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Human Gene Expression in Rodent Cells after Uptake of Isolated Metaphase Chromosomes

(gene mapping/X-linked genes/hypoxanthine and adenine phosphoribosyltransferases/ human lymphoblasts/mouse A9 cells)

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ABSTRACT **Permanent transfer of genetic information from chromosomes isolated from human diploid cells to recipient cells has been demonstrated. Human metaphase chromosomes were incubated with mouse A9 fibroblasts deficient in hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) and adenine phosphoribosyltransferase (AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.-2.7). Colonies of cells containing hypoxanthine phosphoribosyltransferase appeared during growth in a selective medium. The hypoxanthine phosphoribosyltransferase gene product in four independent colonies was identified as human donor species by both gel electrophoresis and isoelectric focusing; hence these colonies did not result from reversion of A9 parental cells. Other X-linked human genes, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NAD$^+$ 1-oxidoreductase, EC 1.1.1.49) and phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3), were not expressed in these same colonies. Dissociation of expression of these X-linked genes probably results from chromosomal fragmentation during uptake, but other mechanisms have not been excluded.**

The stable transfer of genetic information from isolated mammalian metaphase chromosomes into fibroblasts in cell culture has been demonstrated in several laboratories (2–5). This technique could provide a useful method for the mapping of genes in eukaryotic cells which would be complementary, and in some respects superior, to the currently used interspecies somatic cell hybridization. It could also provide a basis for mapping integration sites of oncogenic viruses in mammalian cells (4).

The utility of this technique for genetic mapping will depend to some extent on the amount of information actually transferred. Previous studies, employing isolated Chinese hamster chromosomes and recipient mouse fibroblasts, suggested that only a small amount of genetic information is incorporated and expressed by the recipient cell (2). This suggestion could not be fully evaluated, however, because of inadequate methods for the resolution and identification of the various X-linked gene products under consideration. The amount of information transferred by this technique could be estimated more readily using a system involving the transfer of isolated human metaphase chromosomes into mouse recipient cells. Several human X-linked gene products can be readily distinguished electrophoretically from their murine counterparts (6). Moreover, these X-linked genes have been assigned to relatively specific regions along the human X chromosome (7). Therefore, the present study was undertaken to extend the technique by the use of human donor chromosomes from cell lines of relatively normal karyotype, and to further characterize the unit of genetic information actually transferred in this process.

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8); APRT, adenine phosphoribosyltransferase (AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7); *hprt* or *aprt*, gene directing synthesis of HPRT or APRT, respectively; HAAT, hypoxanthine (50 μM), adenine (50 μM), amethopterin (0.4 μM), thymidine (16 μM), glycine (3 μM); MEM, Eagle's minimal essential medium (1); G6PD, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP$^+$ 1-oxidoreductase, EC 1.1.1.49); PGK, phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3).

MATERIALS AND METHODS

Cell Cultures. Cells used were: (*1*) two human lymphoblastoid cell lines, RAJI (CCL 86) and CCRF-SB (CCL 120), obtained from the American Type Culture Collection; (*2*) HeLa cells; (*3*) mouse fibroblasts (L_{929}); and (*4*) mouse L cells, A9, (8, 9) deficient in hypoxanthine phosphoribosyltransferase (HPRT) and adenine phosphoribosyltransferase (APRT). Fibroblasts were maintained in monolayer cultures at 37° in a gas-flow (7% CO_2–air) humidified incubator, in Eagle's minimal essential medium (MEM) containing twice the usual concentrations of amino acids and vitamins. Lymphoblasts were grown in suspension culture in Eagle's medium without calcium (spinner medium). All media were supplemented with 10% fetal calf serum, 4 mM glutamine, penicillin (50 μg/ml), and streptomycin (50 μg/ml).

Isolation and Purification of Metaphase Chromosomes. Human chromosomes were isolated under sterile conditions using a modification of the procedure of Mendelsohn *et al.* (10). Washed cells (2×10^6/ml) were incubated for 20 min at room temperature in 0.075 M KCl. After centrifugation, the cells (10^7/ml) were lysed in pH 3 buffer with a Waring Blendor®. The chromosomes were separated from cellular debris and nuclei by differential, isopycnic, and unit gravity sedimentation (3).

