Overexpression of an unstably inherited gene in cultured mouse cells

(somatic cell genetics/L cells/chromosome-mediated gene transfer/gene dosage effect/position effect)

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Contributed by Edward A. Adelberg, June 23, 1977

ABSTRACT The specific activity of hypoxanthine-guanine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) is increased up to 58-fold in unstable gene transfers produced by the transfer of cell-free chromosomal material from one mouse L cell line to another; the specific activity of this enzyme returns to normal levels when the transferred gene becomes stabilized. This phenomenon, which is not observed in comparable heterospecific transfers, may be an effect of gene dosage (multiple copies of the transferred genetic fragment in the unstable gene transfers), or it may represent an escape of the unstably inherited gene from the normal regulatory mechanisms of the recipient cell.

Previously we reported the transfer of a gene from one mouse L cell to another, the donor material being a mixture of metaphase chromosomes, nuclear fragments, and nuclei (1). The presence of the transferred gene, hprt, enables progeny cells to synthesize active hypoxanthine-guanosine phosphoribosyltransferase (HPRTase; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) and thus to grow in medium containing hypoxanthine, aminopterin, and thymidine (HAT). The transferred gene was an unusual mutant allele present in a strain (H29) isolated by Sharp et al. (2); it encodes a protein with reduced affinity for the toxic substrate analog, 8-azaguanine (AG). Gene transfers* carrying this allele are able to grow in AG medium and yield extracts containing AG-resistant HPRTase activity. In contrast, all spontaneous HPRT+ revertants of strains carrying our hprt allele possess the wild-type enzyme, rendering them unable to grow in AG medium and yielding extracts containing AG-inhibitable HPRTase activity (1).

Detailed study of the gene transfers showed that (i) the initial transfer event leads to an unstably inherited gene, lost at about 0.1 per cell per generation; (ii) under selective conditions the cells can be carried for at least 200 generations in continuous culture in the unstable state; and (iii) stabilized subclones can be isolated from each unstable subclone. The stabilization process is rare, occurring on the order of $1 \times 10^{-9}$ per cell per generation. Similar phenomena have also been observed in heterologous crosses between mouse and Chinese hamster and between human and rodent cells (1, 3, 4).

In addition, we have found that the transferred gene in our homologous system shows a striking feature not reported in any of the heterologous systems: a pronounced overproduction of gene product (HPRTase) when the gene is unstably inherited, which is abolished when the gene becomes stable. This interrelationship between the heritable state of the hprt locus and the cellular level of HPRTase is the subject of this report. A preliminary report of this work has appeared (5).

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MATERIALS AND METHODS

Cell Lines. The derivation of the cell lines used in these studies is shown in Fig. 1. The original recipient strain, 501-1, is a chloramphenicol-resistant derivative of the HPRT- mouse L cell line, A9 (6); H29, as discussed above, is an L cell line possessing an AG-resistant HPRTase (2). The isolation of the unstable gene transferent, GT427, is described in our previous paper (1); it arose from a culture of 501-1 cells that had been treated with metaphase chromosomes prepared from strain H29.

GT490 is phenotypically identical to 501-1; it is a "cured," ex-gene-transferent derived from GT427 by the spontaneous loss of the unstable hprt allele and selection by growth for several weeks in medium containing 6-thioguanine. (The hprt allele contributed by H29 makes cells sensitive to 6-thioguanine.) Control experiments with GT490 have yielded no revertants to HPRT+ (HAT-resistance).

GT801 and GT802 are independent gene transfers isolated after treatment of $1 \times 10^{9}$ GT490 cells with H29 metaphase chromosomes by the method described previously (1). The donor material was $1 \times 10^{9}$ cell equivalents of chromosomes plus $0.2 \times 10^{9}$ nuclear fragments and nuclei. The recipient cells were partially synchronized (mitotic index = 0.76) by 8.5 hr of treatment with Colcemid (0.1 μg/ml) and selective detachment. The yield of transferent clones was 2 per $10^{7}$ recipient cells.

ULU-1 and ULU-8 are independent fusion hybrids between H29 and 501-1, induced by polyethylene glycol (7) and selected in HAT medium containing chloramphenicol.

Culture Conditions. Cells were grown in plastic flasks (Corning) at 37°C in a humidified atmosphere of 5% CO2/95% air. The medium was α modification of minimum essential medium (Flow Laboratories) supplemented with 9% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The composition of HAT medium and other details have been reported (1).

