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Comparison of Phenotypic Expression with Genotypic Transformation by Using Cloned, Selectable Markers

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The frequency of phenotypic expression of the herpes simplex virus type 1 *tk* and *Escherichia coli gpt* genes was compared with the frequency of genotypic transformation after calcium phosphate-mediated DNA transfection of a number of tk^- and $hprt^-$ cell lines. In three of the five lines tested, the frequency of phenotypic expression was at most 10-fold higher than that of genotypic transformation as indicated by frequency of HAT resistance. The remaining two lines showed phenotypic responses which were 50- to 100-fold greater than the genotypic responses. The data indicate that the efficiency of DNA-mediated transformation with some cell lines can be limited by events after the uptake and expression of transfected DNA.

The detection of DNA sequences by virtue of their transformation activity has facilitated the molecular cloning of nonabundant eucaryotic genes (8, 13). However, the usefulness of transformation assays is limited by the fact that they generally are rather inefficient, with frequencies seldom greater than 10^{-5} for single-copy genes. Also, they are useful only for genes for which selective systems exist. As many interesting genes such as those for hormone receptors or genetic disease markers are not readily selectable, methods for detecting transforming sequences independent of selection would be clearly advantageous.

Pellicer et al. (11) described a novel means for detecting the activity of transfected markers independent of selection. These authors measured the production of immune precipitable β_2-microglobulin at various times after DNA transfection of Daudi cells. They were able to detect expression of the polypeptide at levels approaching 0.5% of the wild type. In a separate study Chang et al. (1) detected high frequencies (up to 10%) of transient expression of the human leukemic surface markers My-1 and OK-T3 by L cells after DNA transfection. These observations suggested that monitoring phenotypic expression might be a more sensitive means of detecting DNA sequences than monitoring genotypic transformation and also might be useful for detecting genes for which no selection exists.

To determine whether this methodology is generally useful for detecting transfected markers, we initiated studies to examine the efficiency of phenotypic expression of genes encoding two well-characterized enzyme activities. The two markers chosen for this work were cloned genes which have a selectable phenotype, thereby making it possible to compare simultaneously phenotypic expression and genotypic transformation. In this report, we compare the efficiency of these processes in a number of cell lines differing widely in their susceptibility to DNA-mediated transformation.

MATERIALS AND METHODS

Cell culture. The cell lines used in this study are described in Table 1. All cells were maintained in alpha minimal essential medium supplemented with 10% fetal bovine serum.

Plasmids. Two recombinant plasmids were used. pX1 is an 8-kilobase recombinant plasmid constructed by inserting the 3.5-kilobase *Bam*HI fragment containing the herpes simplex virus type 1 *tk* gene into the unique *Bam*HI site of pBR322 (4). pSV2-*gpt* is a 5.1-kilobase plasmid containing the ampicillin resistance marker and origin of replication derived from pBR322, and the bacterial gene XGPRT, whose expression in mammalian cells is governed by a simian virus 40 promoter and splice sequences (10). Initial samples of both plasmids were furnished by Peter Ray. Subsequently, plasmid DNAs were prepared by standard procedures from *Escherichia coli* strains containing pX1, provided by Alex Joiner, and pSV2, provided by Paul Berg.

Preparation of high-molecular-weight DNA. High-molecular-weight DNA, used as the carrier during transformation experiments, was prepared essentially as described by Pellicer et al. (12), except that the nuclei isolation step was omitted. When tk^- lines were to be transformed with pX1, Ltk^- DNA was used as the carrier. DR31 DNA was used as the carrier with $hprt^-$ lines.

DNA transfer. The protocol used for DNA transfer was essentially that of Wigler et al. (15), as modified by Lewis et al. (6). The indicated amount of circular plasmid DNA was mixed with carrier DNA and then

