Amplification of Hypoxanthine-Guanine Phosphoribosyltransferase Genes in Chromosome-Mediated Gene Transferents

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Hypoxanthine-guanine phosphoribosyltransferase (HPRT) enzyme activities may be elevated in genetically unstable chromosome-mediated gene transferents selected for transfer of the HPRT gene. Increased levels of HPRT polypeptides in unstable mouse L cell gene transferents were demonstrated by two-dimensional gel electrophoresis and immunoprecipitation. No additional polypeptides were found to be overexpressed. HPRT mRNA levels were elevated 10- to 15-fold in the unstable gene transferent GT427C. Southern blot hybridization experiments showed that overexpression of HPRT correlated with a 5- to 15-fold amplification of HPRT gene sequences in two unstable cell lines. Stabilized gene transferents displayed reduced HPRT copy numbers. The amplification of HPRT gene sequences in the unstable transferent GT427C was associated with the presence of multiple minute chromosome fragments. An average of 9.6 fragments was found per metaphase, but the variation was considerable, ranging from 0 to 53. We conclude that genomic DNA sequences may be amplified in unstable chromosome-mediated gene transferents and that such amplification may be associated with the occurrence of multiple chromosomal fragments.

The technique of chromosome-mediated gene transfer can be used for transfer of genetic material between mammalian cells in culture. (For a review, see reference 14.) This procedure usually results in the transfer of subchromosomal fragments containing the gene selected, usually hypoxanthine-guanine phosphoribosyltransferase (HPRT) or thymidine kinase. The expression of the transferred genome may be stable in the absence of selection or may be dependent on continuous selective pressure. In stable transferents, the donor genome is associated with recipient cell chromosomes (12). Unstable gene transferents may contain cytologically undetectable subchromosomal fragments and lose the transformed phenotype at a rate of 6 to 10% per day (6, 11). Such unstable gene transferents have been reported to overexpress HPRT (6) or thymidine kinase (11) enzyme activities, whereas stabilized cell lines showed enzyme activities that had returned to normal. A second class of unstable gene transferents has been observed in interspecies combinations. These cells possess larger chromosomal fragments and have a lower level of expression of the selected gene (11). Gene transferents from this group are more stable in the absence of selective pressure than transferents containing smaller fragments, possibly because of the presence of centromeres on the transferred chromosomes. Cotransfer of closely linked genes has been demonstrated in the class of transferents containing larger fragments (12, 29, 30), making chromosome-mediated gene transfer a potentially useful tool in gene mapping (23).

The present study was undertaken to examine the mechanism of enzyme overproduction in unstable chromosome-mediated gene transferent (CMGT) cells. For this purpose, we have examined a series of previously isolated (6) CMGTs that were generated by transferring chromosomal material from one mouse L cell line to another. The cell line used as chromosome donor, H29, carried an altered HPRT that showed a decreased substrate specificity for 8-azaguanine.

These cells, nevertheless, have high levels of HPRT activity and are capable of growth in HAT medium (24). It was then possible to distinguish between gene transferents and revertants by analysis of 8-azaguanine sensitivity of isolated HAT-resistant cells. Some of the isolated gene transferent cell lines were found to be genetically unstable and lost the transferred gene at a rate of ca. 10% per cell generation in the absence of selection. These unstable lines showed an overexpression of HPRT enzyme activities at 10- to 50-fold above the donor cell line and could reproducibly be ranked GT427C > GT427D ~ GT802 > GT801, with respect to their HPRT enzyme activities. The present experiments demonstrated that unstable CMGT cell lines overexpressed HPRT polypeptides and HPRT mRNA. Nucleic acid hybridization studies revealed that HPRT gene sequences were amplified in two unstable CMGT cell lines. Amplification of HPRT genes was found to be associated with the occurrence of multiple minute chromosomal fragments in GT427C cells.

MATERIALS AND METHODS

Cell lines and cell cultures. The origins of the cell lines used in this study have been described previously (5) and are summarized in Table 1. All lines are derivatives of mouse L929 cells and were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Gene transferent cell lines were maintained in HAT (HAT: hypoxanthine-aminopterin-thymidine) medium (26). Unstable lines were never kept in culture for more than 2 months, after which new ampoules were thawed.

