clone (pdhfr-21) (22) that shares homology with the hamster *dhfr* gene was used as probe in these experiments. DNAs from A29, from transformants, and from *dhfr*-amplified mouse cells were cleaved with *Hind*III, electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose filters. These filters were hybridized with high-specific activity ³²P-labeled nick translated pdhfr-21 and subjected to autoradiography. This procedure visualizes restriction fragments of genomic DNA homologous to the *dhfr* probe. Prominent bands were observed at 15, 3.5, and 3 kilobases (kb) for *dhfr*-amplified mouse DNA and at 17, 7.9, 3.7, and 1.4 kb for *dhfr*-amplified hamster DNA (Fig. 1). The restriction profiles of these two species were sufficiently different to permit us to detect the hamster gene in the presence of an endogenous mouse gene.

Four Ltk⁻ aprt⁻ cell transformants resistant to Mtx were examined in this way (Fig. 1). In each transformed cell line, we observed the expected profile of bands resulting from cleavage of the endogenous mouse *dhfr* gene although at the decreased

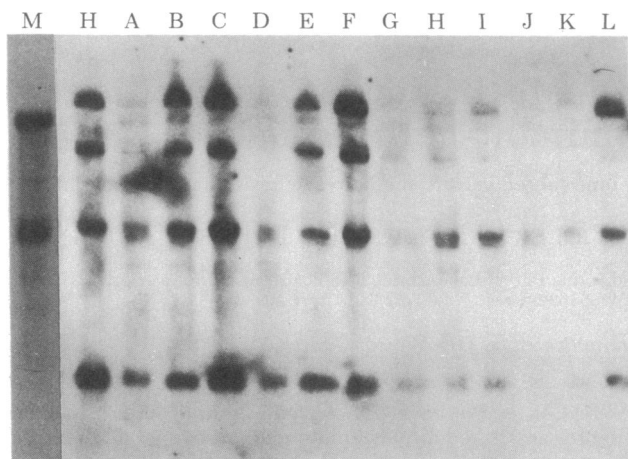


FIG. 1. Chinese hamster *dhfr* sequences are present in mouse cells. Mouse Ltk⁻ aprt⁻ cells were transformed to Mtx resistance and their DNAs were examined for the presence of CHO sequences by molecular hybridization to ³²P-labeled pdhfr-21 DNA. The hybridization profiles of 20 μ g of *Hind*III-cleaved DNA from *dhfr*-amplified mouse (lane M) and the *dhfr*-amplified CHO line A29 (lane H) are shown along with the *Hind*III patterns from four Mtx-resistant cell lines derived after transformation and selection at 0.1 μ g of Mtx per ml (lanes A, D, G, and J). Each of these cell lines was also grown at Mtx levels of 1 μ g/ml (lanes B, E, H, and K) and 10 μ g/ml (lanes C, F, I, and L) and scored for amplification of *dhfr* sequences.

intensity of unamplified *dhfr*. A series of additional bands were observed whose molecular weights were identical to those of restriction endonuclease-cleaved hamster *dhfr* gene. The 17.9-, 7.9-, and 1.4-kb bands observed in hamster DNA are diagnostic for the presence of the hamster *dhfr* gene and were present in all transformants although in disproportionate intensities. In similar studies of NIH 3T3 Mtx-resistant transformants, 12 of 12 contained bands diagnostic of hamster sequences (data not shown).

In initial experiments, we chose the lowest concentration of Mtx (0.1–0.2 μ g/ml) that would decrease survival of the mouse cells to $<10^{-7}$. Previous studies (18) suggested that the presence of a single mutant *dhfr* gene can render cells resistant to this concentration of Mtx. Comparison of the band intensities of the hamster *dhfr* gene fragments of transformed cell DNA with A29 suggest that our transformants contain fewer Mtx-resistant hamster genes than do donor A29 cells.

