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Transfer of the Human Gene for Hypoxanthine–Guanine Phosphoribosyltransferase via Isolated Human Metaphase Chromosomes into Mouse L-Cells

(human gene map/gene transfer/somatic cell genetics)

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ABSTRACT **We have transferred the human gene for hypoxanthine-guanine phosphoribosyltransferase (HPRT, EC 2.4.2.8; IMP:pyrophosphate phosphoribosyltransferase) via isolated metaphase chromosomes from human HeLa S3 cells into murine A9 cells which lack functional murine HPRT activity, using the technique of McBride and Ozer (*Proc. Nat. Acad. Sci. USA* 70, 1258–1262, 1973). Three transformed clones were isolated which contained human HPRT activity as determined by electrophoretic and immunochemical assays. Twenty human isozymes other than HPRT whose genes have been assigned to 14 human chromosomes were found to be absent in our transformed clones. Moreover, the human isozymes of glucose-6-phosphate dehydrogenase (EC 1.1.1.49; D-glucose 6-phosphate:NADP 1-oxidoreductase) and phosphoglycerate kinase (EC 2.7.2.3; ATP:3-phospho-D-glycerate 1-phosphotransferase), whose genes have been linked with the HPRT gene to the long arm of the human X chromosome, were also absent. On the basis of the known linkage relationships of the three markers, we thereby suggest that the transferred piece of human genetic material is smaller than 20% of the human X chromosome or less than 1% of the human genome. This estimate assumes a normal syntenic relationship for the long arm of the X chromosome in HeLa S3 cells. In agreement with this conclusion, no human chromosomes could be detected in our transformed clones. When grown under nonselective conditions about 3% of the gene transfer cells lost the human HPRT marker per cell generation. Transformants that had lost human HPRT activity were subjected to hypoxanthine–aminopterin–thymidine selection. The frequency of revertants to the HPRT$^+$ phenotype was less than 1×10^{-6}, and two revertants that were obtained possessed the mouse electrophoretic phenotype. These results argue against a stable integration of the human donor genetic material into the mouse recipient genome.**

The discoveries of gene transfer systems in bacteria more than 20 years ago were the starting points for the rapid development of bacterial genetics and molecular biology. Likewise, a general gene transfer system for human or mammalian cells can be expected to considerably speed up the process of human gene mapping and our understanding of regulation of gene activity. In several previous attempts, purified DNA was used for the transfer of genetic information between mammalian cells in culture (1–3). In these experiments, however, it was not possible to distinguish between actual transfer of genetic information and reversion of the recipient genome. In 1973, McBride and Ozer described a system in which this last condition was fulfilled (4, 5), using purified metaphase chromosomes from Chinese hamster cells for the transfer of the gene for hypoxanthine–guanine phosphoribosyltransferase (HPRT) into murine cells. McBride and Ozer showed by electrophoretic and chromatographic methods that after chromosomal gene transfer, Chinese hamster HPRT activity was expressed in three murine clones, thus ruling out a reversion mechanism.

We were interested in using this system for the mapping of human genes. If one can transfer a human chromosome or part of it into mouse cells under conditions where it will be replicated and expressed, one could use the resulting transformed clones for rapid mapping of human genes. In order to take advantage of this situation one must know, however, how much of the donor genetic material is functionally maintained in the recipient cells. Since the human gene map is now relatively well known (6), we decided to transfer human chromosomes into mouse cells. In this paper, we describe the successful transfer of the human HPRT gene into mouse cells. From isozyme analyses of the gene transfer clones we estimate that less than 1% of the human genetic material is transferred and maintained in the recipient cells. A preliminary report of our results was presented at a recent conference (7).