TABLE 1. Cell lines used

Designation	Parental strain	Genotype	Reference
A9	L929	*hprt*$^-$	7
DR31	CHO-K1	*hprt*$^-$ *aprt*$^-$	1a
Ltk$^-$	L929	*tk*$^-$	5
2F3	CHO-Toronto	*tk*$^-$	Laboratory strain (unpublished)
IT-22	3T3	*tk*$^-$	3

with 2.5 M $CaCl_2$ to obtain a final concentration of 20 μg of DNA per ml and 0.25 M $CaCl_2$. The $CaCl_2$-DNA solution was added dropwise, with vigorous mixing achieved by bubbling air, to an equal volume of twice-concentrated HEPES-buffered saline (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.12 ± 0.05], 250 mM NaCl, 0.5 mM $Na_2HPO_4 \cdot 12H_2O$). The calcium phosphate precipitate was allowed to stand for approximately 30 min before being added to the cells. To measure genotypic transformation, 2 ml of precipitate was added to a 75-cm^2 flask containing 10^6 cells in 20 ml of medium. When phenotypic expression was measured, 60 μl of precipitate was added to each chamber of a four-well Lab-tek dish containing 0.6 ml of medium plus 3×10^4 cells. The cell densities achieved on the 75-cm^2 flasks and the Lab-tek slides were approximately equivalent. Cells were routinely incubated with DNA at 37°C for 24 h, at which time the medium was changed. At 48 h after the addition of DNA, cells were either placed in selective medium to assess genotypic transformation or assayed for phenotypic expression.

Measurement of genotypic transformation. Genotypic transformation by both *tk* and *gpt* genes was measured by determining the frequency of HAT resistance. At 48 h after the addition of DNA, the contents of each flask (0.5×10^7 to 1×10^7 cells) were plated in selective HAT medium (alpha minimal essential medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine, plus 10% dialyzed fetal calf serum). Colonies were allowed to grow undisturbed for 14 days, at which time they were stained

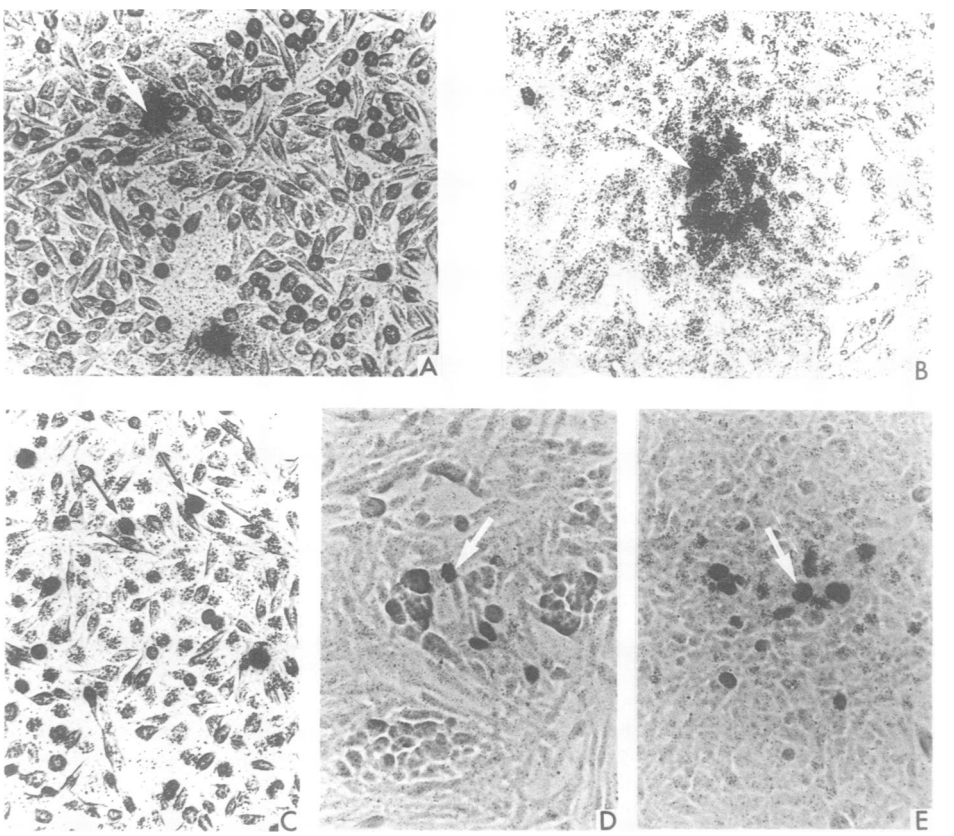

FIG. 1. Phenotypic expression of *gpt* and *tk* genes. The *hprt*$^-$ lines A9 (A) and DR31 (B) were treated with DNA precipitates containing 2.5 μg of pSV2 DNA per 10^6 cells. The *tk*$^-$ lines Ltk$^-$ (C), 2F3 (D), and IT22 (E) were treated in the same fashion, but with 2.5 μg of pX1 DNA per 10^6 cells. Phenotypic expression of both markers was detected as described in the text. After autoradiography, the cells were examined at low power and photographed unstained to show individual cell morphology. Contrast between labeled and unlabeled cells was improved by first wetting the slides with isopropanol to reduce cellular refractility. Arrows indicate position of labeled cells.

with methylene blue and scored. The total number of colonies formed is expressed as a fraction of the 10^6 cells inoculated per flask at the beginning of the experiment.

