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Genetics

Co-transfer of human X-linked markers into murine somatic cells via isolated metaphase chromosomes

(gene transfer/dimethyl sulfoxide/somatic cell genetics/gene mapping)

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ABSTRACT **Transformation frequencies of 4×10^{-5} were obtained in chromosome-mediated gene transfer experiments using human cell line HeLa S3 as donor and mouse cell line A9 as recipient. This high frequency of interspecific transformation was achieved by treating the recipient cells with dimethylsulfoxide in addition to other facilitators. The high frequency of transformation correlated positively with transgenome size on the basis of both co-transfer of linked markers and chromosome analysis. The syntenic human markers glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) and phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) were sometimes transferred together with the selected X-linked prototrophic marker hypoxanthine phosphoribosyltransferase (IMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) into murine somatic cells. Donor human chromosome material could be demonstrated cytologically in some of the transformed cell lines. Transformants exhibited various rates of loss of the human hypoxanthine phosphoribosyltransferase marker when grown under nonselective conditions. These results reveal a broader range of possible interspecific transgenome sizes than has been recognized in the past. The largest transgenomes consist of cytologically detectable donor fragments and contain syntenic markers that are not closely linked to the selected marker.**

In mammalian systems, the metaphase chromosome is a biologically significant subunit of the genome that can be isolated and used as a vehicle to transfer selectable markers (1–5). In interspecific systems, which provide the most reliable biochemical and cytological data, co-transfer of loci tightly linked to a single selectable marker—thymidine kinase—has been demonstrated (6–8). The paucity of known systems that are both highly selectable and tightly linked, as well as the technical difficulties in dealing with infrequent transfer events, has limited the applications of a potentially powerful technique.

In lower organisms, transfer of DNA fragments in viral packages has been used to map small multigenic segments of the bacterial genome (9). Similarly, the ability to transfer mammalian DNA in subchromosomal size packages would provide structural and functional information on the genes within the chromosomally defined syntenic unit. The development of a chromosome-mediated gene transfer system by McBride and Ozer (10) in 1973 generated a great deal of interest, even though the transfer frequency of selectable markers was only 10^{-7} event per cell, which approaches the frequency of spontaneous mutations. In a few interspecific transformants analyzed by several groups, human hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) was transferred without the flanking markers phosphoglycerate kinase (PGK; ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) and (G6*P*D; D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.49) (11–13). Only the tightly linked markers thymidine kinase and galactokinase (GalK; ATP:D-galactose 1-phosphotransferase, EC 2.7.1.6) glucose-6-phosphate dehydrogenase have been co-transferred. It was generalized from these observations that the transferred genetic material ("transgenome") represents less than 1% of the donor haploid genome. Chromosome material was never detected cytologically in transformants, so indirect methods had to be used to locate the transgenome. It appeared that the stable transgenome was nonspecifically associated with single chromosomes (14–16). The unstable transgenome has not yet been located.

In 1977, Spandidos and Siminovich (15) utilized a calcium phosphate transfer technique and found increased chromosome-mediated gene transfer frequencies on the order of 10^{-6}–10^{-5} event per cell in an intraspecific Chinese hamster system. Although this increased frequency facilitated the analysis of individual loci, properties of the entire transgenome could not be assessed because the necessary biochemical and cytological markers were absent in this *intra*specific combination.

We here report that post-treatment with dimethyl sulfoxide (Me_2SO) increased the frequency of chromosome-mediated gene transfer in an *inter*specific system by an additional order of magnitude. The syntenic human markers G6*P*D and PGK were sometimes co-transferred together with the selected HPRT prototrophic marker into murine somatic cells. In some cases, donated human chromosome material could be demonstrated cytologically in "transformed" cell lines. The findings of Wullems *et al.* (17), to be discussed below, differ from our own in terms of both the hybrid system used and the results obtained. Implications of our findings and their potential applications are discussed.