MATERIALS AND METHODS

The following cell lines were used: as reference cells for isozyme analyses, UM 61, WI-L2a-AGC2, and Dean Lazar (all lymphoid lines, obtained from Drs. A. D. Bloom, J. D. Eisenstadt, and J. E. Seegmiller, respectively), CH-V79 (Chinese hamster cell line, obtained from Dr. W. O. McBride), L929 (murine cell line, obtained from the American Type Culture Collection, CCL 1), D 98S (human cell line, obtained from the American Type Culture Collection, CCL 18). HeLa S3 cells (8) were obtained from Dr. B. P. Dorman. A9 is an L-cell derived murine cell line (9) that is defective for hypoxanthine–guanine phosphoribosyltransferase (HPRT, EC 2.4.2.8; IMP:pyrophosphate phosphoribosyltransferase) and adenine phosphoribosyltransferase (APRT, EC 2.4.2.7) and that was extensively used in this laboratory for somatic cell hybridization experiments. The HPRT defect in A9 cells has a reversion rate in the order of 10^{-8} (10) and some evidence has been reported that it consists of a structural gene mutation (11). The HeLa S3 and the A9 cell lines were maintained as monolayer cultures at 37°. HeLa S3 was carried in RPMI 1640 medium (Gibco) with 10% fetal bovine serum (Flow), whereas A9 cells were kept in Dulbecco's modified Eagle's Medium with 10% fetal bovine serum. All media contained penicillin

Abbreviations: HPRT, hypoxanthine–guanine phosphoribosyltransferase; G6PD, glucose-6-phosphate dehydrogenase; PGK, phosphoglycerate kinase; HAT, hypoxanthine–aminopterin–thymidine.

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(50 units/ml) and streptomycin (50 μg/ml). To assure logarithmic growth conditions (21 hr doubling time), the suspension cultures were diluted with fresh medium before they had reached 3×10^5 cells per ml. The suspension cultures were synchronized by thymidine block (2 mM)–release technique (12) and blocked in mitosis by addition of 0.3 μg/ml of Velban (vinblastine sulfate, Eli Lilly) for 4–6 hr. The yield of mitotic cells was 60–80% under these conditions.

Chromosomes for gene transfer experiments were prepared according to McBride and Ozer (5) with some modifications. The mitotically arrested HeLa S3 cells from a synchronized suspension culture were collected by centrifugation at $800 \times g$ and washed once at room temperature in Puck's saline A (13). The cells were resuspended at 0° in a solution of 15 mM Tris·HCl, pH 7.0, and 3 mM $CaCl_2$ to about 2×10^6 cells per ml; then, a Triton X-100 solution was added to give a final concentration of 1%. The cell suspension was homogenized in a tight-fitting Dounce homogenizer (Kontes) until all cells were broken. The homogenate was 2-fold diluted and centrifuged in a swing-out rotor for 7 min at $120 \times g$. The pellet was resuspended in 15 mM Tris·HCl, pH 7.0, 3 mM $CaCl_2$, and centrifuged again for 7 min at $120 \times g$. This step was twice repeated, after which the pellet was practically free of chromosomes. All supernatant aliquots were combined. They contained the chromosomes, and about one nucleus per 400 chromosomes (about 7 cell equivalents). In order to remove fine cell debris and detergent, the combined supernatants were centrifuged for 25 min at $1000 \times g$. The pellet was resuspended in the Tris–$CaCl_2$ buffer and then recentrifuged. The resulting pellet was separated from the supernatant and kept in an ice bath. Immediately before incubation with recipient cells, the chromosomes were resuspended at room temperature to about 10^8 chromosomes per ml by rapid pipetting in Eagle's Spinner Minimum Essential Medium with 5% fetal bovine serum.

Logarithmically growing A9 cells were trypsinized with Viokase (Gibco), centrifuged, and resuspended at a concentration of 2×10^6 cells per ml in Eagle's Spinner Minimum Essential Medium containing 5% fetal bovine serum and 20 μg/ml of poly(L-α-ornithine)·HBr (molecular weight 120,000, Sigma) (4, 5). The suspended cells were equally divided into 25 cm^2 Falcon culture flasks. To the first flask the chromosomes were added to give a final concentration of about 50 chromosomes per recipient cell. The second part of the suspended cells received no donor chromosomes, but was diluted with an equal amount of the same medium. Both plastic flasks were placed on a slowly moving (12 rpm) gyratory shaking platform (New Brunswick) and incubated at 37° for 2 hr. Afterwards the cells were diluted with Dulbecco's modified Eagle's Medium [containing 10% fetal bovine serum and in addition gentamycin (50 μg/ml)] in 75 cm^2 Falcon culture flasks to give about 7×10^5 cells per flask, and then incubated at 37°. After 3 days the medium was replaced by the same medium containing hypoxanthine (13.6 μg/ml), aminopterin (0.19 μg/ml), and thymidine (3.9 μg/ml) (HAT) (14). Cells with and without chromosomes were treated identically. Colonies were isolated with stainless steel cylinders after more than 3 weeks, and then grown up in HAT medium for electrophoresis and karyotype analyses. Only those colonies that grew up in different culture flasks were considered to be of independent origin. We isolated four independent clones which were designated as CT 11A, CT 11B, CT 11C, and CT 11D.

