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Transfer of Genetic Information by Purified Metaphase Chromosomes

(Chinese hamster and mouse fibroblasts/HeLa cells/hypoxanthine phosphoribosyl transferase)

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ABSTRACT **Transfer of genetic information from isolated mammalian chromosomes to recipient cells has been demonstrated. Metaphase chromosomes isolated from Chinese hamster fibroblasts were incubated with mouse A_9 cells containing a mutation at the hypoxanthine–guanine phosphoribosyl transferase (*hprt*) locus. Cells were plated in a selective medium, resulting in death of all unaltered parental A_9 cells. However, colonies of cells containing hypoxanthine phosphoribosyl transferase (EC 2.4.2.8) appeared with a variable frequency of about 10^{-6} to 10^{-7}. The enzyme from these cells was indistinguishable from that from Chinese hamster cells, as shown by DEAE-cellulose chromatography and gel electrophoresis, and differed clearly from the mouse enzyme. The colonies, thus, did not result from reversion of A_9 parental cells to wild type, but appeared to represent progeny of individual cells that had ingested chromosomes, replicated, and expressed the *hprt* gene. These colonies differed from each other in stability of expression of the transferred gene.**

A means for genetic mapping of mammalian chromosomes is provided by a combination of the technique of cell fusion and karyotypic analysis of resultant hybrid clones by the recently developed quinacrine (3) and Giemsa banding procedures (4). Techniques for the direct transfer of genetic information from subcellular particles to cells could provide a complementary method for genetic mapping. This would eliminate the necessity of awaiting segregation of chromosomes, thereby reducing the possibility of chromosomal rearrangements. Mammalian metaphase chromosomes appear eminently suitable for this purpose since a meaningful biological fractionation of genes is present in chromosomes, and numerous methods (5–7) have been described for isolation of these particles. Chromosomal DNA might be somewhat better protected from degradation during cellular uptake than free DNA due to its compact structure and its association with proteins and RNA. The introduction of intact chromosomes into cells could circumvent problems of integration of DNA into the host genome; subsequent replication and expression of chromosomal genes should be analogous to the steps following cell fusion.

Evidence exists that isolated metaphase chromosomes can penetrate into mammalian cells *in vitro* (8–16), but most of the chromosomal DNA is subsequently degraded (8–12). Previous information suggesting that mammalian chromosomes can be replicated after uptake is extremely sparse (14–16), and no evidence has been provided for expression of this new genetic information by the host cell.

Abbreviations: HPRT, hypoxanthine phosphoribosyl transferase (EC 2.4.2.8); *hprt*, gene directing synthesis of HPRT; HAT, hypoxanthine–amethopterin–thymidine, selective growth medium of Littlefield (1); MEM, Eagle's minimal essential medium (2).

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This paper presents the first evidence that both replication and expression of chromosomal genes can occur after the uptake of mammalian metaphase chromosomes. Moreover, permanent transfer of this new genetic information results, although the frequency of this gene transfer is low.

MATERIALS AND METHODS

Cell Cultures. Cells used were: (*1*) wild-type Chinese hamster fibroblasts (V-79), recently cloned; (*2*) mouse fibroblasts (L_{929}); (*3*) HeLa cells; and (*4*) mouse L-cell lines A_9 and B_{82}, deficient in hypoxanthine phosphoribosyl transferase (HPRT; EC 2.4.2.8) and thymidine kinase (EC 2.7.1.21), respectively (17). Cells 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 concentration of amino acids and vitamins. Cells were also grown in suspension culture, in Eagle's medium without calcium, or in Ham's F-10 medium (18). 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. Chromosomes were isolated under sterile conditions from [^{3}H]-dT-labeled cells (0.2 mCi/liter) as described (19), by slight modifications of either the procedure of Mendelsohn *et al.* (5) at pH 3 or the method of Maio and Schildkraut (6) at pH 7. Chromosomes were subsequently separated from intact cells and most debris by ultracentrifugation through a layer of 80% sucrose (w/v). Nuclei were then removed by unit-gravity sedimentation at pH 7, essentially as reported for fractionation of nuclei (20). Final chromosome preparations contained about one nucleus per thousand cell equivalents of chromosomes (i.e., one nucleus per 25,000 chromatid pairs). For each preparation, the molecular weight of dissociated, single-stranded, chromosomal DNA was determined by velocity sedimentation in alkaline sucrose density gradients (21), with ^{14}C-labeled simian virus 40 (SV40) DNA I and II (22) as markers. The molecular weight of chromosomal DNA decreases progressively on storage of the chromosomes, even at 5°, although chromosome morphology remains good. Thus, chromosomes were used in gene-transfer experiments immediately after isolation.