Immunoprecipitation and two-dimensional gel electrophoresis. Cells (10^6) were labeled overnight with 30 μ Ci of [35 S]methionine (Amersham Corp., Arlington Heights, Ill. or Searle) per ml in 4 ml of methionine-free medium containing 10% fetal calf serum. Cytoplasmic lysates were prepared by lysing cells in 10 mM Tris-hydrochloride (pH 7.4)-10 mM NaCl-3 mM $MgCl_2$ -0.5% Nonidet P-40 (RSB-NP40), followed by low-speed centrifugation. Supernatants were treated with RNase (50 μ g/ml) at +4°C for 15 min and mixed with lysis buffer (20); urea was added to saturate the samples.

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TABLE 1. CMGT cell lines

Cell line	Type	Marker(s) ^a	HPRT activity ^b
H29	Donor	HPRT ⁺ AzG ^r	1.0
501-1	Recipient	HPRT ⁻	
GT427A	Transferent (stable)	HPRT ⁺ AzG ^r	1.8
GT427C	Transferent (unstable)	HPRT ⁺ AzG ^r	30-58
GT471	Transferent (stable)	HPRT ⁺ AzG ^r	2.0-2.6
GT427D	Transferent (unstable)	HPRT ⁺ AzG ^r	14-25
GT478	Transferent (stable)	HPRT ⁺ AzG ^r	0.5-0.6

^a AzG, 8-azaguanine.^b These values are from reference 5.

Total cellular lysates were prepared as previously described (13). Immunoprecipitation of radiolabeled HPRT was carried out essentially as described (18), with protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Cells were lysed in 1% Triton X-100-1% deoxycholate-0.1% sodium dodecyl sulfate-0.3 M NaCl-50 mM Tris hydrochloride (pH 7.5)-1 mM EDTA. Preadsorption was carried out with Bio-Gel A-1.5m (exclusion limit, 1,500,000 daltons). Antiserum to human erythrocyte HPRT was kindly provided by William Kelley, University of Michigan, Ann Arbor. This antiserum was found to effectively precipitate mouse HPRT (not shown). Cellular lysates and immunopurified HPRT polypeptides were separated by two-dimensional gel electrophoresis (20). pH 3.5-10 Ampholines (LKB, Bromma, Sweden) or pH 3.5-10 and pH 5-7 Ampholines mixed 1:4 were used in the isoelectric focusing gel. In some experiments, non-equilibrium pH gel electrophoresis (NEPHGE) gels (21) containing 3.5-10 Ampholines were used in the first dimension. Second dimension sodium dodecyl sulfate-slab gels were 11.5% acrylamide. Slab gels were stained with Coomassie blue, dried, and exposed to Kodak X-Omat film. Exposure times were calculated from the number of counts per minute applied to the gels or used for immunoprecipitation.

RNA dot blot analysis. Cytoplasmic RNA was isolated by phenol-chloroform extraction from low-speed centrifuge supernatants of cells lysed in RSB-NP40. The integrity of isolated RNA was verified by agarose gel electrophoresis. RNA samples were brought to 1.1 mg/ml and dotted onto nitrocellulose membranes (BA85; Schleicher & Schuell Co., Keene, N.H.) that had been pretreated with 20× SSC (1× SSC: 0.15 M sodium chloride plus 0.015 M sodium citrate). Filters were baked at 80°C and hybridized with ³²P-labeled mouse HPRT cDNA plasmid pHPT-2 (1), kindly provided by Thomas Caskey.

Southern blot hybridization. Cellular DNA was isolated by phenol-chloroform extraction after proteinase K treatment and was digested with *Eco*RI or *Hind*III (New England Biolabs, Beverly, Mass.). Digestions were carried out overnight in the presence of 4 mM spermidine with 3 to 5 U of enzyme per µg of DNA. DNA was separated on 1% agarose gels, denatured, and blotted onto nitrocellulose (25). Filters were hybridized with nick-translated pHPT-2 probe. Nick-translation was carried out by using a kit obtained from Amersham or by using DNA polymerase I (Boehringer Mannheim Corp., New York) in the absence of DNase I. Filters were washed in 0.1× SSC at 60°C, dried, and autoradiographed with X-ray screens. Restriction fragment sizes were determined by mixing ³H-labeled *Hind*III-digested λ DNA (Bethesda Research Laboratories, Rockville, Md.) with restricted cellular DNA. λ DNA was visualized by fluorography of the nitrocellulose blots.