Electrophoretic analyses were carried out by procedures previously described (15, 16).

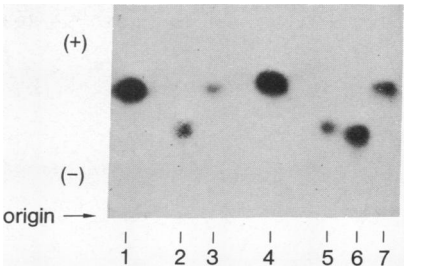

FIG. 1. Autoradiograph of HPRT activity in different cell lines after electrophoretic separation. Cell extracts were separated on starch gels in a citrate–phosphate buffer system, pH 6.8 (15). HPRT activity was detected by incubating the gel after electrophoresis with a substrate mixture containing [^{14}C]hypoxanthine and precipitating the reaction product, [^{14}C]inosine monophosphate, with lanthanum chloride, followed by autoradiography (16). This figure shows an autoradiograph of HPRT activity in extracts of clones isolated from the same gene transfer experiment: (*1*) CT 11B, (*2*) CT 11A, (*3*) CT 11C, (*4*) CT 11D. Control extracts (*5*) LM(TK$^-$), (*6*) CH-V79, and (*7*) D 98S are of mouse, Chinese hamster, and human origin, respectively.

Chromosomes for karyotype analyses were prepared after arresting the cells in mitosis with Velban (0.3 μg/ml) for 3 hr. Standard techniques were followed for Giemsa banding (17, 18) and fluorescence (19).

The immunochemical assays were performed in other laboratories. Viable cell populations or cell extracts were coded and then sent to Dr. R. DeMars at the laboratory for Genetics, University of Wisconsin, Madison, Wisc.; and to Dr. W. Kelley at the Department of Medicine, Duke University, Medical Center, Durham, N.C. The DeMars laboratory employed a method of immunoprecipitation described in detail elsewhere†. Dr. W. Kelley and his associate Dr. A. Leyva used a variety of methods including immunodiffusion, immunoprecipitation, and antibody competition against purified human HPRT (20).

Immunoprecipitation data obtained with the Held method† is reported here (Fig. 2). In their procedure, reaction mixtures in hemagglutination buffer contained rabbit anti-human HPRT, nonspecific rabbit serum, and test cell lysate. Activity of residual nonprecipitated HPRT in 20 μl aliquots of supernatant was assayed by a standard assay procedure†.

RESULTS

We incubated 1.2×10^7 recipient A9 cells with 5×10^8 metaphase chromosomes isolated from HeLa S3 cells. After 25 days in selective medium (HAT) four independent clones were isolated and characterized. No colonies grew up from control cultures.

Three of our A9 derived clones (CT 11B, CT 11C, and CT 11D) clearly showed HPRT activity of the same electrophoretic mobility as the human control cells (Fig. 1). The fourth clone, CT 11A, however, displayed HPRT activity that co-migrated with the murine form of HPRT. The obvious differences in intensity depicted in Fig. 1 were due to different amounts of cells used. We conclude from our data that the clones CT 11B, CT 11C, and CT 11D expressed the genetic information for human HPRT, whereas CT 11A was most probably a revertant.

† K. R. Held, B. Kahan, and R. DeMars (1975), manuscript submitted for publication.

