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Genetics

DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells

(biochemical transformation/unique genes/mutant cells)

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ABSTRACT **In this report, we demonstrate the feasibility of transforming mouse cells deficient in adenine phosphoribosyltransferase (aprt; AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) to the aprt$^+$ phenotype by means of DNA-mediated gene transfer. Transformation was effected by using unfractionated high molecular weight genomic DNA from Chinese hamster, human, and mouse cells and restriction endonuclease-digested DNA from rabbit liver. The transformation frequency observed was between 1 and 10 colonies per 10^6 cells per 20 μg of donor DNA. Transformants displayed enzymatic activity that was donor derived as demonstrated by isoelectric focusing of cytoplasmic extracts. These transformants fall into two classes: those that are phenotypically stable when grown in the absence of selective pressure and those that are phenotypically unstable under the same conditions.**

The DNA-mediated transfer of cellular genes, discovered (1) and previously exploited in the prokaryotes, has recently been extended to eukaryotes. Early studies on the transformation** of eukaryotic cells were restricted to viral genes (2, 3). Recently, transformation of yeast spheroplasts with recombinant DNA molecules has permitted the isolation and characterization of genes coding for selectable biochemical markers (4). In our laboratories we have transferred cellular genes from complex vertebrate genomes to cultured mammalian cells. In initial studies, we demonstrated transfer of the thymidine kinase (tk) gene of herpes simplex virus to mutant mouse Ltk$^-$ cells (5). After optimizing the conditions for transformation in this model system, we were able to transfer the cellular tk gene by using unfractionated high molecular weight genomic DNA from various species as donors (6). The transformed cell expressed tk activity encoded by donor DNA.

The potential usefulness of this observation depends to a large extent on its generality. In principle, transformation should be detectable for all genes for which selection conditions are available. In this study, we demonstrate the transfer of the gene coding for adenine phosphoribosyltransferase (aprt; AMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) to mutant cells lacking this enzyme (Ltk$^-$ aprt$^-$). Transformants express aprt activity with the characteristics of the organism from which the transforming DNA was derived. Taking these results together with our previous results, we have demonstrated that DNA-mediated gene transfer in animal cells can provide a bioassay for dominant-acting genes present at concentrations of one part per haploid genome.

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MATERIALS AND METHODS

Cell Culture. Murine Ltk$^-$ aprt$^-$ cells are derivatives of Ltk$^-$ clone 1D cells (7) and were originally isolated and characterized by R. Hughes and P. Plagemann. They were generously provided by R. Hughes. Cells were maintained in growth medium [Dulbecco's modified Eagle's medium containing 10% calf serum (Flow Laboratories, Rockville, MD)] supplemented with diaminopurine at 50 μg/ml. Prior to transformation, cells were washed and grown for three generations in the absence of diaminopurine. HEp-2 (human), HeLa (human), CHO (Chinese hamster ovary), and Ltk$^-$ cells were grown in growth medium. For CHO, medium was supplemented with 3× the usual concentration of nonessential amino acids. LH2b, a derivative of Ltk$^-$ transformed with herpes simplex virus tk DNA, was maintained in growth medium containing hypoxanthine at 15 μg/ml, aminopterin at 0.2 μg/ml, and thymidine at 5.0 μg/ml (HAT) (5). All culture dishes were Nunclon (Vangard International, Neptune, NJ) plastic.

Extraction and Restriction Endonuclease Cleavage of Genomic DNA. High molecular weight DNA was obtained from cultured cells (CHO, LH2b, and HeLa) or from frozen rabbit livers as previously described (6). High molecular weight salmon sperm DNA was obtained from Worthington. Restriction endonucleases were obtained from New England BioLabs. Restriction endonuclease cleavage (*Bam* I, *Hin*dIII, *Kpn* I, and *Xba* I) was performed in buffer containing 50 mM NaCl, 10 mM Tris·HCl, 5 mM $MgCl_2$, 7 mM mercaptoethanol, and bovine serum albumin at 100 μg/ml (pH 7.9). The enzyme-to-DNA ratio was at least two units/μg of DNA, and reaction mixtures were incubated at 37°C for at least 2 hr (one unit is the amount of enzyme that digests 1 μg of DNA in 1 hr). To monitor the completeness of digestion, 1 μl of nick-translated adenovirus-2 [^{32}P]DNA was incubated with 5 μl of reaction volume for at least 2 hr, cleavage products were separated by electrophoresis in 1% agarose gels, and digestion was monitored by exposing the dried gel to Cronex 2DC x-ray film.