Measurement of phenotypic expression. Cells grown in four-well Lab-tek chambers were incubated for 24 h with either [^{3}H]thymidine (New England Nuclear Corp.; 25 μCi/ml, 20 Ci/mmol), when *tk*$^-$ cells were used as recipients, or [^{3}H]hypoxanthine (New England Nuclear; 25 μCi/ml, 10 Ci/mmol) when *hprt*$^-$ lines were used. Alpha minimal essential medium supplemented with 10% dialyzed fetal bovine serum was used for labeling. After a 24-h incubation period at 37°C, the monolayers were washed thoroughly with phosphate-buffered saline, fixed with 3.7% Formalin in phosphate-buffered saline, and washed again with phosphate-buffered saline. Slides were then placed briefly in cold 5% trichloroacetic acid, washed with trichloroacetic acid and water, and finally dried from methanol. Autoradiography was performed with Kodak NTB2 emulsion and an exposure time of 2 to 3 days. The number of isotopically labeled cells per well was determined as described above and is expressed as a fraction of the 3×10^4 cells inoculated per well at the start of the experiment.

RESULTS

Phenotypic expression of the herpes simplex virus *tk* and *E. coli gpt* genes, after their introduction into appropriate *tk*$^-$ or *hprt*$^-$ recipients, was easily monitored by the autoradiographic assay described above. As shown in Fig. 1, cells expressing *tk* or *gpt* incorporated acid-insoluble [^{3}H]thymidine or [^{3}H]hypoxanthine and could be distinguished by autoradiography. Phenotypic expression of the *tk* gene was observed in three different *tk*$^-$ lines, two of murine origin (Ltk$^-$ and IT-22) and one of Chinese hamster origin (2F3). Likewise, the phenotypic expression of *gpt* was observed in *hprt*$^-$ lines of murine (A9) and Chinese hamster (DR31) origin.

With some lines, most notably DR31, cells which took up the isotope tended to occur in clusters. The clusters most likely resulted from the radial diffusion of labeled metabolites from a single *gpt*-expressing cell to its neighbors during the lengthy labeling period, because when cells were trypsinized and replated immediately before the initiation of the labeling period, clusters still were evident. (It is well known that nucleotide products of the *hprt* and *tk* loci are transferred between cells grown at high density; see reference 2.) When scoring the frequency of phenotypic expression, any group of contiguous, heavily labeled cells was considered to be a single transfection event. The IT22 and 2F3 lines were also affected in this fashion, but less so.

The time course of phenotypic expression of the *tk* locus with Ltk$^-$ cells and of the *gpt* locus with A9 cells is shown in Table 2. A substantial fraction of the cells expressed *tk* or *gpt* in the 24-h period after removal of the DNA precipitate. Thereafter, the number of cells expressing either marker continued to increase, but at a reduced rate, until the cells became totally confluent (between 96 and 120 h after DNA addition). Previous work from this laboratory (6) established that the optimal time for imposing selective conditions when obtaining genotypic transformants was approximately 48 h after DNA addition. We routinely employed this condition to obtain stable transformants, and for comparative purposes we decided to measure phenotypic expression after the same expression time.

Typical dose-response curves for the phenotypic expression of *tk* and *gpt* in different cell lines are shown in Fig. 2. For all of the cell lines tested, the frequency of both phenotypic expression and genotypic transformation for both markers increased in a manner roughly proportional to the amount of plasmid DNA added. The frequency of phenotypic expression of *tk* and *gpt* was consistently higher than the corresponding frequency of genotypic transformation at a given plasmid DNA concentration. The CHO *tk*$^-$ line (2F3) showed the greatest difference (ca. 60-fold) between phenotypic expression and genotypic transformation.