MATERIALS AND METHODS

Human HeLa S3 cells (18) were maintained in Dulbecco–Vogt modified Eagle's medium (Gibco) containing 10% fetal calf serum (IBL). Murine A9 cells (19) — L-cells defective for HPRT and adenine phosphoribosyltransferase (AMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.7), were maintained in α modified Eagle's medium without ribonucleosides and deoxyribonucleosides (αMEM, Gibco) + 10% fetal calf serum. They were periodically passaged through medium containing 6-thioguanine (10 μg/ml) and 8-azaguanine (30 μg/ml) to select against HPRT revertants, which appear at a

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); PGK, phosphoglycerate kinase (EC 2.7.2.3); G6*P*D, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); GalK, galactokinase (EC 2.7.1.6); Me_2SO, dimethyl sulfoxide; αMEM, α modified Eagle's medium without ribonucleosides and deoxyribonucleosides; HAT, hypoxanthine/aminopterin/thymidine; HT, HAT without aminopterin.

rate of $\sim 10^{-8}$ (20). All cells were grown in monolayers in Corning T flasks at 37° and were harvested with Viokase (Gibco).

HeLa S3 cells were harvested by selective detachment after a 16-hr mitotic arrest in vinblastine sulfate (Eli Lilly) at 0.08 μg/ml. The initial cell pellet was washed once with 0.075 M KCl/vinblastine sulfate (0.1 μg/ml) at 37°. Chromosomes were prepared according to Willecke and Ruddle (11). The final preparation contained no detectable intact cells or nuclei.

Recipient cells were treated with chromosomes by using a modification of the method of Spandidos and Siminovitch (15, 21). A9 cells (2×10^6) were plated in 75-cm² flasks 26 hr in advance, which produced recipient populations of approximately 4.75×10^6 cells per flask. Before addition of chromosomes, the medium was removed and each flask was treated with 3 ml of a solution of 0.75 μg of colchicine (Sigma), 0.75 μg of Colcemid (Gibco), and 2.00 μg of cytochalasin D (Sigma) per ml in αMEM/10% fetal calf serum for 5 min at 37°. This solution then was replaced with 2 ml of chromosomes suspended in 250 mM $CaCl_2$. Gentamicin was added to a final concentration of 50 μg/ml, and the flasks were kept at room temperature for 30 min. Chromosomes were omitted from control flasks. Then 20 ml of αMEM/10% fetal calf serum/gentamicin (50 μg/ml) was added to each flask, and the flasks were incubated at 37°. After 4.25 hr, 10% Me_2SO (Sigma) was added to some of the flasks. All flasks were kept at room temperature for 0.5 hr. The medium was then completely replaced and the cultures were returned to 37° for 18 hr prior to replating.

Cells were distributed to flasks containing αMEM/10% fetal calf serum/gentamicin (50 μg/ml) plus hypoxanthine (13.6 μg/ml), aminopterin (0.19 μg/ml), and thymidine (3.9 μg/ml) (HAT) (22). Cells were plated at 0.5×10^6 cells per 75 cm², 0.5×10^5 cells per 25 cm², and, in some cases, 5.0×10^6 cells per 75 cm². Colonies could first be detected 5 days after replating. At 10 days, colonies in all flasks were counted. Individual colonies were isolated with stainless steel cylinders at 10–11 days. Gentamicin was removed from the medium at this time. One, or occasionally two, colonies per flask were picked and the remainder were treated with Wright's stain and recounted. Clones have been designated D(+) (Me_2SO post-treatment) and D(−) (no Me_2SO post-treatment). Clones isolated from the same flask were given the same numerical designation followed by the letter "A" or "B".

Starch gel electrophoresis was carried out as described (23, 24). Colonies were screened for the X-linked markers HPRT, G6PD, and PGK, as well as isocitrate dehydrogenase [*threo*-D_S-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42; human chromosome 2], nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1, human chromosome 4), GalK (human chromosome 17), and glucosephosphate isomerase (D-glucose-6-phosphate ketal-isomerase, EC 5.3.1.9; human chromosome 19). Alkaline Giemsa staining (25) was used to detect human chromosomal material against the mouse background of the recipient cells. The stability of transferred material was examined by carrying cell lines in nonselective αMEM/HT medium with determination of plating efficiency in αMEM/HAT and in αMEM/HT at regular intervals.