Transformation and Selection. The transformation protocol was as described (8) with the following modifications. One day prior to transformation, cells were seeded at 0.7 × 10^6 cells per

Abbreviations: tk, thymidine kinase; aprt, adenine phosphoribosyltransferase; HAT, hypoxanthine/aminopterin/thymidine.

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** We define transformation as a change in the genotype of a recipient cell mediated by the introduction of purified DNA. Transformation can frequently be detected by the stable and heritable change in the phenotype of the recipient cell that results from an alteration in either the biochemical or the morphological properties of the recipient.

dish. The medium was changed 4 hr prior to transformation. Sterile, ethanol-precipitated high molecular weight or restriction endonuclease-cleaved eukaryotic DNA dissolved in 1 mM Tris (pH 7.9)/0.1 mM EDTA was used to prepare DNA/$CaCl_2$, which contains DNA at 40 μg/ml and 250 mM $CaCl_2$ (Mallinkrodt). Twice-concentrated Hepes-buffered saline (2× HBS) was prepared; it contains 280 mM NaCl, 50 mM Hepes, and 1.5 mM sodium phosphate, pH adjusted to 7.10 ± 0.05. DNA/$CaCl_2$ solution was added dropwise to an equal volume of sterile 2× HBS. A 1-ml sterile plastic pipette with a cotton plug was inserted into the mixing tube containing 2× HBS, and bubbles were introduced by blowing while the DNA was being added. The calcium phosphate/DNA precipitate was allowed to form without agitation for 30–45 min at room temperature. The precipitate was then mixed by gentle pipetting with a plastic pipette, and 1 ml of precipitate was added per plate, directly to the 10 ml of growth medium that covered the recipient cells. After 4-hr incubation at 37°C, the medium was replaced and the cells were allowed to incubate for an additional 20 hr. At that time, selective pressure was applied. For tk^+ selection, medium was changed to growth medium containing HAT. For $aprt^+$ selection, cells were trypsinized and replated at lower density (about 0.5×10^6 cells per 10-cm dish) in medium containing 0.05 mM azaserine and 0.1 mM adenine. For both tk^+ and $aprt^+$ selection, selective media were changed the next day, 2 days after that, and subsequently every 3 days for 2–3 weeks while transformant clones arose. Colonies were picked by using cloning cylinders and the remainder of the colonies were scored after formaldehyde fixation and staining with Giemsa. For characterization, clones were grown into mass culture under continued selective pressure. A record was kept of the apparent number of cell doublings for each clone isolated.

Enzyme Assays. Extracts were prepared by resuspending washed cell pellets (approximately 10^7 cells) in 0.1 ml of 0.02 M potassium phosphate, pH 7, containing 0.5% Triton X-100. The supernatant (cytoplasm) obtained after 25 min of 700 × *g* centrifugation was used for the quantitation of enzymatic activity and for electrophoresis. aprt and protein were assayed as previously described (9). Inclusion of 3 mM thymidine triphosphate, an inhibitor of 5′-nucleotidase (10), in the reaction mixture did not increase AMP recovery, indicating that the nucleotidase was not interfering with the measurement of aprt activity. Isoelectric focusing of aprt was carried out essentially as described for hypoxanthine phosphoribosyltransferase (11) with the following exceptions: The polyacrylamide gel contained an Ampholine (LKB) mixture of 0.8% pH 2.5–4, 0.8% pH 4–6, and 0.4% pH 5–7. For assaying enzymatic activity, [2-^{3}H]adenine [0.04 mM, 1 Ci/mmol, New England Nuclear (1 Ci = 3.7×10^{10} becquerels)] was substituted for hypoxanthine.

