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Transformation of the Gene for Hypoxanthine Phosphoribosyltransferase

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Abstract—*Purified DNA from wild-type Chinese ovary (CHO) cells has been used to transform three hypoxanthine phosphoribosyltransferase (HPRT) deficient murine cell mutants to the enzyme positive state. Transformants appeared at an overall frequency of 5×10^{-8} colonies/treated cell and expressed CHO HPRT activity as determined by electrophoresis. One gene recipient, B21, was a newly isolated mutant of LMTK$^-$ deficient in both HPRT and thymidine kinase (TK) activities. Transformation of B21 to HPRT$^+$ occurred at 1/5 the frequency of transformation to TK$^+$; the latter was, in turn, an order of magnitude lower than that found in the parental LMTK$^-$ cells, 3×10^{-6}. Thus both clonal and marker-specific factors play a role in determining transformability. The specific activity of HPRT in transformant extracts ranged from 0.5 to 5 times the CHO level. The rate of loss of the transformant HPRT$^+$ phenotype, as measured by fluctuation analysis, was 10^{-4}/cell/generation. While this value indicates stability compared to many gene transferents, it is much greater than the spontaneous mutation rate at the indigenous locus. The ability to transfer the gene for HPRT into cultured mammalian cells may prove useful for mutational and genetic mapping studies in this well-studied system.*

INTRODUCTION

The transfer of active cellular genes by treatment of cultured mammalian cells with purified DNA (transformation) has recently been demonstrated. The initial experiments involved the transfer of a viral gene, that specifying the thymidine kinase (TK, EC 2.7.1.75) of herpes simplex virus (1–4). This was soon followed by the extension of this phenomenon to cellular *tk* (5) and then to the genes specifying adenine phosphoribosyltransferase (5) and dihydrofolate reductase (6). From this limited number of examples, it

appears that transformation may have general application as a method of gene transfer in cultured cells of higher organisms.

Transformation can be used to detect the presence of specific genes in a preparation of DNA. This bioassay could be employed to monitor the purification of a particular gene by a combination of biochemical and cloning techniques. Until now, gene cloning has been limited to random fragments of genomic DNA or to those genes for which mRNA or cDNA probes have been available. The latter class is in turn limited to loci coding for an abundant gene product. The transformation bioassay does not depend on the abundance of a gene product; rather it depends on the availability of an appropriate selective system. Most loci that have been subjected to somatic cell genetic analysis encode nonabundant but selectable products. Transformation offers a possible approach to the cloning of such genes.

One locus of considerable interest is that governing the enzyme hypoxanthine phosphoribosyltransferase (HPRT, EC 2.4.2.8). Powerful methods have been developed for the selection of forward and reverse mutants at this locus. The X-linkage of the *hprt* gene facilitates the isolation of enzyme deficient mutants, since only one copy of each X-linked gene is functional in most mammalian cells (7). Consequently a large number of well characterized *hprt* mutants exists (for review see 8).

Structural analysis of the effects of mutation at the *hprt* locus would be aided by the availability of cloned DNA sequences representing the *hprt* wild-type and mutant genes. Such cloned sequences might also be useful for the study of X-inactivation at this locus. Transformation of the *hrpt* gene represents one approach to the cloning of this sequence. For these reasons we undertook the development of a transformation system for *hprt* in cultured mouse cells.

While this work was in progress, Willecke and his colleagues (9) described the isolation of a clone of mouse cells with the characteristics of an intraspecific *hprt* transformant. We report here the interspecific transformation of three HPRT-deficient mouse cell lines using purified Chinese hamster DNA. The HPRT-positive clones isolated arise at a low frequency, but exhibit the enzymatic and stability characteristics expected of transformants.

MATERIALS AND METHODS

Cells. The mouse L-cell derivatives LMTK$^-$ (10), lacking TK activity, and A9 (11), lacking HPRT activity were provided by S. Silverstein and S. Shin, respectively. An HPRT-deficient derivative of mouse 3T6 cells, 3T6TG8 (12), was provided by C. Basilico, and human KB (13) cells by G. Zubay. The K1 clone of CHO cells (14) was used.