RESULTS

Frequency of Transfer. Post-treatment with Me_2SO produced a 10-fold increase in the frequency of chromosome-mediated gene transfer. Six flasks of A9 cells were exposed to HeLa S3 chromosomes and three of these flasks were subsequently treated with Me_2SO. When the cells were replated in selective HAT medium, colonies appeared within 5–10 days. Without post-treatment, we observed 0.29×10^{-5} colonies per replated cell; Me_2SO post-treatment produced 3.3×10^{-5} colonies per cell replated at the same density (Table 1). When the quantity of cells replated per flask was decreased by 1 order of magnitude, the Me_2SO-treated recipients still produced colonies in 12 to 15 flasks, whereas no colonies were present in the 12 flasks without Me_2SO treatment. The frequencies are equivalent to 0.45×10^{-5} colonies per A9 recipient cell without

Table 1. Statistical analysis of transfer frequencies

Selection flasks*	Counted,† colonies/cells	Frequency,‡ colonies/cell × 10^5	Poisson test§
	A9 + chromosomes + Me_2SO		
75 cm²; 12 flasks; 5×10^5 cells	$240/60 \times 10^5$	4.00 ± 0.90	$\chi^2 = 10.70$, df = 11 $0.250 < P < 0.500$
25 cm²; 15 flasks; 0.5×10^5 cells	$25/7.5 \times 10^5$	3.33 ± 2.69	$\chi^2 = 1.01$, df = 14 $P > 0.995$
	A9 + chromosomes		
75 cm²; 3 flasks; 50×10^5 cells	$29/150 \times 10^5$	0.19 ± 0.09	$\chi^2 = 4.41$, df = 2 $0.100 < P < 0.250$
75 cm²; 9 flasks; 5×10^5 cells	$13/45 \times 10^5$	0.29 ± 0.20	$\chi^2 = 5.47$, df = 8 $0.500 < P < 0.750$
25 cm²; 12 flasks; 0.5×10^5 cells	$0/6 \times 10^5$	0	NA
	A9 + control + Me_2SO		
75 cm²; 6 flasks; 5×10^5 cells	$0/30 \times 10^5$	0	NA

From the data of the flasks plated at 5×10^5 cells per 75 cm², Z = 13.73; from the data of the flasks plated at 0.5×10^5 cells per 25 cm², Z = 5.00, therefore, the probability of events with chromosomes + Me_2SO being the same as events with chromosomes but no Me_2SO is $\ll 0.001$ (26). Therefore, the Me_2SO enhancement is statistically significant.

* Refers to the size of the flask, the number of flasks, and the number of cells plated in each flask, for each selection set.

† Indicates total number of colonies in each selection set.

‡ Expressed in colonies per cell, this is based on colonies per flask. Data are shown as mean ± SD.

§ Test of equality of mean and variance (27); it shows no significant deviations from the Poisson distribution in any selection set. NA, not applicable (no colonies observed).

Table 2. Expression of X-linked isozymes in transformed cell lines*

	Clone	Original transfer flask†	G6PD	HPRT	PGK
1.	D(+) 21-01	I	M	H	M
2.	D(+) 21-02A	I	M	H	M
3.	D(+) 21-02B	I	M	H	M
4.	D(+) 21-03	I	HM	H	HM
5.	D(+) 21-04	I	HM	H	M
6.	D(+) 21-05	I	M	H	M
7.	D(+) 21-06	II	M	H	M
8.	D(+) 21-07	II	HM	H	M
9.	D(+) 21-08	II	M	H	M
10.	D(+) 21-09	II	M	H	M
11.	D(+) 21-10A	II	HM	H	HM
12.	D(+) 21-10B	II	M	H	HM
13.	D(+) 21-11	III	M	H	M
1.	D(−) 21-53A	IV	M	H	M
2.	D(−) 21-53B	IV	M	H	M
3.	D(−) 21-54A	V	HM	H	M
4.	D(−) 21-54B	V	M	H	M
5.	D(−) 21-55	VI	M	H	M
6.	D(−) 21-56	IV	M	H	M
7.	D(−) 21-57	IV	HM	H	M
8.	D(−) 21-58	IV	M	H	M
9.	D(−) 21-60	V	M	H	M
10.	D(−) 21-62	V	M	H	M
11.	D(−) 21-64	VI	M	H	M
12.	D(−) 21-65	VI	M	M	M

* M = mouse isozyme; H = human isozyme.

† Indicates clonal origin in one of six independent chromosome adsorption flasks. Cell extracts were analyzed by starch gel electrophoresis.