Fluorescence staining of metaphase chromosomes. Metaphase spreads were prepared after exposure of cells to colcemid (0.2 µg/ml) for 6 h. Slides were stained with 4'-6-diamidino-2-phenylindole (DAPI; Boehringer Mannheim) at 0.2 µg/ml or mithramycin (Sigma Chemical Co., St. Louis, Mo.) at 20 µg/ml as described previously (3). Metaphases were examined by fluorescence microscopy and the staining intensity of individual minute fragments was estimated by microfluorimetry (3).

RESULTS

Overexpression of HPRT polypeptides in unstable gene transferents. A series of CMGT mouse cell lines selected for transfer of HPRT genes were examined with respect to their synthesis of cellular polypeptides. The properties of these different lines are summarized in Table 1. Gene transferent cell lines were labeled with [³⁵S]methionine and cellular polypeptides were separated by two-dimensional gel electrophoresis. One polypeptide with an isoelectric point in the basic range and with a molecular weight of 26,000 was found to be synthesized at higher levels in the unstable CMGT cell line GT427C, as compared with its stabilized derivative GT471 (Fig. 1A). The two-dimensional gel migration properties of this polypeptide were those expected from HPRT subunits (16, 17). Immunoprecipitation of radiolabeled cellular lysates with anti-human HPRT antiserum confirmed this polypeptide to be HPRT (Fig. 1B). The H29 mouse cell line, used as donor in the chromosome transfer experiment, displayed one major precipitated polypeptide and a series of minor components, whereas the HPRT⁻ recipient cell line 501-1 did not show any immunoprecipitable HPRT. The unstable CMGT cell line GT427C had increased levels of immunoprecipitable HPRT, whereas its stabilized derivative, GT471, showed lower levels. Another unstable line, GT427D, also showed elevated levels of immunoprecipitable HPRT and its stable derivative GT478 had normal levels (data not shown). Immunoprecipitated HPRT from all CMGT cell lines and H29 donor cells had identical migration behavior on two-dimensional gels as determined by coelectrophoresis of unlabeled L cell cytoplasmic proteins.

Examination of autoradiograms of two-dimensional gels of the unstable CMGT lines GT427C and GT427D, compared with gels of their stable derivatives and the recipient 501-1 cell line, did not reveal any other reproducible differences in the polypeptide pattern other than the 26-kilodalton polypeptide. Polypeptides were separated both by isoelectric focusing (Fig. 1C) or NEPHGE (data not shown) in the first dimension. The pH gradient of isoelectric focusing gels run in the presence of urea is unstable in the basic range (21); therefore, HPRT is not present on the gels in Fig. 1C.

Levels of HPRT mRNA. Total cytoplasmic RNA was extracted from H29, GT427C and GT471 cells. RNA was dotted onto nitrocellulose filters and hybridized with a ³²P-labeled mouse HPRT cDNA plasmid (1). This analysis demonstrated a 10- to 15-fold increase in HPRT mRNA levels in GT427C (Fig. 2). HPRT mRNA from GT427C was subjected to Northern blot hybridization (27) and was found to migrate as H29 HPRT mRNA on agarose gels (data not shown).

Amplification of HPRT gene sequences in unstable CMGT cell lines. DNA was isolated from CMGT cell lines and from the H29 donor cell line. The DNA was digested with *Eco*RI or *Hind*III and subjected to Southern gel blot hybridization (Fig. 3). The HPRT cDNA clone pHPT-2 was used as a hybridization probe. The unstable transferents GT427C and

FIG. 1. Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled cellular polypeptides. (A) Total cytoplasmic extracts from GT427C (upper) or GT471 (lower); only portions of the autoradiograms are shown. Note the increased levels of the 26-kilodalton polypeptide in the unstable transferant GT427C. (B) Immunoprecipitation of HPRT from parental and gene transferant cell lines. Labeled HPRT was immunoprecipitated with an anti-human erythrocyte HPRT serum, mixed with unlabeled L cell cytoplasmic proteins, and separated in two dimensions by using pH 3.5-10 Ampholines in the isoelectric focusing gel. Gels were stained and autoradiographed. Only the relevant portions of the autoradiograms are shown. (C) Total cellular extracts of [^{35}S]methionine-labeled 501-1 cells (upper), GT427C (middle) and GT427D cells (lower) were separated by two-dimensional gel electrophoresis.