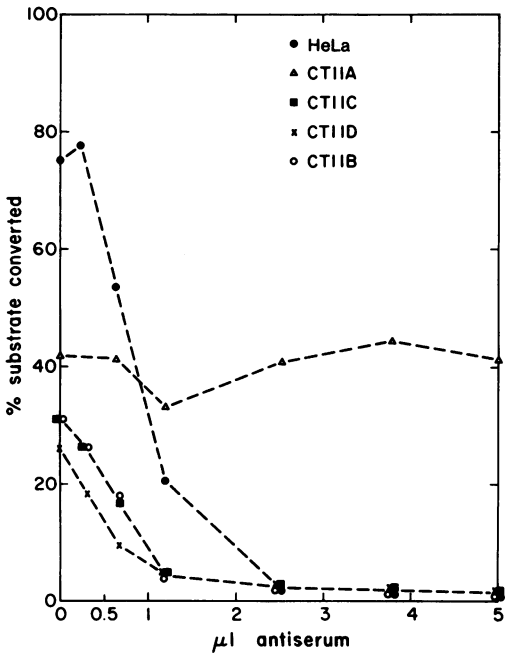

FIG. 2. Immunochemical characterization of HPRT. The figure shows the reduction of HPRT activity in the supernatant following immunoprecipitation with rabbit anti-human HPRT. The ordinate indicates the percentage of [^{14}C]hypoxanthine converted in a standard assay. The abscissa shows the concentrations of added antiserum. The specific activities of the original samples calculated as picomoles of substrate converted per microgram of cell protein per hr were HeLa S3 (2870), CT 11A (1060), CT 11C (519), CT 11D (86.2) and CT 11B (82.4). These data were prepared by Dr. R. DeMars and Mrs. B. Trend (see *Materials and Methods, Acknowledgments*, and †).

This conclusion is also supported by the immunochemical assays. Coded samples were sent to two independent investigators: R. DeMars and W. Kelley (see *Materials and Methods*, and *Acknowledgments*). Their immunological evaluation of the samples, which included several control extracts as well as the experimental HAT-resistant clones, showed that human HPRT activity was present in clones CT 11B, CT 11C, and CT 11D, but not in CT 11A. The basic immunoprecipitation data obtained with the Held method† are shown in Fig. 2. These results are consistent with the electrophoretic characterizations of HPRT in the HAT-resistant clones, and

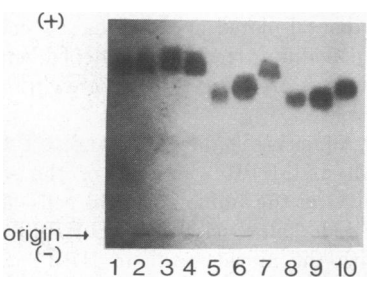

FIG. 3. Electrophoretic separation of G6PD activity in different cell lines. Cell extracts were run on starch gel in a Tris–EDTA–borate buffer, pH 8.6 (15). Clones from the gene transfer experiment: (*1*) CT 11D, (*2*) CT 11C, (*3*) CT 11B, and (*4*) CT 11A. Controls: (origin of the cell lines in parentheses) (*5*) UM 61 (human), (*6*) HeLa S3 (human), (*7*) L929 (mouse), (*8*) Dean Lazar (human), (*9*) WI-L2a-AGC2 (human), and (*10*) D98S (human).

TABLE 1

Enzyme	EC no.	Corresponding human chromosomes in normal karyotype	Activity found
Hypoxanthine-guanine phosphoribosyltransferase	2.4.2.8	X	+
Glucose-6-phosphate dehydrogenase	1.1.1.49	X	–
Phosphoglycerate kinase	2.7.2.3	X	–
Phosphoglucomutase-1	2.7.5.1	1	–
UDPG pyrophosphorylase	2.7.7.9	1	–
Peptidase C	3.4.3.X	1	–
Adenylate kinase-2	2.7.4.3	1	–
Isocitrate dehydrogenase (NADP)	1.1.1.42	2	–
Malate dehydrogenase (cytoplasmic)	1.1.1.37	2	–
Malic enzyme (cytoplasmic)	1.1.1.40	6	–
Mannose phosphate isomerase	5.3.1.8	7 or 15	–
Glutamate oxalacetate transaminase	2.6.1.1	10	–
Lactate dehydrogenase A	1.1.1.27	11	–
Lactate dehydrogenase B	1.1.1.27	12	–
Triose phosphate isomerase	5.3.1.1	12	–
Nucleoside phosphorylase	2.4.2.1	14	–
Adenine phosphoribosyltransferase	2.4.2.7	16	–
Galactokinase	2.7.1.6	17	–
Peptidase A	3.4.3.X	18	–
Glucosephosphate isomerase	5.3.1.9	19	–
Adenosine deaminase	3.5.4.4	20	–