The frequency of phenotypic expression for both markers was higher in the L cell lines (Ltk$^-$ and A9), but did not vary much more than 20-fold between any *tk*$^-$ or *hprt*$^-$ lines tested. The *tk*$^-$ lines IT-22 and 2F3 showed nearly identical responses for phenotypic expression, but differed 10- to 50-fold in the frequency of genotypic transformation. A greater than 100-fold differ-

TABLE 2. Time course of phenotypic expression of *tk* and *gpt* after DNA transfection[a]

Marker	Labeling period (h after DNA addition)	Frequency of phenotypic expression ($\times 10^2$)	
		Without plasmid	With plasmid
tk	24–48	0.007	0.18
	48–72	0.007	0.26
	72–96	0.003	0.32
	96–120	0.03	0.42
gpt	24–48	0.003	0.74
	48–72	0.003	1.2
	72–96	<0.003	2.1
	96–120	0.003	2.1

[a] DNA precipitates with or without 0.1 μg of pX1 or 0.7 μg of pSV2 per 10^6 cells were added to Ltk$^-$ or A9 cells, respectively. After 24 h the precipitate was removed, fresh medium was added, and incubation at 37°C was continued. At the indicated times, [^{3}H]thymidine or [^{3}H]hypoxanthine was added, and the frequency of phenotypic expression of the appropriate marker during a subsequent 24-h period was determined as described in the text. The preparation of pX1 used in this experiment had less transforming activity than the one used in the other experiments reported in this paper.

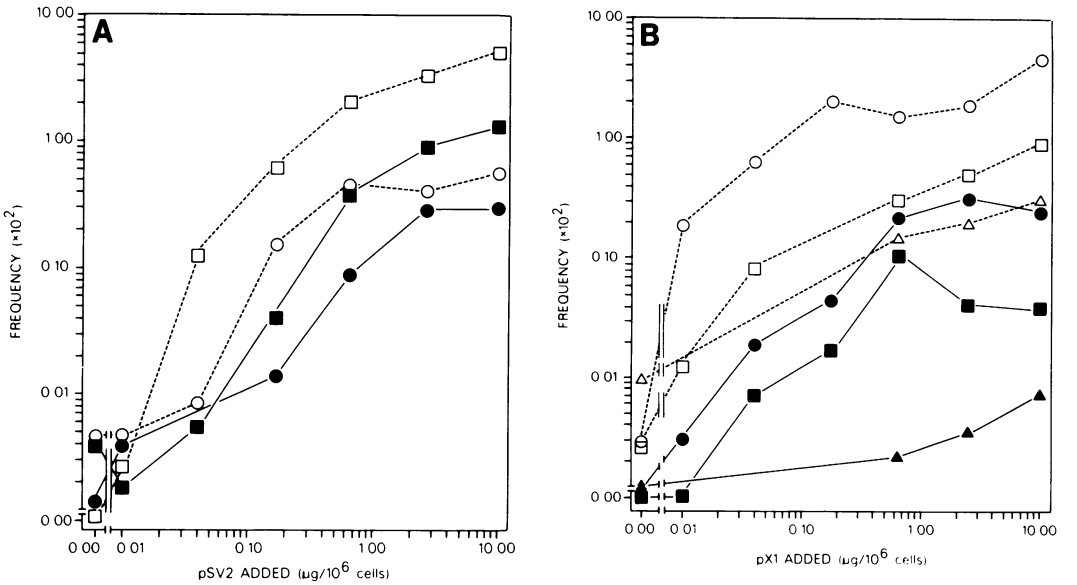

FIG. 2. Dose-response curves for phenotypic expression and genotypic transformation with *hprt*⁻ and *tk*⁻ cell lines. Phenotypic expression (-----) and genotypic transformation (——) were assessed as described in the text. (A) The *hprt*⁻ lines DR31 (○, ●) and A9 (□, ■) were treated with precipitates containing the indicated amounts of pSV2. (B) The *tk*⁻ lines Ltk⁻ (○, ●), 2F3 (△, ▲) and IT22 (□, ■) were treated with precipitates containing the indicated amounts of pX1.

ence was observed in the frequency of genotypic transformation between the 2F3 line and the Ltk⁻ line. The relative advantage of using L cells rather than CHO cells as recipients for DNA transfer was observed previously for the transfer of single-copy genes from genomic DNA (14).