Cell Culture. Cells were routinely grown as monolayers at 37° in 5%

CO_2 in a mixture of F12 and Dulbecco's modified Eagle's medium (15) with a final glucose concentration of 4 g/liter and lacking hypoxanthine and thymidine. This growth medium was supplemented with 10% (v/v) fetal calf serum.

Selective media were formulated with the following modifications. Selection for the $HPRT^+$ phenotype was carried out in either HAT medium (100 μM hypoxanthine, 0.7 μM amethopterin, and 15 μM thymidine) or HAS medium (30 μM hypoxanthine, 20 μM azaserine, and 3 μM thymidine). It was necessary to use HAS medium for selections using derivatives of $LMTK^-$, since these cells cannot grow in HAT medium due to their TK deficiency. Selection for TK^+ was accomplished either in HAT or in AAT. The latter is identical to HAT except for the substitution of 50 μM adenine for hypoxanthine. AAT was used in all TK selections involving $HPRT^-$ cells. Adenine phosphoribosyltransferase is still present in these cells and allows the salvage of adenine as the sole purine source when de novo synthesis is blocked with amethopterin.

Mutants of $LMTK^-$ cells deficient in HPRT activity were selected on the basis of their resistance to purine analogs as described by Sharp et al. (16). Cells were mutagenized with ethyl methanesulfonate (360 μg/ml) and allowed 6 days' growth (17) before being challenged in medium containing 8-azaguanine and 6-thioguanine (3 μg/ml each). Fourteen million mutagenized cells gave rise to 4 colonies that were recloned in selective medium. No drug-resistant colonies were obtained from 6×10^6 nonmutagenized cells.

DNA Purification. Late log-phase suspension (spinner) cultures of CHO or KB cells, or nearly confluent monolayers of A9 cells, were harvested and DNA was extracted and purified as described by Pellicer et al. (18). The preparations were of high molecular weight as judged by slower migration in 0.4% agarose gels than a Bam H1-linearized plasmid pLC7-21-$ColE_1$ marker (15.7 kb, kindly provided by C. Squires).

Transformation Procedure. Most experiments were carried out according to Wigler et al. (6), with the following modifications: Recipient cells, $0.5–1.0 \times 10^6$/100-mm dish, were preplated in nonselective growth medium 14–20 h before the addition of DNA. Four hours before DNA addition this medium was replaced with 9 ml of Dulbecco's modified Eagle's medium containing 0.4% glucose, 10 mM HEPES (pH 7.1), and supplemented with 5% calf serum (exposure medium).

DNA was coprecipitated with calcium phosphate (6) in siliconized glass tubes in a final volume of 1–3 ml at 30 μg DNA/ml. Double-strength HEPES-buffered saline stocks were periodically tested for retention of pH 7.05–7.10. Additions involving DNA were done with disposable plastic 1-ml or 5-ml pipettes. Dispersion of the DNA during initial mixing was accomplished by a combination of gentle hand agitation and bubbling air through

the delivery pipette with a Pipet-aid (Drummond). Precipitated DNA (1 ml) was added directly to the 9 ml of exposure medium. Cells were exposed to DNA for 3.5 h at 37°, then were shifted to room temperature. After 30 min, 2.5 ml of 30% (v/v) dimethyl sulfoxide [final concentration 6%, a modification of the procedure of Miller and Ruddle (19)] in exposure medium was added and incubation at room temperature continued for 30 min. The medium was then replaced with growth medium and the dishes returned to the 37° incubator. An atmosphere of 5% CO_2 was maintained with the use of a gassed sealed box during room temperature manipulations. After a 24- to 28-h expression period in nonselective medium, cells were challenged in selective medium either by direct replacement of the medium, or by trypsinization and replating (see Table 1). Selective medium was renewed twice a week. After 2–3 weeks' growth, transformant or revertant colonies were recloned in selective medium.

Enzyme Measurements. Cell monolayers were harvested by trypsinization, washed twice with PBS, and stored as frozen cell pellets at −20°. For enzyme extraction, cell pellets were thawed in 20 mM potassium phosphate, pH 7.0, containing 0.5% Triton X-100, at a concentration of about 1.5×10^8 cells/ml. After centrifugation at 12,000 *g* for 25 min, the resulting supernatant solution was used as a crude cytoplasmic extract.