Extracts of the cell lines CT 11B, CT 11C, and CT 11D were electrophoretically analyzed according to published methods (15). The chromosome assignment of the listed marker enzymes refers to conditions in the normal diploid human karyotype (6). No human isozyme was detected in the presumptive revertant line CT 11A.

clearly support the gene transfer interpretation. Dr. Kelley's laboratory confirmed these findings in a series of tests using different immunochemical methods (see *Materials and Methods*).

All clones from the gene transfer experiment clearly exhibited the murine forms of glucose-6-phosphate dehydrogenase (G6PD EC 1.1.1.49; D-glucose 6-phosphate:NADP 1-oxidoreductase) (Fig. 3) and phosphoglycerate kinase (PGK, EC 2.7.2.3; ATP:3-phospho-D-glycerate 1-phosphotransferase). Therefore, out of three isozyme markers that had been assigned to the long arm of the human X chromosome in the normal karyotype, only human HPRT was found in our transformed clones.

Table 1 summarizes the results of electrophoretic analyses of 20 constitutive isozymes. Except for the human form of HPRT only murine isozymes were found in gene transfer clones. These data suggest that the human genetic material to which these isozyme markers had been assigned was either absent or not expressed. Furthermore, these results make it very un-

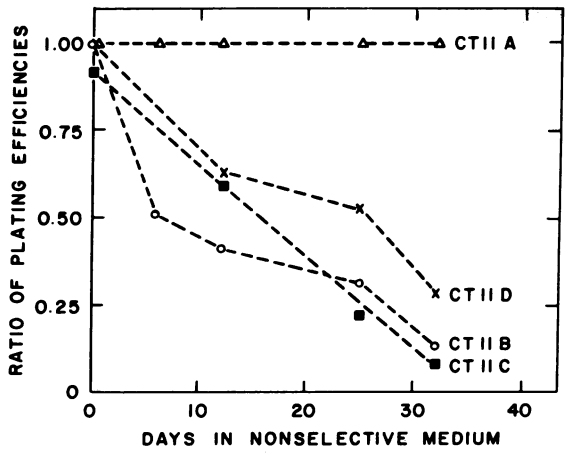

FIG. 4. Stability of the HPRT marker under nonselective conditions in clones isolated from the gene transfer experiment. At time 0 all 4 cell lines that were derived from the gene transfer experiment and that had been maintained in selective medium (HAT) were transferred to nonselective medium. Aliquots were removed at the indicated times and tested for plating efficiencies in selective and nonselective media. The ordinate gives the ratio of colonies in selective medium (HAT) over the colonies in nonselective medium.

likely that the isolated gene transfer clones may be derived from spontaneous fusion events between recipient mouse cells and human nuclei that were present in our chromosome preparations as contaminants.

How stable is the expression of HPRT activity after release of the selective pressure in those clones that had been isolated from the gene transfer experiment? To answer this question, we transferred and maintained aliquots of these cells in nonselective medium (Dulbecco's modified Eagle's Medium with 10% fetal bovine serum) for about a month. Aliquots of 200 cells were withdrawn at regular intervals and tested for their ability to grow in: (*a*) Dulbecco's modified Eagle's Medium with 10% fetal bovine serum, (*b*) the same medium plus HAT, and (*c*) the same medium plus 8-azaguanine (3 μg/ml) and 6-thioguanine (6 μg/ml) (21). Fig. 4 illustrates our results. Three of our clones that contained the human form of HPRT showed a relatively rapid decrease (about 3% HPRT loss per cell generation) in their ability to grow in selective medium (HAT). The fourth clone (CT 11A), which had been shown to contain mouse HPRT activity, produced an equal number of colonies under conditions (*a*) and (*b*), even after 30 days. The number of colonies in azaguanine- and thioguanine-containing medium [condition (*c*), data not shown] was always less than the theoretically expected difference between (*a*) and (*b*). This might have been due to reduced plating efficiency under these selective conditions.