For determining relative HPRTase levels, the strains to be compared were grown in HAT medium in parallel cultures. H29 (the stable HPRT+ donor parent) was always included and served as a normalization standard for the enzyme levels of the other strains. Equal inocula of each strain were seeded into triplicate 150-cm² flasks and fed simultaneously at intervals of 1 to 2 days. When cells approached confluency, extracts were

Abbreviations: HPRTase, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8); HAT, hypoxanthine/aminopterin/thymidine; AG, 8-azaguanine; G6PDase, glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

* As before, we will use the term "gene transferent" to describe a recipient cell that has taken up and expressed a donor cell's gene. The term "transformant," used in microbial genetics, has other connotations in mammalian cell genetics.
made of all strains simultaneously. The donor parent, H29, generally outgrew the gene transferents and yielded extracts of somewhat higher protein concentration. Stable and unstable gene transferents generally grew at equal rates and gave comparable levels of protein in their extracts.

The presence of HAT in the culture medium for these cells prevented the accumulation of HPRTase-negative ("cured") ex-gene-transferents, which arise when an unstable transferent is cultured in nonselective medium (1). However, it did not exclude the possibility that genetic stabilization of the test population had occurred. This possibility was minimized by starting each experiment with unstable cells frozen at an early passage and was ruled out by testing each thawed population to confirm its genetic instability.

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Preparation of Extracts. Enzyme extracts were prepared from cell monolayers as described (1), with the following modifications: (i) monolayers were prewashed three times with phosphate-buffered saline at 4°C and drained thoroughly after each wash; (ii) except in experiment 1, extraction and dialysis buffers were supplemented with 0.1 mM Na 5-phosphoribose 1-pyrophosphate/10 μM NADP/1.0 mM phenylmethylsulfonyl fluoride/60 μM thymidine; and (iii) dialysis was continued for approximately 24 hr. All strains in a single experiment were carried through the steps of extraction, dialysis, and enzyme and protein assays simultaneously. After dialysis, extracts were stored at 0°C until all assays were completed. The enzyme activities are stable under these conditions for several weeks.

Enzyme Assays. HPRTase was assayed as described (1), with N-2-hydroxyethylpipеразине-N'-2-ethanesulfonic acid hydrochloride (pH 7.0) substituted for Tris-HCl where noted. For each extract, the linearity of activity with dilution was verified experimentally; triplicate assays were performed with and without 2 mM AG.

Glucose-6-phosphate dehydrogenase (G6PDase; D-glucose-6-phosphate:NADP+ 1-oxidoreductase, EC 1.1.1.49) was assayed by the spectrophotometric methods of Marks (8); extracts were diluted to give activities that were linear with both concentration and time. Extracts containing approximately 40 μg of protein per 2.5 ml of reaction mixture were incubated 5 min at 30°C in a recording spectrophotometer. In one experiment (experiment 2, Table 1) both single-substrate and dual-substrate assays were performed; the dual-substrate assays, which correct for the extra reduction of NADP catalyzed by 6-phosphogluconate dehydrogenase [6-phospho-D-glucolate:NAD(P) 2-oxidoreductase, EC 1.1.1.43], showed that

Table 1. Gene expression and stability of inheritance in donor and transferentsa

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>HPRTase b</th>
<th>G6PDase c</th>
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<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
<td>Exp. 1</td>
</tr>
<tr>
<td></td>
<td>Normalized specific activity</td>
<td>AG-resistant activity</td>
<td>Normalized specific activity</td>
</tr>
<tr>
<td>H29</td>
<td>1.0</td>
<td>73%</td>
<td>1.0</td>
</tr>
<tr>
<td>GT427A</td>
<td>Transferent (stable)</td>
<td>ND f</td>
<td>ND f</td>
</tr>
<tr>
<td>GT427C</td>
<td>Transferent (unstable)</td>
<td>58.0</td>
<td>74%</td>
</tr>
<tr>
<td>GT471</td>
<td>Transferent (stable)</td>
<td>2.6</td>
<td>71%</td>
</tr>
<tr>
<td>GT427D</td>
<td>Transferent (stable)</td>
<td>25.0</td>
<td>86%</td>
</tr>
<tr>
<td>GT478</td>
<td>Transferent (stable)</td>
<td>0.6</td>
<td>75%</td>
</tr>
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</table>

a H29 is the stable donor (D-S). Arrows indicate the origin of a stable transferent (T-S) from an unstable transferent (T-U) precursor.