Information Transfer to A9 Cells by Chromosome Uptake. Purified human metaphase chromosomes were incubated with mouse A9 fibroblasts (6×10^6/ml) for 2 hr at 37° in Eagle's MEM spinner medium containing 12 μg/ml of poly(L-ornithine) (molecular weight 70,000), as described previously

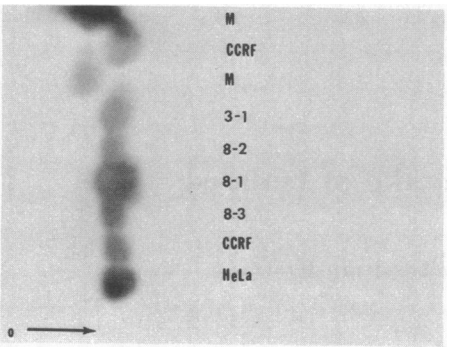

FIG. 1. Starch gel electrophoresis of HPRT activity. M = mouse L_{929} extract. Colonies are labeled numerically as in *text*. CCRF and HeLa are extracts from the respective human cell lines. O = origin; arrow points to anode.

(2). Aliquots (5×10^5 cells) were plated in 100 mm plastic dishes (Falcon) containing 10 ml of MEM. The medium was replaced with hypoxanthine/adenine/amethopterin/thymidine/glycine (HAAT) selective medium after 3 days, and plates were refed twice weekly for 6 weeks. (It is possible to simultaneously select for the non-linked *hprt* and *aprt* loci in this medium.) Colonies appearing on separate plates were cloned and subcultured in HAAT medium. Large numbers of A9 fibroblasts ($>10^8$ cells per exp.), which had not been exposed to chromosomes, were similarly cultured in HAAT for 6 weeks to determine the frequency of spontaneous reversion to the wild-type phenotype for *hprt* and *aprt*.

Enzyme Extracts and Assays. Cells were washed and sonicated (0°) in 0.01 M Tris·HCl, pH 7.4 (5×10^7 cells per ml). High-speed supernatants (100,000 × *g* for 1 hr) were assayed for HPRT and APRT activity by the method of Harris and Cook (11). Extracts for glucose-6-phosphate dehydrogenase (G6PD) analysis were similarly prepared, except that the "extraction buffer" of Bakay and Nyhan (12) was employed, and the assay procedure was that of Motulsky *et al.* (13). Phosphoglycerate kinase (PGK) extracts were prepared from sonicates of washed cell pellets suspended in equal volumes of buffer containing 0.1 M Tris·HCl, pH 7.0; 1.1 mM 2-mercaptoethanol; 1 mM Na_2EDTA; and 1 mM ATP (14).

Gel Electrophoresis. Mobility of APRT was determined by disc electrophoresis in 5% polyacrylamide gel slabs using a slight modification of the procedure of Bakay and Nyhan (15). Slabs were photopolymerized in the presence of riboflavin and a 4% stacking gel was added. Extracts were electrophoresed at 5–15° for 3.5 hr at 300 V until the bromophenol blue tracking dye reached the bottom of the separating gel. The gel was reacted with substrate and the [^{14}C]AMP product was precipitated with $LaCl_3$ and detected by autoradiography (2). Electrophoresis of HPRT was performed at 4° in 12% starch gels at 120 V for 14–18 hr and evaluated by the method of Nichols and Ruddle (16). Electrophoresis of G6PD was carried out on Cellogel® (Chemetron) according to Meera Khan (6), with the addition of NADP (3 mg/liter) to the electrophoresis buffer. Electrophoresis of PGK was performed at 4° for 18–24 hr at 150 V in 12% starch gels containing 3 mM ATP (17). The sliced gel was reacted for 1 hr at 37° with an 0.5% agar overlay containing a reaction mixture slightly modified from Omenn and Cohen (14). The position of PGK activity appeared in long-wave ultraviolet light as an absence of fluorescence (conversion of NADH to NAD) or it was observed in visible light by flooding the gel-agar overlay with a phenazine methosulfate/MTT tetrazolium mixture (6). Photographs of G6PD and PGK gels were made with a Polaroid® camera using visible light.

TABLE 1. *Colonies arising in gene transfer and in reversion experiments*

Exp. no.	No. of cells plated* ($\times 10^{-7}$)	No. of chromosomes added, donor†	Positive plates/ total plates‡	DNA§ mol. wt. ($\times 10^{-6}$)
A. Gene transfer experiments				
1	1.2	1.8 × CCRF	2/24	—
2	2.4	2 × RAJI	7/48	—
3	1.8	5 × HeLa	4/36	5.5
4	1.8	1 × CCRF	4/36	—
5	1.8	2 × RAJI	2/36	—
6	0.6	1.2 × RAJI	0/12	2.0
7	1.2	0.4 × RAJI	4/12	2.6
8	4.6	2 × CCRF	8/96	11.6
9	3.7	1 × CCRF	9/68	11.9
10	1.8	1 × CCRF	0/36	7.0
11	1.8	1.3 × HeLa	5/36	14.0
12	7.2	1.3 × HeLa	11/44	19.5
B. Reversion experiments				
A	26	0	4/57	—
B	31	0	3/100	—
C	51	0	3/90	—
D	38	0	2/106	—
E	21	0	4/48	—
F	37	0	0/100	—

* Total number of A9 cells incubated and subsequently plated.