Me_2SO and 4.35×10^{-5} colonies per A9 recipient cell with Me_2SO when recipient cells are counted at the time of chromosome adsorption rather than at replating in selective medium, using the frequencies of the flasks plated at 5×10^5 cells per 75 cm^2. No colonies arose from control cells, which received similar treatment but with chromosomes omitted. Statistical analysis indicates that independent primary clones were being scored, rather than multiple colonies from single transfer events.

Human Gene Expression in Transgenotes. These were 13/13 D+ clones and 11/12 D− clones that clearly showed HPRT activity of the same electrophoretic mobility as that of human control cells (Table 2). Therefore, the human selectable marker had been transferred into recipient cells. The remaining clone, D(−) 21-65, displayed HPRT activity that comigrated with the murine form of HPRT (Fig. 1). It is assumed to be a revertant. None of the 25 clones expressed the human form of the autosomal isozymes isocitrate dehydrogenase, nucleoside, phosphosylase, GalK, and glucosephosphate isomerase.

PGK and G6PD, which are syntenic to the selected marker HPRT, were also analyzed. The human form of PGK was expressed in 3/13 D+ clones. Human G6PD was expressed in 4/13 D+ clones and in 2/12 D− clones. This includes two D+ clones that expressed all three human X-linked markers that were assayed. There were no instances in which two clones from a single isolation flask were identical in their expression of these human X-linked markers. Human PGK and G6PD were expressed strongly in some cases and weakly in others.

Cytological Detection of Human Chromosomal Material in Transgenotes. If the transgenome exists as a chromosome fragment, the presence of human material in metaphase spreads of co-transfer lines can be anticipated. Interspecific transfer of cytologically detectable chromosome material has not been previously reported with nonhybrid recipients. Alkaline Giemsa staining revealed human chromosome material in 7 of the 15 clones analyzed (Fig. 2). No more than one human chromosome fragment has been detected in a single line. This survey, which included five lines from preliminary experiments, was biased in favor of clones expressing human X-linked isozymes in addition to HPRT. Four lines that co-expressed human HPRT and PGK all contained visible human chromosome material in