The frequency of phenotypic expression in Ltk⁻ cells at a given concentration of plasmid DNA could be increased slightly (ca. 30%) when pX1 was first linearized by cleavage at the unique *Hin*dIII site (4) (data not shown). The genotypic transformation of Ltk⁻ cells at a given plasmid concentration was approximately 4- to 10-fold higher when linearized plasmid DNA was used for transfection.

DISCUSSION

The present work was initiated to determine whether the high frequency of phenotypic expression observed by Pellicer et al. (11) and Chang et al. (1) could be generalized to other markers. Our data suggest that this is unlikely. To achieve the level of phenotypic expression reported by Pellicer et al. (i.e., 0.5%, with genomic DNA used as the donor) with Ltk⁻, approximately 40 ng of pX1 must be added per 10^6 cells, a level corresponding to the addition of approximately 5,000 copies of *tk* per cell. In contrast, in a standard DNA transfection assay in which approximately 30 μg of genomic DNA is added to 10^6 cells, only about five copies of a single-copy gene are added per cell.

It was of interest to determine whether unique genes such as *tk* and *hprt* could indeed be detected by phenotypic expression. In preliminary experiments, we were unable to measure greater than background frequencies of phenotypic expression of *tk* or *hprt* after transfection of Ltk⁻ or DR31 cells with wild-type *tk*⁺ or *hprt*⁺ genomic DNA, respectively. We also were unable to detect phenotypic expression of *gpt* in DR31 cells after transfection with DR31 DNA containing multiple integrated copies of the marker (M. Breitman and L. Tsui, unpublished data).

It is not clear how the sensitivity of phenotypic expression assays might be increased to allow the detection of unique genes like *tk* and *hprt*. The amount of pX1 which produces an acceptably high signal with Ltk⁻ used as a recipient (i.e., 5 to 10 times the background frequency of 1.5×10^{-4} to 3×10^{-4}) is 0.25 to 0.5 ng per 10^6 cells. This corresponds to approximately 30 to 60 copies of the *tk* gene added per cell. This gene dosage might be achieved simply by increasing the amount of genomic DNA added during a transfection assay. In practical terms, however, DNA concentrations can only be increased to approximately 100 μg of DNA per 10^6 cells, an increment not likely to represent a major improvement. The frequencies we observed for phenotypic expression are in good agreement with those reported by Milman and Herzberg (9), who used a different transfection procedure.

The difference between our results and those

of Pellicer et al. (11) and Chang et al. (1) may reflect an intrinsic difference between the *tk* and *gpt* genes, both of which are required for DNA synthesis, and genes coding for more specialized or differentiated products such as β_2-microglobulin or the MY-1 or OK-T3 antigens. Genes responsible for cell cycle-specific functions such as DNA synthesis normally are expressed in a complex and highly controlled fashion. It is possible that the requirements for proper regulation are too stringent to allow the efficient phenotypic expression of *tk* or *gpt*. Perhaps expression of the differentiated products is under less stringent control and hence is more efficient. The assumption by Pellicer et al. of wild-type levels of β_2-microglobulin expression may also be incorrect. If cells expressing exogenously added β_2-microglobulin do so at higher than wild-type levels, the actual frequency of cells producing the product in their experiments may have been considerably less than 0.5%. It is noteworthy that in our experiments the intensity of labeling of L cells phenotypically expressing *tk* or *gpt* was similar to that observed with wild-type cells (tk^+ or $hprt^+$).

Our data shed some light on factors governing the efficiency of DNA-mediated transformation of mammalian cells. In both $hprt^-$ and one of the tk^- lines (IT-22) tested, the frequency of phenotypic expression of *tk* or *gpt* genes at the time of initiation of selection (i.e., at 48 h after DNA addition) was at most 10-fold higher than the frequency of genotypic transformation. The Ltk^- and 2F3 lines, however, gave frequencies of phenotypic expression 50- to 100-fold higher than those for HAT resistance, suggesting that events subsequent to the uptake and expression of exogenous DNA sequences also can limit the efficiency of genotypic transformation. It is also of interest that whereas all of the tk^- lines tested showed phenotypic responses which differed by little more than 10-fold, the 2F3 line gave frequencies of HAT resistance 100-fold lower than the Ltk^- line. The relatively poor ability of CHO cell lines to serve as recipients for DNA transfer (6) may result from the inability of these lines to maintain, rather than express, transfected DNA sequences.

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