Incubation of A_9 Cells with Chromosomes. Purified metaphase chromosomes (about 1 cell equivalent per recipient cell) from Chinese hamster cells were dispersed with A_9 mouse fibroblasts (6×10^6/ml) in complete Eagle's MEM spinner medium, containing 12 μg/ml of poly-L-ornithine (molecular weight 70,000; Mann Research) in a sterile, siliconized, glass culture tube. The tube was equilibrated with 5% CO_2–air and incubated for 2 hr at 37° while rolling in a nearly horizontal position at 10 rpm. Aliquots of 5×10^5 cells were transferred to 100-mm plastic dishes (Falcon) containing 10 ml

of complete MEM. After 3 days of incubation, the medium was replaced with HAT medium, and the plates were refed with this selective medium at 3- to 4-day intervals for 6 weeks. Colonies that appeared during this interval were cloned in metal cylinders, removed by treatment with trypsin, and recultured in HAT medium.

Enzyme Assays. Cells were washed with 0.15 M NaCl, suspended in 0.01 M Tris·HCl (pH 7.4) (6×10^7 cells per ml), and lysed by freezing and thawing. The assay for HPRT activity was basically that described by Harris and Cook (23), involving conversion of [8-^{14}C]hypoxanthine substrate to [^{14}C]IMP product, which was collected on DEAE-cellulose disks (Whatman DE-81). The reaction product of the hamster and wild-type mouse HPRT, as well as that of extracts of the experimental clones, was confirmed to be [^{14}C]IMP by thin-layer chromatography on cellulose; both solvent systems B and C of Ciardi and Anderson (24) were used. Purity (>97–99%) of the [^{14}C]hypoxanthine substrate was ascertained in the same manner.

DEAE-Cellulose Chromatography. Micro-granular DEAE-cellulose (Whatman DE-52) was washed (25), equilibrated with starting buffer [0.01 M Tris·HCl (pH 8.7)], and packed in a 5 × 140 mm column. Enzyme extract (about 5 mg of protein) was applied to the column at 5° and sequentially eluted at a constant flow-rate (8 ml/hr) with 4 ml of starting buffer, a 60-ml linear (0–225 mM) NaCl gradient followed by 3 ml of 0.4 M NaCl (both containing starting buffer), and finally with 6 ml of 2 M NaCl–0.1 M Tris·HCl (pH 8.8). 1-ml Fractions were assayed immediately for HPRT activity and later for protein concentration, conductivity, and pH.

Gel Electrophoresis. Gels were prepared by a modification of the procedure of Bakay and Nyhan (26). An 8% polyacrylamide gel (70 × 100 × 2 mm) was formed between two glass plates, and a 5% stacking gel was added. A cellulose acetate strip containing sample slots was pushed into the upper gel. Protein extracts (about 15 μg of protein) were mixed with bovine-serum albumin (25 μg per application) and sucrose (10% w/v) and layered under upper-tray buffer in the slots. Bromphenol blue (0.05 ml saturated solution per liter) was included in the upper tray as a tracking dye. Electrophoresis was conducted at 300 V until albumin migrated to the bottom of the running gel. The gel was reacted with substrate (30 min at 37°), and the [^{14}C]IMP product was precipitated with 0.1 M $LaCl_3$–0.1 M Tris·HCl (pH 7.0) (26). Autoradiography was performed after repeated washing and dehydration of the gel (27).