† Number of cell equivalents of chromosomes from the respective donor incubated per A9 cell. Cell equivalent = number of chromosomes isolated from a single mitotic cell.

‡ Number of plates with one or more colonies per total number of plates inoculated.

§ Molecular weight of single-stranded DNA in the chromosome preparations (3).

Isoelectric Focusing. Five percent polyacrylamide gel slabs, 115 × 250 × 1 mm, containing 2% Ampholines® (LKB) were prepared (18). Enzyme extracts were either applied in cylindrical wells molded into the gel or directly onto the gel surface using square (6 × 6 × 2 mm) glass tubing chambers. Electrophoresis was performed at 5° along the long axis of the gel for 10–16 hr up to a voltage of 1200 V (maximum power of 5 W). Suitable controls demonstrated focusing of the proteins within this time period. After focusing, disks (6 mm diameter) were cut from one edge of the gel and extracted overnight before pH measurement at room temperature. Positions of HPRT were determined on the remainder of the gel by reaction with [^{14}C]hypoxanthine substrate and autoradiography (2).

Karyotypes. Cells were arrested with colcemid (0.2 μg/ml) for 5 hr, swollen in 0.075 M KCl at room temperature for 30 min, fixed in 3:1 (v/v) methanol:acetic acid, and spread on cold, moist slides by flaming. Slides were stained with 1% crystal violet and photomicrographed. Total number of chromosomes, number of biarmed chromosomes (metacentric plus acrocentric), and number in other classes (teleocentric

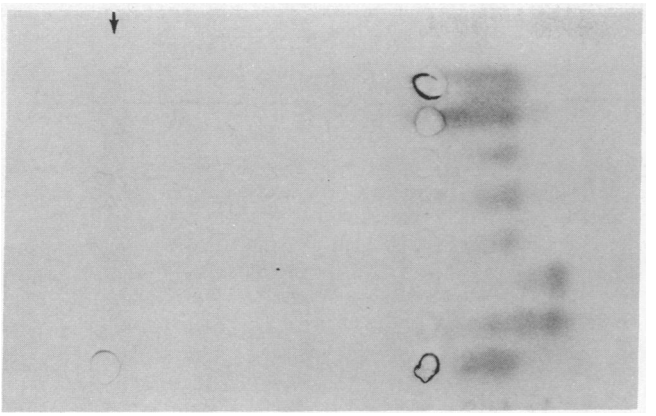

FIG. 2. Polyacrylamide gel isoelectric focusing of HPRT activity. The gel contained 1% Ampholine® pH 4–6 (LKB) plus 1% Ampholine® pH 5–7. The extracts are, from top to bottom: colony 3-1, HeLa, CCRF, colony 8-2, colony 8-1, colony 9-1, mouse L_{929}, colony 3-1. The sample origins (molded wells) are noted by the arrow; the cathode is to the right. The circular patterns in the gel immediately anodal to the autoradiographic spots are artifactual. Approximate pI for human species = 5.9; for mouse species = 6.1.

plus dot forms) were determined by a single observer from photographs of suitable metaphase cells.

RESULTS

Gene Transfer to A9 Cells. Colonies appeared following exposure to HAAT medium in most experiments in which A9 cells had been incubated with human chromosomes (Table 1A). Only colonies that exhibited continued growth in HAAT medium after cloning are reported. However, a large fraction of these colonies were subsequently lost before the gene product could be identified and characterized as to species of origin. Most losses were due to microbial infection. Colonies appeared with a frequency of about 2×10^{-7} per A9 cell incubated with chromosomes; it did not vary significantly with the chromosome donor cell type. In addition, the frequency showed no apparent dependence on the input ratio of chromosomes (cell equivalents) to recipient cells, although this ratio varied little (1–2) in 10 of the 12 experiments. The molecular weight of donor chromosomal DNA did not have a clear effect on the frequency of colony appearance.