We isolated between 20–35 $HPRT^-$ subclones from each transformed clone (CT 11Bat, CT 11Cat, and CT 11Dat) in azaguanine (3 μg/ml) and thioguanine (6 μg/ml) medium. After about 20 generations of growth in this medium, the subclones were tested for their ability to grow in counter-selective medium (HAT). Triplicate populations at densities of 2×10^2, 10^3, 10^4, 10^5, 10^6, and 5×10^6 were backselected in HAT medium. Only two clones grew up from cells originally derived from CT 11C. These may have been related, since they appeared in a single flask. Electrophoretic analysis showed these cells to possess the murine form of HPRT. We conclude from these results that the human HPRT marker

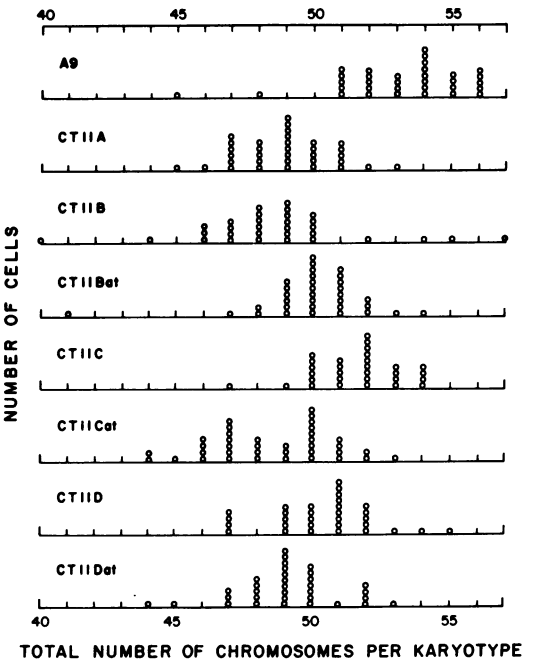

FIG. 5. Total chromosome number in metaphase cells of different cell lines. At least 30 karyotypes (each represented by one circle) of every cell line were counted. CT 11A, CT 11B, CT 11C, and CT 11D are clones from the gene transfer experiment. A9 is the parental line. CT 11Bat, CT 11Cat, and CT 11Dat were isolated as subclones from the corresponding gene transfer clones in azaguanine- and thioguanine-containing medium.

activity had been lost, and was not modulated in these cells (22).

The total number of chromosomes per cell of all clones was slightly less than in the parental A9 cell line (Fig. 5). This was also true for three subclones that were derived from the three original gene transfer clones and that had been isolated and maintained in azaguanine- and thioguanine-containing medium.

About 15 chromosome spreads of each clone were monitored after Giemsa-trypsin banding (17, 18) and after staining with the Hoechst 33258 benzimidazole derivative (19). Fig. 6 illustrates the karyotype of our gene transfer clone CT 11D as a representative example of our chromosome preparations. No human chromosomes or their parts could be detected in any of the A9-derived clones.

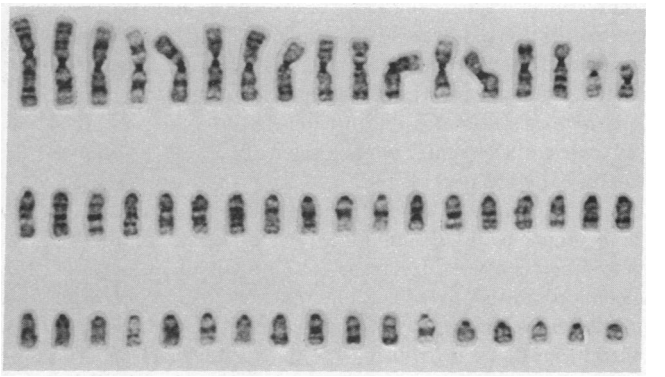

FIG. 6. Representative karyotype of the A9-derived gene transfer clone CT 11. The karyotype is representative of our chromosomal analyses. Only mouse, and no human, chromosomes could be detected in these cells.