TABLE 1. *HPRT-positive colonies after incubation of A_9 cells with chromosomes*

Experiment	Total no. of cells[a] (×10^{-6})	Positive plates/ total plates[b]	DNA[c] mol. wt. (×10^{-6})
1	6	5/12	30
2	25	2/50[d]	3
3	25	2/49[e]	30
4	50	0/100[f]	20
5	6	1/12	25
6	10	0/20[f]	1.5–30[g]
7	9	1/18	30
8A	10	1/20[h,i]	30–130
8B	10	3/20[h]	30–130

Chromosomes used in experiments 2, 4, and 8 were isolated at pH 7; otherwise chromosomes were isolated at pH 3. [a] Total number of A_9 cells incubated and subsequently plated. [b] Number of plates with one or more colonies per total number of plates inoculated. [c] Molecular weight of single-stranded DNA in the chromosome preparations (see *Methods*). [d] Colonies not confirmed by cloning and growth in HAT medium. [e] HeLa chromosomes incubated with A_9 recipient cells. [f] Incubation medium contained 2 mM $CaCl_2$ (monolayer medium). [g] Very heterogeneous molecular weight. [h] Ratio of cell equivalents of chromosomes to recipient cells was 10:1 in experiment 8B and 1:1 in experiment 8A; the ratio was about 1:1 in the other experiments. [i] This colony was a revertant.

Other Assays. Protein concentration was determined by the procedure of Lowry *et al.* (28) with bovine-serum albumin as the standard, and most assays were kindly performed by Mr. Miles Otey with a Technicon Autoanalyzer. Conductivity and pH measurements were performed at room temperature (24°). Particle concentrations were determined with an electronic counter (Celloscope).

Karyotypes. Cells were exposed to colcemid (0.2 μg/ml) for 3 hr, swollen in 1% Na citrate (20 min at 37°) or 75 mM KCl (20 min at 25°), and fixed with methanol–acetic acid (3:1). The fixed cells were applied to a cold, moist slide and spread by flaming before staining with crystal violet.

RESULTS

Isolation of HPRT-Positive Colonies after Incubation of A_9 Cells with Chromosomes. Isolated Chinese hamster chromosomes were incubated in suspension (see *Methods*) with mouse A_9 cells before plating the cells and the subsequent addition of selective medium (Table 1). Colonies appeared at a relatively low frequency of 10^{-6} in experiment 1 and about 10^{-7} in the combination of all experiments. A positive result was also obtained (experiment 3) with chromosomes isolated from HeLa cells. Since migration of cells may occur, resulting in satellite colonies, only one colony was scored for any plate, irrespective of the actual number of colonies observed, and each colony that was further analyzed was cloned from a separate plate. Local overgrowth of unaltered cells may also occur early before the addition of HAT, and slowly regress or persist for long intervals. Therefore colonies were cloned and cultured in the same selective medium and colonies lost in the cloning process were considered false positives. In two experiments designed to detect gene transfer from hamster chromosomes to A_9 cells, we used inactivated Sendai virus to mediate the transfer, but were unsuccessful.

Reversion of A_9 Cells. A control incubation performed in experiment 1 (Table 1), by use of identical procedures without chromosomes, resulted in no colonies. Larger numbers of cells (1.37×10^9) have also been plated in selective HAT medium, and only two revertant colonies were found. Thus, the A_9 cells have a very low rate of reversion that appears to be lower than the gene-transfer frequency, even considering that under the experimental conditions, cells could have doubled about three times before the HAT selective medium was added.