RESULTS

Transformation to the $aprt^+$ Phenotype. Biochemical transformation occurs with low frequency and is usually detected by the ability of the rare transformed cell to grow under appropriate selective conditions. The development of a transformation system therefore requires a recipient cell that is both competent for transformation and sensitive to selection. In addition, the frequency at which the recipient cell spontaneously reverts to selection resistance must be lower than the frequency of transformation. Previous results indicated that Ltk^- cells (deficient for tk) are competent recipients for cellular DNA and undergo transformation to the tk^+ phenotype at a rate of 1–10 colonies per 10^6 cells per 20 μg of donor DNA (6). The $aprt^-$ variant of Ltk^- cells (Ltk^- $aprt^-$) grows in the presence of diaminopurine. $aprt^+$ cells are selected in media containing azaserine and adenine. Azaserine blocks *de novo* purine biosynthesis, and adenine can be utilized for the synthesis of purine nucleotides only by $aprt^+$ cells. Ltk^- $aprt^-$ cells show a low rate of spontaneous reversion to the $aprt^+$ phenotype as judged by their cloning efficiency in azaserine/adenine (unpublished studies).

Table 1. Gene transfer with total genomic DNA from various species

Donor DNA	Total tk^+ colonies/ total plates	Average/ plate	Total $aprt^+$ colonies/ total plates	Average/ plate
Chinese hamster (CHO cells)	22/5	4.4	20/14	1.4
Human (HeLa cells)	42/4	10.5	95/14	6.8
Mouse (LH2b cells)	100/5	20.0	24/15	1.6
Salmon (testes)	0/5	0.0	0/15	0.0
None	0/5	0.0	0/15	0.0

High molecular weight DNA was prepared and coprecipitated with calcium phosphate; 20 μg of precipitated DNA (in 1 ml) was added to each plate. Transformants were scored for either the tk^+ or $aprt^+$ phenotype after selection.

Ltk^- $aprt^-$ cells are therefore appropriate recipients for the transfer of the aprt gene. High molecular weight DNA was prepared from human, hamster, and wild-type mouse cultured cells. A calcium phosphate/DNA coprecipitate was added to Ltk^- $aprt^-$ cells under a modification of the transformation conditions described by Graham and van der Eb (8). After 24 hr, cells were exposed to the selection media.

Colonies were scored after 2–3 weeks. The results of one experiment are shown in Table 1. The data demonstrate that transformation to the tk^+ and $aprt^+$ phenotypes can be effected with DNA preparations from hamster, human, and mouse. The frequency of transformation in each case was about 10 colonies per 10^6 cells per 20 μg of DNA. No transformants resulted from treatment with salmon DNA and no $aprt^+$ colonies arose in untreated cultures. This is in accord with the observation that the reversion of this line to $aprt^+$ occurs with a frequency of 3×10^{-8} under these conditions (unpublished studies).

Individual transformant colonies were picked, in cloning cylinders, from separate plates to ensure that they represented independent transformation events. These colonies were grown

Table 2. aprt activities of parental and transformant clones

Cell line	Donor DNA	aprt activity, nmol AMP/min per mg
Donors		
HEp-2 (human)	—	2.19
CHO (Chinese hamster)	—	6.52
Ltk^- (mouse)	—	2.09
Recipient		
Ltk^- $aprt^-$	—	<0.005
Transformants		
MA-1	Mouse	1.94
MA-4	Mouse	2.29
HA-1	Human	1.72
HA-4	Human	1.37
CA-3	Chinese hamster	0.86
RA-1*	Rabbit	1.62

* This clone is a mouse cell revertant isolated after presumptive transformation with rabbit liver DNA.

into mass cultures under selective conditions (azaserine/adenine). Cytoplasmic extracts were prepared from individual clones and assayed for aprt activity. As shown in Table 2, Ltk$^-$ aprt$^-$ cytoplasmic extracts had negligible activity. In contrast, all transformants displayed enzymatic activities in the range of wild-type mouse Ltk$^-$ cells.