b HPRTase was assayed as described in Materials and Methods. In Exp. 1, 111 mM Tris-HCl pH 7.0 buffer was used; in Exp. 2, 111 mM N-1-hydroxyethylpipеразине-N'-2-ethanesulfonic acid hydrochloride, pH 7.0, was used. AG-resistant activity is the percentage of activity retained when AG was present at 2 mM, relative to simultaneous assays in the absence of AG. For wild-type and revertant cells, the equivalent value is 10–20% resistance in Exp. 1 and 5–10% in Exp. 2 conditions. For determination of the proportion of unstable cells in the population, see Materials and Methods. This is a minimum estimate because loss of the gene was followed for only a limited period (approximately 6 weeks).

c The glucose-6-phosphate dehydrogenase (G6PDase) assay described in Materials and Methods was carried out on the same extracts used for HPRTase assays.

d All enzyme specific activities were calculated on the basis of arbitrary units per microgram of protein and then normalized to the values for H29 extracts processed at the same time. The values of H29 specific activities, when converted to picomoles of product formed per minute per microgram of protein, are (approximately): for Exp. 1, HPRTase 0.03 and G6PDase 70; for Exp. 2, HPRTase 0.09 and G6PDase 100.

e These strains were found, in previous tests, to be stable HPRTa—i.e., their plating efficiencies were not significantly different in medium with and without HAT.

f Not determined.
Table 2. Gene expression and stability of inheritance in donor, transferents, and hybrids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>HPRTasea</th>
<th>G6PDaseb</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Exp. 3</td>
<td>Exp. 4</td>
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<tr>
<td>H29</td>
<td>Donor (stable)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>GT801</td>
<td>Transferent (unstable)</td>
<td>6.8</td>
<td>7.2</td>
</tr>
<tr>
<td>GT802</td>
<td>Transferent (unstable)</td>
<td>14.1</td>
<td>20.5</td>
</tr>
<tr>
<td>H29</td>
<td>$hprt^+$ parent (stable)</td>
<td>1.0c</td>
<td>1.0</td>
</tr>
<tr>
<td>ULU-1</td>
<td>Fusion hybrid (stable)</td>
<td>0.16</td>
<td>0.30</td>
</tr>
<tr>
<td>ULU-8</td>
<td>Fusion hybrid (stable)</td>
<td>0.18</td>
<td>0.27</td>
</tr>
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</table>

a HPRTase assay buffer for Exp. 1 is defined in Table 1. Buffer for Exp. 3 and Exp. 4 was as in Exp. 2 in Table 1. All other details are the same as in Table 1.
b See Table 1, note c.
c See Table 1, note d. The values of H29 specific activities not given are, in picomoles of product formed per minute per microgram of protein (approximately): for Exp. 1, HPRTase 0.1; for Exp. 4, HPRTase 0.15 and G6PDase 90. d Not determined.

26.1% (SD, 6.5%; n = 6) of the NADPH formed in the single-substrate assay was due to the activity of the latter enzyme. This percentage did not vary significantly from strain to strain and was used to correct the values obtained in single-substrate assays in all experiments.

Miscellaneous. Protein in cell extracts was determined as described (1). Before measurement of the absorbance, the samples were recentrifuged after the color had developed, to remove precipitated detergent. Sources not given above or in ref. 1 were: fetal bovine serum, Flow Laboratories and International Biological Laboratories; [5-3H]hypoxanthine (13 Ci/mmol), Schwarz/Mann; remaining biochemicals, Sigma.

RESULTS

The results obtained in two sets of experiments (carried out 6 months apart) are shown in Table 1, in which the specific enzyme activities are normalized with respect to those of the donor strain, H29. The stable gene transferent GT427A had a relative HPRTase specific activity of 1.8, close to that of H29; in contrast, the unstable gene transferents GT427C and GT427D had HPRTase activities ranging from 14 to 58 times the activity of H29. More significantly, the two stable subclones that arose from GT427C and GT427D, respectively, had activities closer to the activity of H29. Thus, the stabilization events were accompanied by a 15- to 28-fold decrease in HPRTase specific activity, which was shown to be the H29 type (AG-resistant) in all of the extracts. As a control, the specific activity of G6PDase was measured and found to be constant in all strains. Like HPRTase, G6PDase is a soluble enzyme determined by an X-linked gene.

To gain more information about the generality of HPRTase overexpression and the instability of the hprt gene in the early stages after its transfer, we carried out similar tests on two independent, unstable gene transferents, GT801 and GT802, whose isolation is described in Materials and Methods. These strains were tested 44 days after gene transfer (experiment 3) and again after 65 additional days in culture (experiment 4). As shown in Table 2, both strains overproduced HPRTase (7- to 20-fold over H29), which is consistent with their genetic instability. The G6PDase controls were again normal.