The apparent spontaneous reversion rate was about 0.8×10^{-8} per A9 cell exposed to HAAT (Table 1B). This is approximately 3-fold lower than that seen in Exps. 1–12, assuming that the A9 cells in the latter experiments double about three times prior to their exposure to selective medium.

Electrophoresis of APRT Extracts. Colonies were cloned as soon as they appeared, and they were expanded in HAAT medium, usually as spinner cultures. Cell-free extracts from 10 colonies were assayed for HPRT and APRT activity. No extract contained both phosphoribosyltransferase activities. Lysates from five colonies (2-1 from Exp. 2; 3-2 from Exp. 3; and 8-4, 8-5, and 8-6 from Exp. 8) contained APRT activity and each was characterized by polyacrylamide gel electrophoresis. The two parental species of APRT are clearly resolved by this technique, with the mouse species exhibiting a significantly lesser electrophoretic mobility than the three identically migrating human cell types. The APRT in all five extracts was indistinguishable from the wild-type mouse

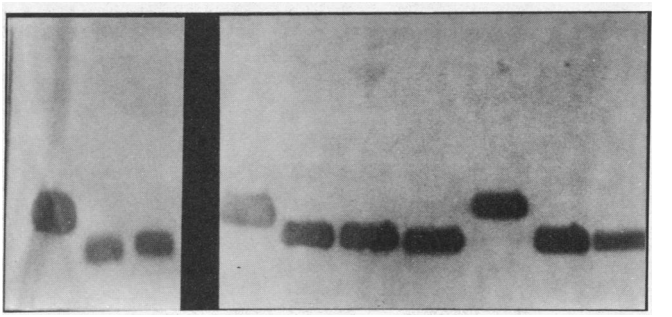

FIG. 3. G6PD electrophoresis. Two separate Cellogel® electrophoreses are shown; direction of migration is toward the anode (bottom). Spots represent extracts of (from left to right): HeLa (G6PD isozyme A), colony 3-1, mouse A9, HeLa, colony 3-1, mouse A9, colony 8-1, CCRF (G6PD isozyme B), colony 8-2, colony 8-3.

(L_{929}) species. Therefore, all five colonies were considered spontaneous revertants.

Electrophoresis and Electrofocusing of HPRT Extracts. Lysates of five colonies contained HPRT activity. Preliminary studies indicated that adequate resolution of the parental species of HPRT could not be obtained by the use of unmodified procedures from a previous study (2). However, satisfactory resolution of these species was obtained by two different methods. Starch gel electrophoresis of four of these extracts (from colonies 3-1 of Exp. 3; and 8-1, 8-2, and 8-3 of Exp. 8) showed a single band of HPRT activity with the same mobility as the human species of HPRT; this property clearly differed from that of the mouse species of this enzyme (Fig. 1). These extracts were also characterized by isoelectric focusing (Fig. 2). The HPRT in these extracts has the same isoelectric point as the human parental species. In contrast, a fifth extract (colony 9-1, Exp. 9) shows the same pattern as the murine species of HPRT. Additional isoelectric focusing experiments, which have included extracts from colony 8-3, have demonstrated identical relative migration patterns to that presented in Fig. 2, although the pI values of these proteins vary slightly (<0.3 pH units) with the pH range of Ampholines® employed, and perhaps with other factors. The combined results of electrophoresis and electrofocusing strongly suggest that 4 independent colonies (3-1, 8-1, 8-2, and 8-3) arose through the uptake and expression of human genetic information by mouse cells, whereas the fifth colony (9-1) resulted from a spontaneous reversion.

Evaluation of Colonies for Human G6PD and PGK Activity. HPRT is well known to be X-linked in man, as are G6PD and PGK (7, 19). It would thus be anticipated that colonies expressing human HPRT should also express human G6PD and PGK if the presence of HPRT resulted from transfer of an intact human chromosome. Therefore, extracts of all four colonies containing human HPRT were examined for their G6PD phenotype (Fig. 3). The murine species of the enzyme was present in each extract, but neither human G6PD nor the expected interspecies hybrid band of intermediate mobility (20) was observed. Similarly, no human PGK activity could be detected in any of these four extracts; the results of a typical electrophoresis are shown (Fig. 4).