GT427D contained *Eco*RI restriction fragments of 10 kilobases (kb), 5.6 kb, and 1.4 kb that hybridized stronger with the HPRT probe than the corresponding fragments in the stable transferants GT427A, GT471 and GT478 and in the donor H29 donor cell line (Fig. 3). A strongly hybridizing 6-kb *Eco*RI fragment in H29 was not found to be amplified. This band was about equally strong in all cell lines in other experiments (Fig. 4 and data not shown). The basis for the variability in the intensity of this band between different experiments is unclear. A doublet band of 3.6 kb was also detected in some experiments (Fig. 4 and data not shown)

but was not found to be amplified in GT427C or GT427D. The 3.6-kb band probably represents pseudogene sequences (see below). DNA digested with *Hind*III displayed a comparable pattern with amplified 13-, 8-, 1.1-, and 1-kb bands and nonamplified 4- and 1.2-kb bands.

The degree of amplification of HPRT gene sequences in unstable gene transferants was quantitated by densitometric scanning of the 10- and 1.4-kb *Eco*RI bands. Compared with the H29 donor, these bands appeared to be 10 to 15 times and 5 times stronger in GT427C and GT427D, respectively. When compared to the recipient 501-1 cell line, these num-

FIG. 2. Dot blot hybridization of total cytoplasmic RNA with ^{32}P -labeled pHPT-2 probe.

bers come to be 15 to 25 times and 8 times, respectively, since H29 appears to have more HPRT hybridizing sequences than 501-1 cells (see below). GT471 and GT478, the stable derivatives of GT427C and GT427D, appeared to contain 2 and 0.8 times the set of H29 HPRT gene sequences. The GT427A cell line that was genetically stable on the first occasion it was tested contained about 1.5 times the H29 set. These numbers are in good agreement with previously reported levels of HPRT enzyme activities in these cell lines (see Table 1). Blot hybridization experiments in which decreasing amounts of GT427C and GT427D DNA were electrophoretically separated and hybridized pointed to ca. 10-fold and 5-fold amplification of HPRT gene sequences, respectively (data not shown).

The organization and abundance of HPRT gene sequences were examined in three different L cell strains (Fig. 4). DNA was digested with *Eco*RI and hybridized with pHPT-2 probe. To obtain improved detection of single-copy DNA sequences, the probe was labeled in the absence of DNase I. Densitometric scanning showed that the 10- and 1.4-kb bands are ca. equal in strength in Ltk⁻ (HPRT⁺) and 501-1 (HPRT⁻) cells, whereas these fragments are somewhat (1.5 to 2 times) stronger in H29 (HPRT⁺, 8-azaguanine-resistant) cells.

A thioguanine-resistant variant of GT427C was isolated. This line displayed 10- and 1.4-kb bands of similar intensity as 501-1 cells (Fig. 4).

Presence of multiple minute fragments in GT427C. Metaphase spreads of the CMGT cell lines GT427C and GT471 were stained with the fluorochrome DAPI and examined by fluorescence microscopy (Fig. 5). Numerous minute fragments were found in GT427C metaphases (Fig. 5A). The distribution of such fragments in 100 metaphase spreads is shown in Fig. 6. Up to 53 fragments were found per metaphase plate; the mean number was 9.6. Most of the fragments appeared as singles (with an average of eight per metaphase), whereas only 1.6 doublets were found per metaphase spread. About one-fifth of the metaphase did not contain a detectable minute fragment. Microfluorimetric measurements indicated a mean staining intensity of an individual fragment of about 2.5% relative to the smallest mouse chromosome. Mithramycin staining resulted in relatively brighter fluorescence suggesting that the fragments consist of guanine-cytosine-rich DNA (28). DNase treatment removed all the fluorescence from both chromosomes and fragments. No minute fragments were detected in GT471 metaphase spreads (Fig. 5B, 100 metaphases examined).