Amplification of the Transferred *dhfr* Gene. Initial Ltk⁻ aprt⁻ transformants were selected for resistance to relatively low levels of Mtx (0.1 μ g/ml). For each transformant, however, it was possible to select cells resistant to increased levels of Mtx by exposing mass cultures to successively increasing concentrations of this drug. In this manner, we isolated cultures resistant to 40 μ g of Mtx per ml, starting from clones that were initially resistant to 0.1 μ g/ml. We next determined if increased resistance to Mtx in these transformants was associated with amplification of a *dhfr* gene and, if so, whether the endogenous mouse or the newly transferred hamster gene was amplified. DNAs from four independent transformants and their highly resistant derivatives were examined by blot hybridization. In each instance, enhanced resistance to Mtx was accompanied by an apparent increase in the copy number of the hamster gene. This is most readily seen by comparing the intensities of the 1.4-kb band (Fig. 1). In no instance did we detect amplification of the endogenous mouse *dhfr* gene. Lastly, different lines selected at equivalent Mtx concentrations appeared to contain different amounts of the *dhfr* gene.

Table 1. Transformation data

DNA source (CHO cells)	Mtx-resistant	
	colonies, no./total no. dishes	tk ⁺ colonies, no./total no. dishes
A29*	56/5† 161/5†	25/5†
Wild type	0/5† 4/5†	30/5†

Twenty micrograms of DNA was used to transform 10⁶ cells per dish. Mtx concentration was 0.2 μ g/ml.

* CHO Pro⁻ mtx^{R111} (18).

† Ltk⁻ aprt⁻ cells were used as recipients.

‡ NIH 3T3 cells were used as recipients.

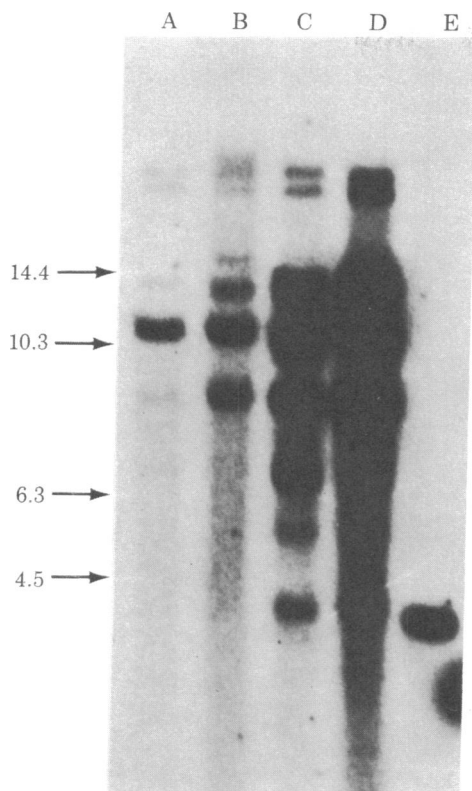


FIG. 2. Presence and amplification of pBR322 sequences in cells transformed with A29-pBR322 ligates. Cells were transformed with the ligation product of *Sal* I-cleaved A29 and pBR322 DNAs. Transformants were selected initially for resistance to 0.1 μ g of Mtx per ml. After cloning, cultures were exposed to increasing concentrations of Mtx, and DNA was extracted, cleaved with *Xba* I, and analyzed for the presence of pBR322 sequences by filter hybridization. Lanes A–D, 10 μ g of DNA from the SS-6 line grown in 0.1, 2, 10, or 40 μ g of Mtx per ml, respectively. Lane E, 50 pg of *Pst* I-cleaved pBR322 DNA.

The *dhfr* Gene as a Generalized Transformation Vector. Selectable genes can be used as vectors for the introduction of other genetic elements into cultured cells. In previous studies, we have demonstrated that cells transformed with the *tk* gene are likely to incorporate other unlinked genes (9). The generality of this approach was tested for the selectable marker, the mutant *dhfr* gene. Total cellular DNA (20 μ g) from A29 was mixed with 1 μ g of *Hind*III-linearized pBR322 DNA. Recipient cells (*Ltk*⁻ *aprt*⁻) were exposed to this DNA mixture and, after 2 weeks, Mtx-resistant colonies were picked. Genomic DNA from transformants was isolated, cleaved with *Hind*III, and analyzed for the presence of pBR322 sequences. Two independent transformants were examined in this way, and multiple copies of pBR322 sequences were present in both cases (data not shown).