Detection of the Product of Gene Transfer In Vitro. The clones obtained in experiment 1 of Table 1 were propagated

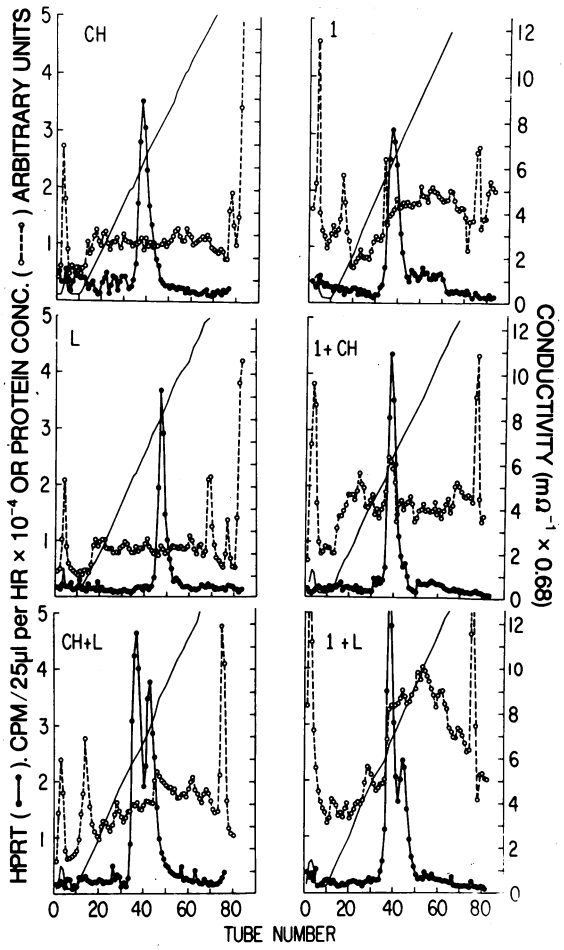

FIG. 1. Chromatography of crude enzyme extracts isolated rom Chinese hamster (*CH*), mouse (*L*), Clone 1 cells, and artificial mixtures of these solutions. Extracts (3–15 mg of protein) were applied to columns of DEAE-cellulose and eluted with a gradient of NaCl in 0.01 M Tris·HCl (pH 8.7). Fractions of 1 ml were collected and assayed for HPRT activity (●——●), protein (O- - -O), and conductivity (——). Enzyme activity is plotted at 0.5 the normal scale for L, 1 +CH, and 1 + L. Each unit of protein concentration (*left ordinate*) represents 100 μg/ml (CH, L, 1 +CH) or 70 μg/ml (CH + L, 1, 1 + L).

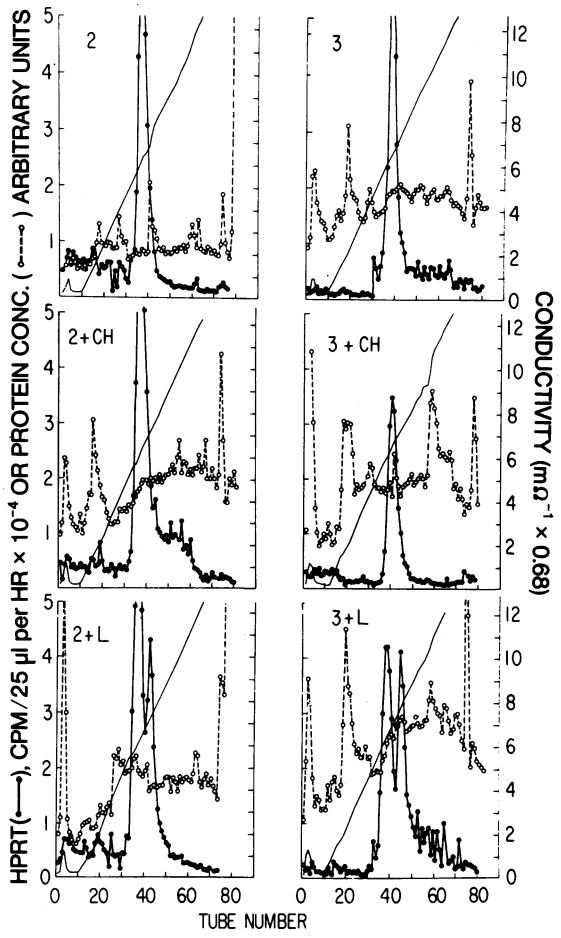

FIG. 2. DEAE-cellulose chromatography at 5° of HPRT extracts isolated from clones 2 and 3, and artificial mixtures of these solutions with Chinese hamster (*CH*) or mouse (*L*) extracts. Enzyme activity is plotted at 0.5 the usual scale for 3 +CH. Each unit of protein concentration (*left ordinate*) represents 100 μg/ml (2, 2 + L, 3 +CH) or 70 μg/ml (2 +CH, 3, 3 + L). See Fig. 1 and *Methods* for further details.