DISCUSSION

The major finding of the present study is that HPRT gene sequences were amplified about 15- and 5-fold in the unstable gene transferents GT427C and GT427D and that amplification was associated with the occurrence of multiple minute chromosome fragments. Two-dimensional gel electrophoresis analysis of unstable CMGT cell lines demonstrated that HPRT polypeptides were overexpressed, corroborating previous findings of increased HPRT enzyme activity in these cells (5). Dot blot hybridization experiments showed that HPRT mRNA levels were elevated 10- to 15-fold in the GT427C line. Measurements of HPRT enzyme activities in the original isolates of GT427C indicated a 30- to 58-fold overexpression. Later measurements of cells from a later passage number have shown a 10- to 20-fold elevation of HPRT activities (data not shown), similar to the levels of HPRT mRNA and gene sequences observed in the present study. There appears, then, to be a good correlation between the level of HPRT expression and the level of HPRT gene amplification, suggesting that the level of HPRT expression is unaffected by the localization of the HPRT gene in minute fragments or in the DNA of the X chromosome.

[^{35}S]methionine-labeled HPRT migrated as a series of spots on two-dimensional gels, similar to the pattern obtained after silver-staining of immunoprecipitated human

FIG. 3. Amplification of HPRT gene sequences in unstable gene transferents. Cellular DNA was digested with *Eco*RI or *Hind*III, electrophoretically separated on 1% agarose gels, and blotted to nitrocellulose. The filter was hybridized with nick-translated pHPT-2 in the presence of 50% formamide and 10% dextran-sulfate at 42°C. A 30- μg amount of DNA was loaded per slot.

erythrocyte HPRT (7, 16). Ghangas and Milman (7) reported that immunoprecipitated HPRT from HeLa cells gave rise to a single spot on two-dimensional gels. Some more acidic polypeptides that were observed in occasional experiments were suggested to be artifacts. Southern blot hybridization experiments demonstrated that three *Eco*RI restriction fragments of 10, 5.6, and 1.4 kb were amplified in GT427C and GT427D, whereas a 6-kb and a 3.6-kb fragment did not appear to undergo amplification. It appears unlikely that only parts of the HPRT gene become amplified since HPRT polypeptides migrating as the wild type on two-dimensional gels and HPRT mRNA of normal size were observed in overexpressing cells. It is more likely that the 6- and 3.6-kb fragments represent non-HPRT gene sequences, possibly pseudogenes or other cross-hybridizing phosphoribosyl-transferase genes (19). Melton and Caskey (15) observed that a 3.5-kb *Eco*RI fragment was not amplified in the mouse neuroblastoma cell line NBR4 and suggested that this sequence represented a pseudogene. The NBR4 cell line has previously been demonstrated to have amplified HPRT gene sequences (1). An X autosome translocation and triplication of the X chromosome has been found in NBR4 cells, but no homogeneously staining region or double minute chromosomes were found. The 6-kb band was found to vary in intensity in different experiments. This was not due to partial restriction enzyme digestions (partial digestions did not result in increases in this band but to hybridizing material on top of the gel). The basis of the variation of the 6-kb band, as well as the nature of this sequence, is unclear.

Staining of GT427C metaphase spreads with the fluorochrome DAPI revealed that these cells contain moderate numbers of minute fragments. These fragments may correspond to double minute chromosomes observed in various tumor cells and drug-resistant cells (for a review, see reference 4). The number of fragments per metaphase varied between 0 and 53. It is possible that some fragments may be lost during the preparation of the spreads, explaining the rather high number of metaphases that appear to lack them.

FIG. 4. HPRT gene sequences in parental L cell lines and in a back-selected gene transferant. Cellular DNA was extracted, digested with *Eco*RI, and subjected to Southern blot hybridization. Hybridization was carried out in the presence of 7% dextran-sulfate at 68°C. A 30- μ g amount of DNA was loaded per slot. TG, Thioguanine.