Electrophoretic Characterization of aprt. Tables 1 and 2 indicate that the appearance of aprt$^+$ colonies was dependent upon the addition of mammalian DNA, suggesting that gene transfer, rather than reversion, had occurred. Direct evidence for gene transfer was obtained by the electrophoretic characterization of the aprt in transformed clones. Isoelectric focusing in polyacrylamide gels clearly separates murine aprt from that of human, rabbit, and Chinese hamster (Fig. 1). Transformed clones derived from treatment with human or Chinese hamster DNA express aprts with isoelectric points characteristic of the donor DNA species. No murine aprt activity is detected in these cells. In contrast, transformants derived after treatment with murine DNA express the murine aprt activity. These results argue strongly that the selected clones do not represent a special class of revertants that reexpress the parental murine aprt rather than the donor DNA aprt. Reversion of the parental Ltk$^-$ aprt$^-$ clone can occur, however, as indicated in one experiment in which rabbit DNA was used as donor. In this case, the aprt$^+$ clone subsequently isolated exhibited *murine* aprt activity.

Stability of the Transformed Phenotype. We next asked if expression of aprt was stable in the absence of selective pressure. Individual transformant clones, grown into mass culture under selective pressure, were subcultured for various times in the absence of selective pressure. The fraction of cells that retained the aprt$^+$ phenotype was determined by measuring cloning efficiencies in selective and nonselective media. The results of these experiments (Table 3) demonstrate that transformants fall into two categories: stable transformants that retain the ability to grow in azaserine/adenine when cultured in the absence of selective pressure (HA-1, HA-4, CA-3); and unstable transformants that do not (CA-1, MA-1, MA-4). The rate of loss of the aprt phenotype can be calculated from these data if one assumes that rate of loss is constant in each generation (see footnote §, Table 3). For the parental Ltk$^-$ (aprt$^+$) cells, a revertant of Ltk$^-$ aprt$^-$ (RA-1), and the stable transformants, the rate of loss was no more than 2% per generation. For the unstable transformants, the calculated rate of loss was as high as 27% per generation (CA-3).

Table 4. Gene transfer with restriction endonuclease-cleaved DNA

Endonuclease	Total aprt$^+$ colonies/ total plates	Average/ plate
*Bam*I	1/9	0.1
*Hin*dIII	104/10	10.4
Kpn I	109/8	13.6

High molecular weight rabbit liver DNA was prepared and cleaved to completion with the indicated restriction endonucleases. Cleaved DNA was used as donor in transformation experiments.

Table 3. Stability of the transformed phenotype

Cell line*	Exp.	Generations in†		Relative cloning efficiency in selective medium‡	Rate of loss of aprt$^+$ phenotype per generation§
		Selective medium	Neutral medium		
Ltk$^-$	1	23	3	0.89	
	2	0	26	0.93	<0.01
CA-1	1	37	2	0.52	
	2	24	16	0.006	0.27
CA-3	1	42	3	0.96	
	2	25	20	0.72	0.02
HA-1	1	54	3	1.00	
	2	47	20	0.84	<0.01
HA-4	1	41	3	0.64	
	2	25	20	0.76	<0.01
MA-1	1	39	4	0.28	
	2	24	26	0.01	0.14
MA-4	1	38	4	0.23	
	2	24	23	0.01	0.15
RA-1	1	47	3	0.88	
	2	31	20	0.96	<0.01

* Ltk$^-$ is the parental line for Ltk$^-$ aprt$^-$. RA-1 is a revertant aprt$^+$ derivative of the latter. All other lines represent aprt$^+$ transformants of Ltk$^-$ aprt$^-$. For derivatives and enzyme characterization see Table 1 and Fig. 1.

† Clones were picked and grown in selective medium for a known number of generations. Cells were then grown in neutral medium for a known number of generations prior to measuring their cloning efficiencies under selective and nonselective conditions.

‡ One hundred cells were plated in triplicate into selective (azaserine/adenine) and nonselective media. The relative cloning efficiency in selective medium is defined as the ratio of the cloning efficiency under selective conditions to the cloning efficiency under nonselective conditions. The latter was generally 50–70%.

§ In these calculations we have assumed that for any given cell line the rate of loss of the aprt phenotype is constant in each generation. With that assumption, the rate of loss per generation may be calculated by using the formula $F_M(1 - X)^{N-M} = F_N$, in which F_M is the relative cloning efficiency in selective medium after M generations in nonselective medium; F_N is similarly defined; and X is the rate of loss per generation.