DISCUSSION

The results presented here provide strong support for gene transfer in mammalian cells as first described by McBride and Ozer (4). Several significant aspects of this system, which are only partially resolved, deserve discussion. These are (*1*) the size of the transferred genetic element, which we shall term the "transgenote", and (*2*) its cellular localization, and, more specifically, its relationship to the host cell and genome.

Our experiments permit us to make some inferences regarding size. Somatic cell genetic studies have provided information on the regional localization of PGK, HPRT, and G6PD genes on the X chromosome long arm (23). It has been shown that the HPRT locus lies between the loci for the other two markers, and that the distance between PGK and G6PD genes represents no more than 1% of the human genome. Therefore, it can be inferred, since neither human PGK or G6PD enzymes are observed, that the transgenote can be no larger than 1% of the human genome, or about 10^7 nucleotide base pairs. This conclusion assumes that the X chromosome constitution of HeLa S3 is normal, an assumption that is buttressed by cytogenetic observations on HeLa S3 (17, 24). These studies, employing Q-banding techniques, report a normal, structurally unmodified X chromosome. In addition to X-linked markers, no autosomal markers were detected in the transformed clones, although 14 autosomes could be monitored using enzyme markers. Thus, we must conclude that only a small part of the genome has been transferred and/or retained. Presumably, most of the transferred genetic material has been degraded, possibly by restriction type enzymes, either during or subsequent to uptake by the recipient cell (25). Chromosome-mediated gene transfer systems may have considerable application for high-resolution mapping, if the size of the transgenote is reproducibly small, and if in future experiments an estimate of its physical size can be obtained.

No definitive answer can yet be given to the question of cellular localization and integration of donor genetic material into the recipient genome. Our results as well as some of those by McBride and Ozer (4) and Schwartz *et al.* (26) suggest that gene transfer clones lose the donor HPRT marker in nonselective medium. Therefore, it is unlikely that the donor gene material is stably integrated into the chromosomes of the recipient cells in a structural sense. In contrast to our results, McBride and Ozer (4) also described two gene transfer clones in which the donor HPRT activity seemed to be stably maintained even under nonselective conditions. Further experiments must clarify whether these results may be explained by evolutionary differences in the homology of DNA between donor and recipient cells. Our present data remind us of the results reported for the genetic transformation of the plant *Arabidopsis thaliana* (27) or the insect *Drosophila melanogaster* (28) where "exosome" models seem to best fit as an explanation of experimental results.

There is one clear consequence from our data: gene transfer clones that harbor a small piece of human genetic material can very likely be used for rapid assignment of human genes in close proximity to selectable markers. As more selectable markers become known, it may be possible to develop the chromosomal gene transfer system in mammalian cells as a useful, high-resolution genetic mapping tool in much the same way as the F prime elements are employed for genetic analysis in *Escherichia coli* (29). The system may also prove useful in the physical isolation of small segments of mammalian genomes.

We gratefully acknowledge the technical help of Ms. Elizabeth Nichols and Ms. Kay Friend and the skillful preparation of the manuscript by Mrs. Mae Roger. The experiments here reported were carried out within the framework of a group of investigators at Yale University, all of whom have collaborative interests in mammalian gene transfer problems. This consortium consists of Drs. E. Adelberg, G. Eisenstadt, L. Hodge, P. Magee, and F. Ruddle. This work owes much to the intellectual support and material aid of this group whose cooperative research is supported by National Institutes of Health Grant GM 19481. We also extend thanks to Drs. W. O. McBride and H. Ozer, who, in addition to stimulating this work, were extremely helpful by sharing with us details concerning their early experiments. We are especially grateful for the help given us by Dr. DeMars and Mrs. Trend at the University of Wisconsin and Dr. Kelley and Dr. Leyva at Duke University in performing immunochemical tests for human HPRT on our samples. These studies were also supported by a grant-in-aid to K.W. from the German Research Association and by National Institutes of Health grant 09966 to F.R.

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