in suspension culture in HAT medium, and the high-speed (100,000 × *g*) supernatant fluids of freeze–thaw lysates were examined directly for HPRT activity (Table 2). The specific activities were similar to those obtained from extracts of Chinese hamster fibroblasts or wild-type mouse L_{929} cells, or the closely related, B_{82} thymidine-kinase-mutant L cells.

DEAE-Cellulose Chromatography of HPRT Extracts. Chromatography demonstrated a single peak of HPRT activity for both the mouse and hamster parental species (Fig. 1). However, the hamster HPRT is adequately resolved from mouse (L) enzyme when compared directly by elution position or conductivity at the point of emergence, or by mixture of the extracts before chromatography (*lower left*, Fig. 1). Clones from experiment 1 of Table 1 were similarly analyzed. Chromatography of an extract of clone 1 revealed a single peak of HPRT activity occurring in the position appropriate for hamster enzyme. The mixture of clone 1 extract with hamster HPRT again resulted in the single peak of activity, whereas the mixture with mouse enzyme disclosed two peaks of HPRT at the appropriate positions for each species.

Similar results are presented in Fig. 2 with two other clones (from experiment 1) alone or as artificial mixtures with hamster or mouse HPRT. Approximately equal quantities (enzyme activity) of each of the two components were used in all mixtures. The elution positions and half-widths as measured by elution volumes or conductivities of eluate for all of these clones, individually or mixed with hamster extract, are virtually identical with that obtained with the hamster enzyme alone. It therefore appears unlikely that the patterns result from three revertant clones that all fortuitously show chromatographic behavior very similar to that of the hamster enzyme. Furthermore, HPRT in extracts of thymidine-kinase-mutant L-cells (B_{82}) and the single A_9 revertant exhibit chromatographic behavior (not shown) identical with that of wild-type mouse HPRT. A fourth clone from experiment 1 (Table 1) was lost before chromatography, but it appeared to contain hamster enzyme, as determined by electrophoresis on cylindrical acrylamide gels. The fifth clone was lost before further study.

Chromatographic analyses (not illustrated) of the clone

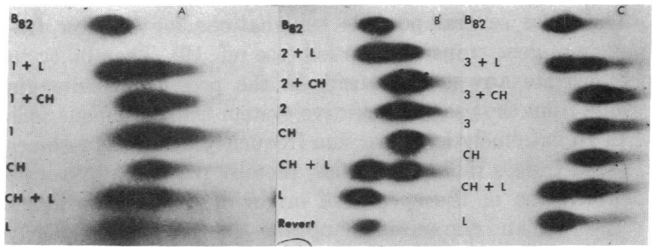

FIG. 3. Gel electrophoresis of HPRT extracts on vertical slabs of polyacrylamide at 5°. The individual extracts and artificial mixtures are identified by the same symbols as Figs. 1 and 2, and the A_9 revertant is also shown (*B*).

from experiment 5 of Table 1 and the three clones from experiment 8*B* of Table 1 also demonstrated the hamster-enzyme profile, while the clone from experiment 8*A* exhibited a profile that is identical with that of mouse HPRT, and therefore represents a revertant.