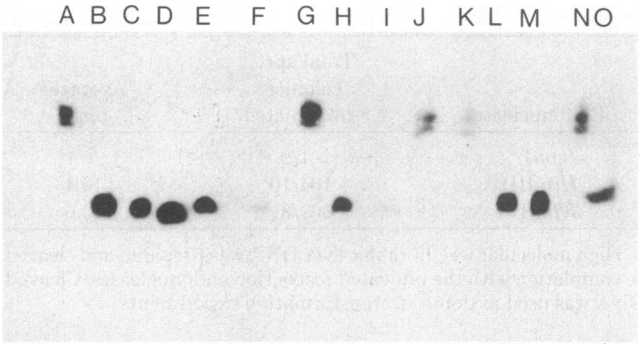

FIG. 1. Isoelectric focusing of aprt. The high-speed supernatants from homogenates of wild-type and mutant cells, transformed cells, and rabbit liver were focused on 4.5% acrylamide gels containing an Ampholine mixture of 0.8% pH 2.5–4/0.8% pH 4–6/0.4% pH 5–7. For development of enzyme activity, [2-^{3}H]adenine was used and the product was blotted onto polyethyleneimine-cellulose and localized by fluorography. A, Ltk$^-$ cell extract; B, rabbit liver homogenate; C, HEp-2 cell extract; D, CHO cell extract; E, extract of Ltk$^-$ aprt$^-$ cells transformed with HeLa cell DNA (HA-1); F, extract from cells transformed with CHO cell DNA (CA-3); G, extract from cells transformed with LH2b cell DNA (MA-1); H, extract from cells transformed with HeLa cell DNA (HA-4); I, extract from cells transformed with CHO cell DNA (CA-1); J, extract from cells transformed with LH2b cell DNA (MA-4); K, extract from an Ltk$^-$ aprt$^-$ revertant (RA-1); L, extract from HEp-2 cells; M, extract from CHO cells; N, extract from Ltk$^-$ cells, O, extract from rabbit liver homogenate.

Transformation with Restriction Endonuclease-Cleaved DNA. It was of interest to determine whether transformation of aprt$^-$ cells could be performed with restriction enzyme fragments of DNA. This requires the use of restriction endonucleases that do not cleave the aprt gene. High molecular weight DNA was therefore prepared from rabbit liver and digested to completion with a variety of restriction enzymes. This restriction endonuclease-cleaved DNA was used in transformation assays. The data are summarized in Table 4. Although *Bam* I destroyed the ability of DNA to transfer aprt, cleavage of rabbit DNA with either *Kpn* I or *Hin*dIII did not result in a reduction in transformation efficiency compared with the efficiency obtained when the transformation was performed with uncleaved DNA. Subsequent experiments indicated that cleavage of rabbit DNA with *Xba* I also does not destroy the aprt gene (data not presented).

DISCUSSION

In this study we have demonstrated the transformation of aprt$^-$ mouse cells to aprt$^+$, using both high molecular weight and restriction endonuclease-cleaved DNA from a variety of species as donors. Stable transformation appears at a frequency 100-fold higher than the spontaneous reversion frequency displayed by this cell line. The frequency of transformation ranges from 1 to 10 colonies per 10^6 cells per 20 μg of donor DNA. These results together with comparisons of the aprt activities from donors and transformants by isoelectric focusing directly demonstrate that the aprt in transformants is derived from the donor DNA and is not due to reversion or reactivation of the murine gene.

The transformants we have isolated fall into two categories: those that retain the ability to express aprt even in the absence of selective pressure and those that do not. In this respect, biochemical transformants obtained after DNA-mediated gene transfer resemble transformants obtained after chromosome-mediated transfer (12, 13). The molecular basis for the observed phenotypic instability is not known at the present time.

The method employed to transfer both the tk and aprt genes can in principle be applied to any gene for which appropriate selective conditions and recipient cells exist. We have, for example, recently succeeded in transferring the gene coding for a methotrexate-resistant folate reductase gene (14) to wild-type cells (unpublished results). The generality of these observations indicates that transformation will facilitate the dissection of complex cellular phenotypes in eukaryotic cells.

To date, the isolation of genes from animal cells has been confined to those loci for which hybridization probes are available. Numerous interesting loci, however, are not transcribed in amounts sufficient to generate hybridization probes. DNA-mediated gene transfer provides a unique bioassay for gene function and a method that could be employed in alternative approaches for gene isolation.

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