FIG. 5. Presence of minute fragments in GT427C. Metaphase spreads were stained with DAPI and examined by fluorescence microscopy. (A) GT427C and (B) GT471.

Alternatively, these are cells that have lost the transferred genome. GT427C cells have been shown to lose the transferred HPRT gene at a rate of about 10% per generation in nonselective medium. A subpopulation of cells that lack the transferred genome but survive with decreasing HPRT enzyme levels for a few generations is to be expected even under continuous selection. The estimates of the number of fragments in GT427C may be too low since cells containing many fragments may grow slowly and may be underrepresented in metaphase spreads (see below).

Microfluorimetric measurements of stained single fragments indicated an average size of about 2.5% of the smallest mouse chromosome which would correspond to a size in the order of 500 to 1,000 kb. This is a similar value to the estimated size of minute chromosomes in methotrexate-resistant cells overexpressing dihydrofolate reductase (9). Wahl and co-workers (quoted in reference 4) have demonstrated that amplification of the gene for the multifunctional CAD protein (carbamyl-phosphate synthetase, aspartate transcarbamylase, dihydro-orotase) is accompanied by amplification of rRNA sequences. In the present study, we were unable to detect increased levels of expression of any other polypeptides in addition to HPRT by two-dimensional gel electrophoresis analysis. Previous experiments have shown that the levels of expression of the linked glucose-6-phosphate dehydrogenase gene were normal in GT427C and GT427D (5). Our data suggest that the transferred chromosome fragments contain the HPRT gene but no other protein-coding sequences.

The various stabilized cell lines appeared to have acquired various numbers of HPRT genes as evidenced by differences in hybridization with the HPRT probe and differences in HPRT enzyme activities. These differences may either be due to various numbers of HPRT genes becoming associated with recipient chromosomes or to karyotypic rearrange-

FIG. 6. Distribution of minute fragments in GT427C. The number of DAPI-stained fragments was counted in 100 metaphases. No distinction was made between single and double fragments. Ms, Minute fragments.

ments that have occurred after the initial stabilization event. In this context, it is interesting that the parental L cell lines differed from what might have been expected in terms of their HPRT gene content. It has been suggested that recessive mutations in polyploid cultured cells arise by a mutational event at one allele and segregation of other alleles (2). The two mutant L cell lines 501-1 and H29 may, therefore, be anticipated to contain fewer copies of the HPRT gene than wild-type Ltk⁻ cells. Repetitive hybridization experiments showed that 501-1 cells contain about the same number of HPRT genes as Ltk⁻ cells and that H29 contains more copies of the HPRT gene. Then, at least in mouse L cells, the HPRT gene content may not necessarily reflect the number of copies present after the original mutation or stabilization event but rather karyotypic rearrangements occurring at later stages.

Olsen et al. (22) have studied the size and number of copies of human X chromosome fragments transferred to mouse cells. One transferant contained a large fragment (15% of the X chromosome) in a single copy. This fragment was lost at a rapid rate in the absence of selection. The reason for the discrepancy between this finding and ours is unclear but could possibly be due to the differences in size of the transferred fragments. The observation that more than 50 minute fragments can be found per cell is surprising, considering previous reports that double minute chromosomes confer a selective disadvantage to cells growing in nonselective medium (10). In the present situation, the selective disadvantage of having a moderate to high number of minute fragments appears to be balanced by the advantage of generating HPRT⁻ daughter cells at a lower frequency. It is not likely, however, that HPRT genes are amplified to provide the cells with more HPRT enzyme that would be needed for survival in selective medium. The H29 cell line and the GT471 and GT478 cell lines express a low level of HPRT and grow well in HAT. This is in contrast to the study of Haber and Schimke (8) who have reported amplification of dihydrofolate reductase genes after chromosome-mediated gene transfer. The transferants were selected for overexpression of dihydrofolate reductase in high concentrations of methotrexate in these experiments. The GT427C and GT427D cell lines were found to contain different numbers of HPRT genes. The GT427C cell line has reproducibly shown higher levels of HPRT enzyme activity than other gene

transferants. The basis for this phenomenon, as well as the structure of the transferred genome, remains to be determined.

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