An alternate approach to generalized transformation involves ligation of a nonselectable DNA sequence to a selectable gene. Because the mutant *dhfr* gene is a dominant-acting gene conferring drug resistance, it can be used as a vector. Furthermore, it may be possible to amplify any genetic element ligated to this vector by selecting cells resistant to increased levels of Mtx. To explore this possibility, restriction endonucleases that do not destroy the *dhfr* gene of A29 were identified by transformation assay. One such restriction endonuclease, *Sal* I, does not destroy the transformation potential of A29 DNA. *Sal* I-cleaved A29 DNA was therefore ligated to *Sal* I-linearized pBR322. This ligation product was subsequently used in transformation experiments with *Ltk*⁻ *aprt*⁻ cells. Mtx-resistant colonies were

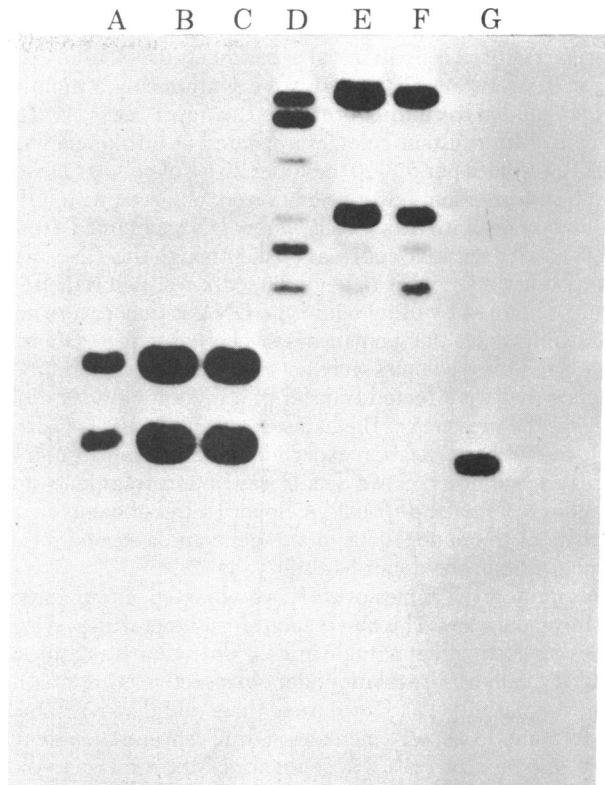


FIG. 3. As in Fig. 2. Lanes A, B, and C, 10 μ g of DNA from cell line SS-1 grown in 0.1, 2, and 40 μ g of Mtx per ml, respectively. Lanes D–F, 10 μ g of DNA from the clone HH-1 grown in 0.1, 2, and 40 μ g of Mtx per ml. Lane G, 50 pg of *Pst* I-cleaved pBR322 DNA.

picked and grown into mass culture in the presence of 0.1 μ g of Mtx per ml. Mass cultures were subsequently exposed to increasing concentrations of Mtx.

DNAs were obtained from mass cultures resistant to 0.1, 2, 10, and 40 μ g of Mtx per ml and the copy number of pBR322 and *dhfr* sequences was determined by blot hybridization. Six independent transformed lines were examined in this fashion. Five of these lines exhibited multiple bands homologous to pBR322 sequences. In four of these transformed clones, at least one of the pBR322-specific bands increased in intensity upon amplification of *dhfr* (Figs. 2 and 3). All pBR322 bands present in transformant SS-6 at 0.1 μ g/ml continued to increase in intensity as cells were selected first at 2 μ g/ml and then at 40 μ g/ml (Fig. 2). We estimate that there was at least a 50-fold increase in copy number for pBR322 sequences in this cell line. In SS-1 (Fig. 3, lanes A, B, and C), two pBR322-specific bands were observed in DNA from cells resistant to 0.1 μ g of Mtx per ml. These bands increased severalfold in intensity in cells resistant to 2 μ g/ml. No further increase in intensity was observed, however, in cells selected for resistance to 40 μ g/ml. In a third cell line, HH-1 (Fig. 3, lanes D, E, and F), two pBR322-specific bands increased in intensity upon amplification, whereas others remained constant or decreased in intensity. Thus, the pattern of amplification of pBR322 sequences we observed in these cells was quite varied. Nevertheless, the mutant *dhfr* gene can be used to introduce and subsequently amplify unselected DNA sequences in cultured animal cells.