Acrylamide Gel Electrophoresis of HPRT. Mouse HPRT has a greater electrophoretic mobility than the hamster enzyme, from which it is adequately separated (Fig. 3). HPRT in extracts of clones 1, 2, and 3 from experiment 1 of Table 1 were all identical in mobility with hamster enzyme, when run alone or when mixed with hamster extract, whereas two bands resulted when artificial mixtures of these extracts with mouse HPRT were subjected to electrophoresis. Furthermore, the HPRT produced by the A_9 revertant (Fig. 3*B*) is not electrophoretically distinguishable from the wild-type mouse enzyme. Evidence (not shown) that the radioactive spots reflect the location of HPRT activity is provided by the fact that the spots were markedly attenuated when the gel was reacted with substrate at 5° rather than 37°, as well as by the fact that no radioactivity could be detected when 5-phosphoribosylpyrophosphate was omitted from the reaction mixture. No radioactivity was observed under any condition when an extract of A_9 cells was subjected to electrophoresis. Gel electrophoresis (not shown) also demonstrated that the HPRT products of the clone from experiment 5 and the three clones from experiment 8*B* of Table 1 were indistinguishable from the Chinese hamster enzyme, whereas the product of the clone from experiment 8*A* had the same electrophoretic mobility as the mouse HPRT.

Karyotypes. Histograms of the numbers of total chromosomes (Fig. 4) and biarmed chromosomes (not shown) in the clones from experiment 1 of Table 1 were closely similar to that of the parental A_9 cells. Karyotypes of all experimental cell lines clearly differ from the Chinese hamster karyotype, which exhibits a narrow mode of 23 chromosomes,

TABLE 2. *HPRT activity*

Cell type	Specific activity*	Cell type	Specific activity*
CH-V-79	196, 226, 354, 322	Clone 2	274, 254
L_{929}	134, 143, 151, 193	Clone 3	149
B_{82}	43, 142, 105	Clone 4	155
A_9	<0.01, <0.02	A_9 revertant	141
Clone 1	65, 160		

* nmol of IMP/hr per mg of protein.

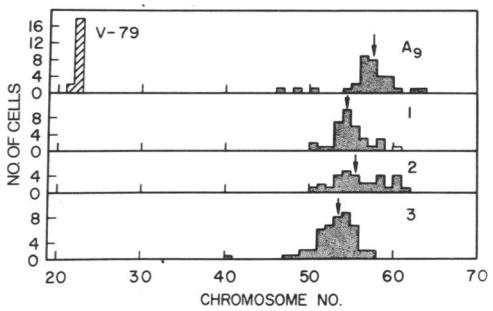

FIG. 4. Histogram of chromosomes in parental cell lines (V-79 and A_9) and in clonal lines (1, 2, and 3) from experiment 1 of Table 1. *Arrows* indicate the median number of chromosomes in each line.

indicating that none of these lines could have arisen by contamination of the cultures with hamster cells.

Stability of Genotype after Chromosome Transfer. The clones of experiment 1 of Table 1 were grown in selective HAT medium for several generations. After a shift to nonselective MEM spinner medium, the growth of each line was continued in suspension cultures for 2 months. Aliquots were removed at intervals for determination of plating efficiencies in MEM, HAT, and 20 μM 8-azaguanine, and the plating efficiencies in HAT relative to those in MEM are shown in Fig. 5. Clones 1 and 2 exhibited no detectible change in plating efficiency in the selective HAT medium during this entire interval, whereas there was a very rapid accumulation of HPRT-deficient cells when clone 3 was cultured in nonselective medium. The curve for clone 3 suggests that about 10–20% of the cells lose the *hprt* gene at each division. Similar reversion behavior has been reported by Schwarz *et al.* (29) for cells containing *hprt* on a chromosome fragment. The instability exhibited by clone 3 would be highly unlikely if it had arisen by reversion (back-mutation) of A_9 cells rather than by gene transfer, unless the parental A_9 cells had very marked selective growth advantage in MEM relative to the revertant.