Karyotypes. Mean chromosome numbers were calculated and classified as shown in Table 2 for colonies 8-1, 8-2, 8-3,

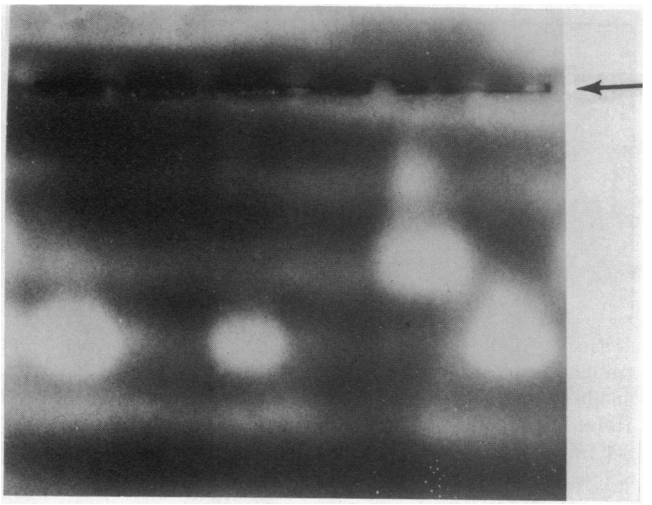

FIG. 4. Starch gel PGK electrophoresis. Arrow denotes origin; anode is at bottom of figure. Spots represent the following extracts (from left to right): mouse A9, colony 8-2, CCRF, mouse A9.

and 3-1; and for CCRF, HeLa, and A9 cells. The means of the total chromosome numbers were compared statistically. A one-way analysis of variance indicated an overall statistical difference among the five means: 8-1, 8-2, 8-3, CCRF, and A9; $F(4,105) = 10.55$, $P < 0.01$. A multiple comparison procedure showed that the mean for CCRF differed from those of the other four ($P < 0.05$). In a similar fashion an overall comparison of colony 3-1, HeLa, and A9 also showed a statistical difference among the means, $F(2,82) = 26.42$, $P < 0.01$, and a multiple comparison procedure revealed the mean of HeLa to be different from those of the other two ($P < 0.01$). Identical analyses of the number of biarmed chromosomes and of the number in other classes yielded essentially the same results.

DISCUSSION

These results strongly suggest that the permanent transfer of a human gene to a recipient mouse cell can occur through uptake of an isolated metaphase chromosome. The fact that colonies appeared with a frequency about 3-fold greater than was anticipated on the basis of reversion adds very little support to the argument for a true genetic transfer, as others have already indicated (21). Proof of genetic transfer at this low frequency requires adequate demonstration that the gene product is specified by the chromosome donor species and that all colonies examined originate through independent events. In these experiments, cell progeny from four independent colonies show HPRT activity that is indistinguishable from that of the chromosome donor species, and clearly different from the recipient species, by two different physical criteria. It must be recognized that a revertant enzyme would not necessarily be identical in physical properties with the wild-type species. However, the fact that the HPRT product from these four colonies cannot be distinguished by either starch gel electrophoresis or electrofocusing from the HPRT in their respective human chromosome donor lines indicates that reversion is a very improbable explanation for these results. Moreover, chromosome donor species of HPRT with different physical properties than either human or wild-type

TABLE 2. *Chromosome content of colonies and parental cell lines*

Cell line	N*	Total no. of chromosomes	No. of biarmed chromosomes†	No. in other classes‡
CCRF	22	45.95 ± 1.13	38.41 ± 4.85	7.54 ± 4.53
8-1	17	51.82 ± 5.36	23.41 ± 1.80	28.41 ± 3.87
8-2	32	55.56 ± 9.91	26.81 ± 5.13	28.75 ± 5.39
8-3	18	53.22 ± 2.65	24.00 ± 1.33	29.22 ± 2.67
A9	21	55.81 ± 1.47	21.86 ± 0.96	33.95 ± 1.77
3-1	18	55.94 ± 9.07	23.83 ± 4.77	32.11 ± 4.64
HeLa	46	64.63 ± 4.88	50.70 ± 5.05	13.93 ± 4.12

Cell lines are identified as in *text* (*Results*). Figures represent mean values per cell ± one standard deviation.

* N = number of metaphase cells counted.

† Biarmed chromosomes include metacentric plus acrocentric forms.