In order to determine whether the observed overproduction of HPRTase was indeed a consequence of the unstable state of the transferred hprt gene rather than a reflection of some regulatory interaction between the parental genomes, we constructed fusion hybrids between H29 and 501-1 as described in Materials and Methods. Two independent hybrids, ULU-1 and ULU-8, were isolated and tested for HPRTase and G6PDase levels. As shown in Table 2, the HPRTase specific activity of the fusion hybrids was depressed, rather than elevated, relative to that of H29.

Finally, assays were done on mixtures of extracts from stable and unstable transferents. The results (data not shown) indicated that the HPRTase activities were additive, ruling out the presence of an enzyme inhibitor in the extracts from stable cells.

DISCUSSION

The overproduction of HPRTase that we have demonstrated in unstable mouse gene transferents did not occur in an analogous interspecies transfer reported by Wullems et al. (9), in which the human hprt gene was transferred to Chinese hamster cells. In their experiments, four well-characterized stable gene transferents were found to contain 9–30% of the HPRTase specific activity of wild-type hamster (DON) cells; similar values (17–29%) were found for three unstable transferents. The low level of gene expression in both classes of transferents is in contrast to our own results, in which the unstable transferents had 7–58 times the specific activity of the donor strain, and the stable transferents had 0.6–2.6 times the donor specific activity. Moreover, Wullems et al. found a large number of gene transferents that had even lower levels of HPRTase, 1–4% of the control.

Such results raise the possibility that interspecific gene transfer may produce defectively expressed genes, perhaps resulting from regulatory incompatibilities arising by evolutionary divergence. In our experiments, the donor and recipient
cell lines were nearly isogenic, both being derived from Littlefield's stock of the L cell line (2, 10). Thus, whatever the abnormal state of the unstable gene may be (see below), its expression is taking place in a compatible system.

From experiments on gene transfer between cells of different mammalian species, the size of the transferred genetic fragment can be inferred to be small. For example, when the human X-linked marker hprt + is transferred to mouse cells, there is no co-transfer of the other known X-linked markers, g6pd and pgk (4). Furthermore, when the autosomal human marker tk is transferred to mouse cells, the closely linked gene gk is co-transfered only about one-fourth of the time (11). We assume that the fragment that is unstably inherited in our own experiments is similarly small. The modal number of chromosomes in our transferees, both stable and unstable, was not significantly different from that of the recipient strain, 501-1.

What the physical state of the unstable gene might be is not at all clear. Although it might be associated with a recipient cell chromosome, the rapidity with which it is lost under nonselective conditions strongly suggests that it is replicated as an extrachromosomal fragment. There is no evidence concerning the composition and structure of the fragment—e.g., whether it consists of a short length of chromatin, or of plasmid-like DNA. It is also not clear how many copies of the fragment are present in the unstable state; however, multiple copies would be consistent with our observation of overproduction of HPRTase, which would thus be simply explained in terms of gene dosage. The multiple copies could be either independent elements or tandemly linked; the latter model would help to explain the rapid loss of the donor phenotype that we have documented.

The stabilization event is most readily explained in terms of the integration of one copy of the fragment into one of the recipient cell's chromosomes. Three types of experiments performed on hprt transferees after stabilization support this: (i) cosedimentation of hprt (assayed by a second cycle of gene transfer) with bulk metaphase chromosomes (12); (ii) cosedimentation of hprt with a particular size class of metaphase chromosome (13); and (iii) coordinate segregation of a particular chromosome and the HPRT + phenotype after somatic cell hybridization (ref 14; F. H. Ruddle, personal communication). The integration of a single copy of the transferred material, accompanied by the loss of multiple extrachromosomal copies, would be consistent with our observation that stabilization is accompanied by a return to normal levels of HPRTase.

As an alternative explanation for our observations, it may be hypothesized that the rate of expression of the hprt locus is greatly increased when it is carried on an extrachromosomal fragment and that the rate of expression returns to normal when the fragment is integrated—i.e., a position effect. Such changes in regulation could occur in any one of a number of ways, depending on the location and physical state of the extrachromosomal fragment. They could represent escape from a non-specific inhibition of transcription, such as might be imposed by the state of chromatin folding in the intact chromosome, or they could represent escape from a regulation mechanism that is specific for the hprt locus.

It will be of particular interest to discover whether the phenomenon of overexpression of an unstably inherited gene is a general one. If so, unstable gene transferees would be particularly suitable as the starting material for the isolation and purification of various mammalian gene products. They would also make possible the detection of closely linked markers in cases in which two or more overproduced gene products were lost or stabilized concurrently.

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