HPRT was assayed by the conversion of [^{14}C]hypoxanthine to [^{14}C]inosine monophosphate under reaction conditions previously described (20). Incubations were carried out in duplicate at 37° in 50 μl total volume and were stopped by the addition of 5 μl of 100 mM EDTA and chilling in ice. Samples of 22 μl were spotted onto 22 × 22-mm squares on a plastic-backed polyethyleneimine cellulose thin-layer chromatography sheet (Bakerflex PEI-F). The sheet was air dried, washed 5 min with 0.5 mM ammonium formate, pH 7; washed 15 min under running tap water, and dried under an infrared lamp. The squares were then cut out and counted in 5 ml of toluene-based scintillation fluid.

Electrophoresis. A combination of previously described methods (21–23) yielded improved resolution of mouse and Chinese hamster HPRT activities in vertical slab polyacrylamide gels. The running gel contained 7.5% acrylamide, 0.188% *N,N'*-methylene-bis-acrylamide (bis), 0.06% *N,N,N',N'*-tetramethylethylenediamine (TEMED), 15 mM NaCl, 8% (w/v) sucrose, and 15 mM glycylglycine, pH 8.7. The spacer gel comprised 6% of the running gel and contained 4% acrylamide, 1% bis, 0.06% TEMED, and 15 mM glycylglycine, pH 7.0. Polymerization was initiated by the addition of ammonium persulfate to a final concentration of 0.7 mg/ml. The reservoir buffer contained 5 mM glyclyglycine and 4 mM glycine, pH 8.7. After 20 min preelectrophoresis at 3 mA/cm^2 at 4°, samples containing 10–50 μg of protein were underlaid and electrophoresis carried out overnight at 4° at

70–80 V for a 14-cm gel. Electrophoresis was terminated when a bromophenol blue–bovine serum albumin marker in a separate channel had run at least 8 cm. Zones of HPRT activity were then detected using the radioactive assay method previously described (20).

Estimation of HPRT$^+$ Segregation Rates by Fluctuation Analysis. Each clone tested was grown in mass culture in HAS medium for at least a week before the analysis. Twenty-four replicate cultures were then started for each clone, by inoculating 25 cells per culture in 24-well culture dishes (Linbro). Ten to 15 colonies formed in each well; these were dispersed after 1 week and allowed to grow to near confluence ($4.5–7 \times 10^5$ cells/culture, 15–16 generations). Samples of 5×10^4 cells/culture (TB23 and TA28) or the total cell yield, 7×10^5 cells/culture (RD91) were then plated in 60-mm dishes in medium containing 25 μM 6-thioguanine. Reconstruction experiments indicated that the high cell density used in the case of RD91 selections does not affect the appearance of 6-thioguanine-resistant segregants. Selection dishes were stained with crystal violet after 10 days.

Segregation rates were calculated using the mean method (equation 8) of Luria and Delbrück (24) and the median method of Lea and Coulson (25) based on the estimated number of colonies per culture. Due to an expected delay in expression of an HPRT$^-$ phenotype (17) as 6-thioguanine resistance, segregation events occurring in the last several days of nonselective growth are probably not represented as colonies. The actual rates of genetic events may be somewhat higher than those calculated in Table 3 as a consequence of this expression lag.

RESULTS

Transformation for Thymidine Kinase (TK). Exposure of LMTK$^-$ cells to DNA from hamster (CHO), human (KB), or mouse (A9) cells led to the appearance of colonies with a TK$^+$ phenotype (HAT$^+$ or AAT$^+$) in more than half (67/122) of the treated dishes representing an overall frequency of 3.7×10^{-6} (Table 1, top). Control dishes, treated either with salmon sperm or bacterial DNA, or with calcium phosphate precipitates containing no DNA, only rarely yielded a colony (4×10^{-8}). The few colonies observed may in fact represent spontaneous mutation to amethopterin resistance, as revertants to TK$^+$ have never been found using LMTK$^-$ (5), and the one colony tested from these experiments was indeed resistant to amethopterin (0.67 μM) and to 5-bromodeoxyuridine (0.1 mM). These results with *tk* transformation are in essential agreement with those previously reports by Wigler et al. (5) and attest to the effectiveness of the DNA preparations and transformation methods used.

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