DISCUSSION

The evidence for transfer of genetic information from ingested metaphase chromosomes to recipient cells and expression of this information by recipient mammalian cells can be summarized as: (1) A relatively high frequency of appearance

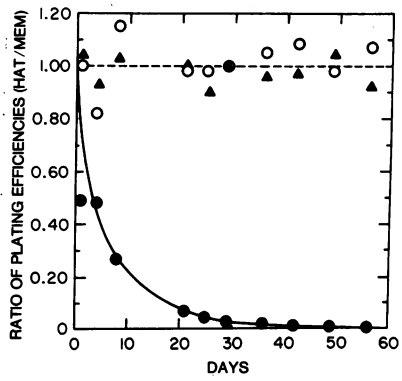

FIG. 5. Stability of the *hprt*$^+$ genotype in colonies after removing selective pressure. The plating efficiency in selective HAT medium compared to that in nonselective medium is plotted as a function of the time interval after the cells were removed from HAT medium. The cell lines depicted are clones 1 (○), 2 (△), and 3 (●——●) from experiment 1 of Table 1.

of colonies in selective medium when chromosomes are present, compared to the very low reversion frequency under similar conditions. (2) The rapid loss of the *hprt* gene by one of the clones (clone 3 of experiment 1), which is unexpected if the colony arose by reversion of parental cells. (3) The physical characterization of the enzyme (HPRT) product as indistinguishable from the chromosomal species and clearly different from the parental species, as shown by DEAE-cellulose column chromatography and acrylamide gel electrophoresis. This last point is the most convincing one.

Other possible explanations that have been considered, but appear extremely unlikely or completely inconsistent with the results, include the following:

(1) Reversion is inconsistent with any of the three points mentioned above and is especially refuted by the physical characterization of the gene product. Some revertants could occur involving mutation at a locus for a charged residue, resulting in a gene product that differed from the parental type. However, the possibility that the product of all revertants could be completely indistinguishable from that of the donor chromosome species by two methods of characterization seems remote. Furthermore, the single authentic revertant that was analyzed produced an HPRT that was not distinguishable from that of the parental (mouse) species. Schwarz *et al.* (29) also reported that an A_9 revertant produced HPRT that was electrophoretically identical to wild-type mouse enzyme.

(2) The possibility that the cultures were contaminated with a few wild-type (L_{929}) mouse cells is excluded by the characterization of the enzyme product and by the absence of similar colonies in control cultures.

(3) Contamination of the cultures with a few Chinese hamster cells or incomplete removal of these cells during the process of chromosome isolation is excluded by the fact that the karyotypes of the resultant clones were similar to that of the mouse species and totally different from that of the hamster species. Furthermore, no viable intact cells were detected under the conditions of chromosome isolation.

(4) It is unlikely that spontaneous fusion of any intact cells (surviving chromosome isolation and purification procedures) or nuclei with A_9 mouse cells is responsible for the observed results in view of the low number of intact cells and nuclei in the preparations. Furthermore, experiments performed under conditions more favorable for cell fusion, involving the use of inactivated Sendai virus, failed to result in colony formation. Also, no evidence for persistence of large numbers of hamster chromosomes was found in karyograms.

(5) Transformation of the cells by naked DNA or nucleoprotein cannot be excluded but it is considered unlikely. Most DNA or nucleoprotein would have been removed during the chromosome isolation by several centrifugations at 1000 $\times g$ for 30 min.

(6) Some nonspecific effect of added chromosomes is excluded by the physical characterization of the enzyme product as hamster type and the absence of gene transfer when chromosomes containing low molecular weight DNA were used (19). Any stimulation by degraded chromosomal products is unlikely since they would be rapidly diluted out during growth.

(7) Contamination of cultures with viruses or mycoplasma coding for an active HPRT is excluded by inability to culture mycoplasma from the clones, failure to observe HPRT-positive colonies in control cultures, and physical characterization of the enzyme product. However, the possibility that a transducing virus was present cannot be excluded.

There are several possible explanations for the low frequency of gene transfer observed (see ref. 19). The ability to demonstrate any gene transfer in the present experiments results from the use of a selective system using recipient cells with an extremely low reversion frequency and chromosomes isolated from a different species, thereby permitting positive identification of the species of origin of the gene product.

Mammalian chromosome uptake *in vitro*, particularly combined with the use of fractionated chromosomes, could provide a powerful tool for genetic mapping. However, the utility of this procedure would be increased by the development of methods for a greater efficiency of transfer and expression of the genetic information. The possible application of this technique to "gene modification" is open to considerably greater skepticism (30).

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