DISCUSSION

The potential usefulness of DNA-mediated transformation in the study of eukaryotic gene expression depends to a large extent on its generality. Cellular genes coding for selectable biochemical functions have previously been introduced into mutant cultured cells (10–13). In the present study, we have

transferred a dominant-acting Mtx-resistant *dhfr* gene to wild-type cultured cells. In initial experiments, DNA from A29 cells, a Mtx-resistant CHO derivative synthesizing a mutant *dhfr*, was added to cultures of mouse Ltk⁻ aprt⁻ cells or NIH 3T3 cells. Mtx-resistant colonies appeared at a frequency of about 10 colonies per 5×10^5 cells per 20 μ g of cellular DNA. Upon transformation, fewer colonies were observed with NIH 3T3 and none with Ltk⁻ cells when DNA obtained from wild-type Mtx-sensitive cells was used, although this DNA was a competent donor of the *tk* gene. The Mtx-resistant NIH 3T3 colonies obtained by using wild-type DNA as donor were not significantly above the spontaneous level of resistance (data not shown), and these colonies were not studied further. Definitive evidence that we effected transfer of a mutant hamster *dhfr* gene was the presence of the hamster gene in mouse transformants in blot hybridization experiments. In all transformants examined, we observe two sets of restriction fragments homologous to a mouse *dhfr* cDNA clone: a series of bands characteristic of the endogenous mouse gene and a second series characteristic of the donor hamster gene.

The number of copies of *dhfr* we observed in our initial transformants is low. This observation is consistent with previous studies suggesting that a single mutant *dhfr* gene is capable of rendering cells Mtx-resistant under our selective criterion (0.1 μ g of Mtx per ml) (18). Exposure of these initial Mtx-resistant transformants to stepwise increases in drug concentration results in the selection of cells with enhanced Mtx resistance. Blot analysis indicates that these cells have increased amounts of the newly transferred mutant hamster *dhfr* gene. In no transformants have we observed amplification of the endogenous mouse gene in response to selective pressure. It is likely that a single mutant gene affords significantly greater resistance to a given concentration of Mtx than does a single wild-type gene. If the frequency of amplification is low, we are merely selecting resistant variants arising from the minimal number of amplification events. It is also possible that newly transferred genes undergo amplification more readily than do endogenous genes.

We have explored the use of the mutant *dhfr* gene as a vector for the introduction and amplification of nonselectable genetic elements into cultured cells. Genomic DNA from A29 cells was cleaved with restriction enzymes and ligated to restriction endonuclease-cleaved pBR322 sequences prior to transformation. Most resulting transformants contained multiple pBR322 sequences. In many cases, amplification of *dhfr* genes resulted in the concomitant amplification of pBR322. The patterns of amplification differed among cell lines. In one transformant, all pBR322 sequences amplified with increasing Mtx concentrations. In other transformants, only a subset of the sequences amplified. In yet other lines, sequences appeared to be lost or rearranged. In some lines, amplification proceeded apace with increasing Mtx concentrations up to 40 μ g/ml whereas, in others, amplification ceased at 2 μ g/ml. It appears that the mechanisms of amplification may be quite varied. Whatever mechanisms are responsible for these complex events, it is clear that the *dhfr* amplification unit extends beyond the limits of the *dhfr* gene itself and this can be exploited to control the dosage of any gene introduced into cultured cells.

Although we have succeeded in transferring Mtx resistance to Ltk⁻ aprt⁻ cells and mouse NIH 3T3 cells, we have not as yet had success with various other cell lines. In these instances either the cells were poor recipients for DNA-mediated transformation or they were already substantially resistant to Mtx. We expect that cloning a Mtx-resistant *dhfr* gene will overcome these difficulties and extend the use of this gene as amplifiable vector for wild-type cells.

We thank Drs. Sol Spiegelman and James Watson for their generous support and C. Fraser and C. Lama for their excellent technical assistance. This work was supported by grants from the National Institutes of Health to R.A. and S.S. (CA-16346, CA-23767, CA-17477) and a Cancer Center Grant to James Watson (CA-13106). S.S. was the recipient of a Research Career Development Award from the National Institutes of Health (CA-00492).

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