‡ Other classes include teleocentric plus dot forms.

mouse enzyme were found previously when A9 cells were incubated with Chinese hamster chromosomes (2). It seems quite implausible that the revertant A9 HPRT enzyme would always fortuitously resemble the chromosome donor species employed in any specific investigation. The authentic revertants of A9 HPRT that have been examined in this laboratory could not be distinguished from the wild-type mouse enzyme by DEAE-cellulose chromatography or polyacrylamide gel electrophoresis. Both the karyographic data and the absence of human G6PD, PGK, or APRT excludes the possibility that these colonies could have arisen through contamination of the cultures with human cells. These observations, particularly the absence of expression of human G6PD and PGK, also diminish the likelihood that colonies arose through spontaneous fusion of A9 cells with human cells or nuclei. Other arguments to support the nature of this transfer have been presented previously (2). Our findings support and extend the results of reported studies (2–4); the combined observations indicate that genetic transfer by this method is not dependent upon the species of the chromosome donor or recipient cells, and it is not restricted to a single marker.

A major objective of this study was to characterize the unit of genetic information transferred to a recipient cell by this technique. Only the murine species of the X-linked markers G6PD and PGK could be detected in any of the colonies that expressed human HPRT. This implies, in terms of functional genetic information, that only a portion of the donor chromosome is actually incorporated into the recipient cell's genome. According to Ricciuti and Ruddle (7), this would correspond to a portion of the long arm of the X chromosome that has lost genetic material that is, with respect to the centromere, both proximal (PGK) and distal (G6PD) to the *hprt* locus. Such a fragment represents less than 1.5% of the total DNA in a diploid human cell (22). Of course, this still corresponds to about 10^8 nucleotide pairs and could contain as many as 10^5 genes coding for proteins with an average molecular weight of 30,000. There is preliminary evidence in a related system—the transfer of thymidine kinase (ATP:-thymidine 5′-phosphotransferase, EC 2.7.1.75) from donor Chinese hamster chromosomes into mouse B82 recipient cells —that more than one gene can be transferred and expressed by this technique (studies in progress in collaboration with F. H. Ruddle); however, the genes in question [thymidine kinase and galactokinase (ATP:D-galactose 1-phosphotrans-

ferase, EC 2.7.1.6)] are in man (26) and, presumably, also in Chinese hamster much more closely linked than are PGK or G6PD to HPRT.

Perhaps a more serious problem relates to the apparent absence of a centromere from the portion of functional X chromosome incorporated into the host cell. Transfer of one chromatid from such an acentric fragment to each daughter cell at mitosis would seem difficult unless this fragment were integrated into a recipient chromosome. Evaluation of the stability of the phenotype in these colonies should help to clarify the problem. A previous study (2) demonstrated two colonies with stable phenotypes as well as one which was unstable; the latter would be compatible with the existence of the HPRT gene in a free chromosome fragment.

The current evidence does not rule out the presence of a much larger non-functional piece of genetic information, perhaps even the entire X chromosome. Methods are available for differentiating human and mouse chromosomes (23) and for the identification of a small piece (about 10^3 to 10^4 genes) of donor DNA integrated into the recipient cell's genome, or existing as a free fragment (24). Neither the biochemical analysis of multiple genetic markers nor the morphologic methods proposed can exclude the presence of many small donor chromosome fragments, originating from one or more chromosomes, incorporated into the recipient cell. This possibility should be amenable to investigation by DNA molecular hybridization studies.

Three of the colonies in the present study were obtained from an experiment employing donor chromosomes from the relatively normal diploid cell line CCRF (25). This is particularly important, since there would be no simple way to exclude the possibility that normal linkages had been lost through translocations in most heteroploid lines such as HeLa. The ideal chromosome donor for mapping studies would be mitogenically stimulated normal human lymphocytes.

There is considerable evidence from morphologic studies and measurements of [^{3}H]dT-labeled chromosome uptake that chromosomes can be incorporated by cells in tissue culture. Most of the chromosomal DNA is rapidly degraded to small molecular weight fragments (27). All available information, including that from gene transfer studies, favors a mechanism of phagocytosis of the donor chromosome followed by its partial or complete digestion by lysosomal enzymes. Genetic transfer then results from isolated instances of escape from the lysosomes of small portions of a donor chromosome and their subsequent incorporation into the recipient cell's genome. A nuclear location for the transferred genes is considered probable, but no proof is yet available. Alternative hypotheses are possible (27). This genetic material is likely integrated into the recipient's DNA. The unit of transfer is considerably smaller than an entire chromosome but it can encompass more than a single gene, and it could consist of thousands of genes. Whether multiple chromosome fragments are incorporated into the host cell genome is not yet clear. More work is required to determine the validity of these speculations. This technique presently does not provide a method for determining a syntenic relationship for genes located at considerable distances from one another. It could be used for mapping genes closely linked to a selective marker and for investigating integration sites of oncogenic viruses. Future developments should further expand the applications of this approach.

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