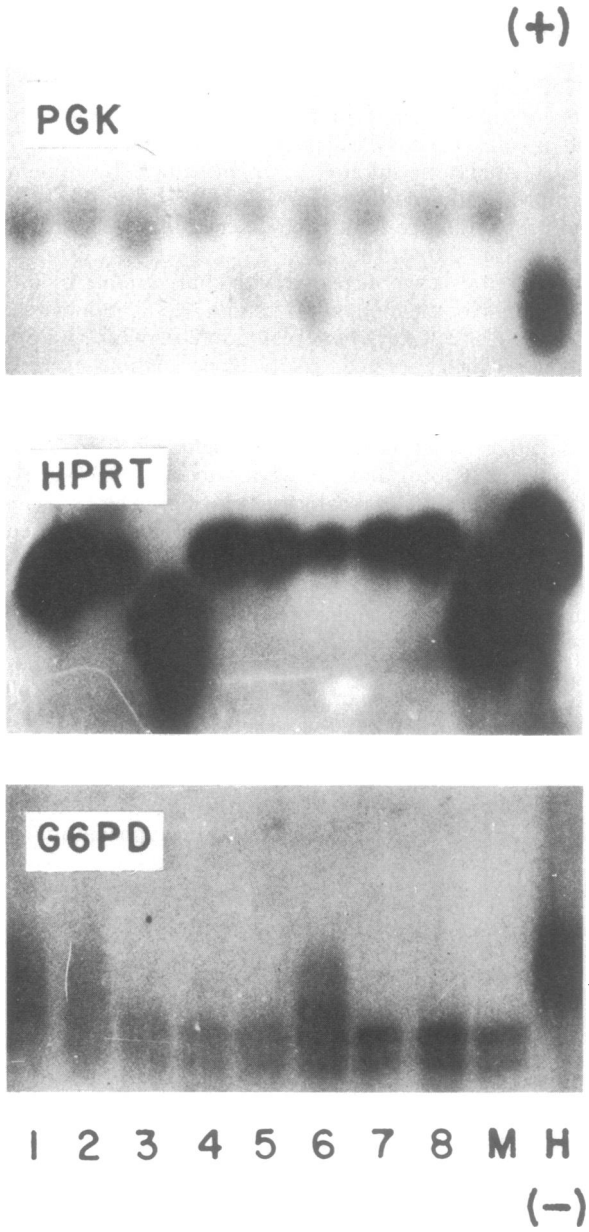

FIG. 1. Starch gel electrophoresis. All channels display mouse (A9) *PGK*. In addition, channel 5 displays weak human (HeLa S3) *PGK* and channel 6 displays a stronger human activity. Seven channels exhibit human (HeLa S3) *HPRT* alone; channel 3 exhibits mouse (liver) *HPRT* alone. A9 (not shown) did not exhibit any *HPRT* activity. Channels 1, 2, and 6 contain a human (HeLa S3)–mouse (A9) heteropolymeric *G6PD* pattern, whereas all other channels contain the mouse *G6PD* activity alone. Channels 1 through 8 show transformants D(−) 21-57, D(−) 21-54A, D(−) 21-65, D(−) 21-53, D(+) 21-10B, D(+) 21-03, D(+) 21-11, and D(+) 37A, respectively. M, mouse; H, human.

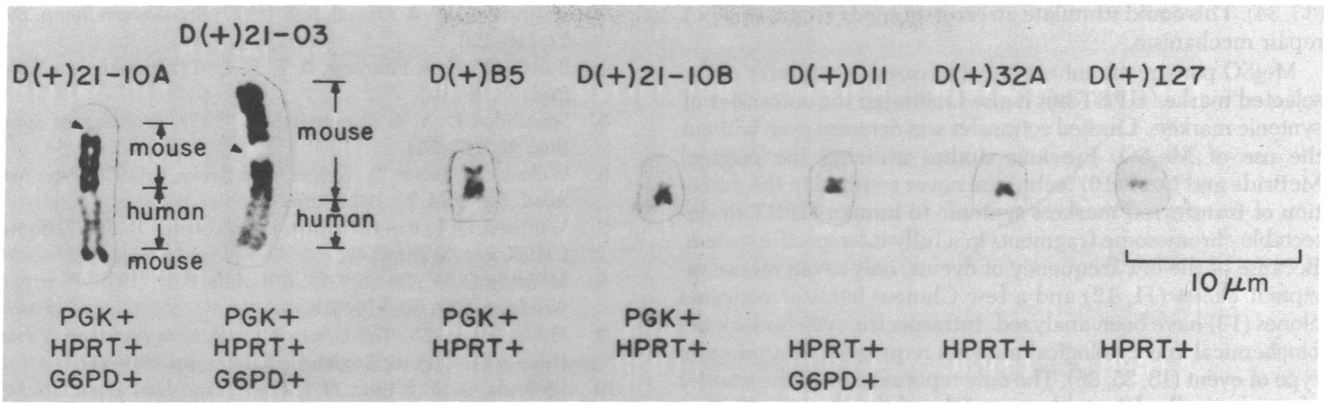

FIG. 2. Representative human staining material in different transformed cell lines detected with alkaline Giemsa. The first two chromosomes contain pale-staining human material and dark-staining mouse material in the arms, with characteristically pale mouse centromere regions (►). The remaining chromosomes consist of pale human-staining material only. Human X-linked isozymes expressed by each transformed cell line are recorded.

metaphase spreads. In the two cases of co-transfer without G6PD, a subtelocentric human fragment was present (Fig. 2). The two lines that expressed all three markers contained cells with long human-staining regions that had been translocated onto a large submetacentric A9 chromosome. Only one of four G6PD–HPRT co-transferrents contained human material detectable under our staining conditions. In this case, a small human fragment that seemed to have a centromeric region was present. A small dot-like chromosome of presumed human origin was present in one of seven lines that expressed HPRT alone; another line contained a slightly larger fragment.

Stability Analysis. Preliminary analyses revealed a range of stabilities in different clonal lines (Fig. 3). The highly unstable clones, which had loss rates greater than 5%/day, include D(−) 21-53, which expressed human HPRT, and D(+) 21-04, which expressed human HPRT and human G6PD. Both lacked visible human chromosome material. Transformant D(+) I27, which contained the smallest cytologically detectable human fragment, had a lower loss rate, slightly greater than 1%/day. Line D(+) 32A, containing a slightly larger human chromosome fragment, was even less unstable, losing the ability to clone in HAT medium at a rate less than 1%/day. D(+) 21-03 retained a high cloning efficiency in HAT medium after a period of 60 days while growing under nonselective conditions. The human donor material is translocated onto a recipient chromosome in this line.

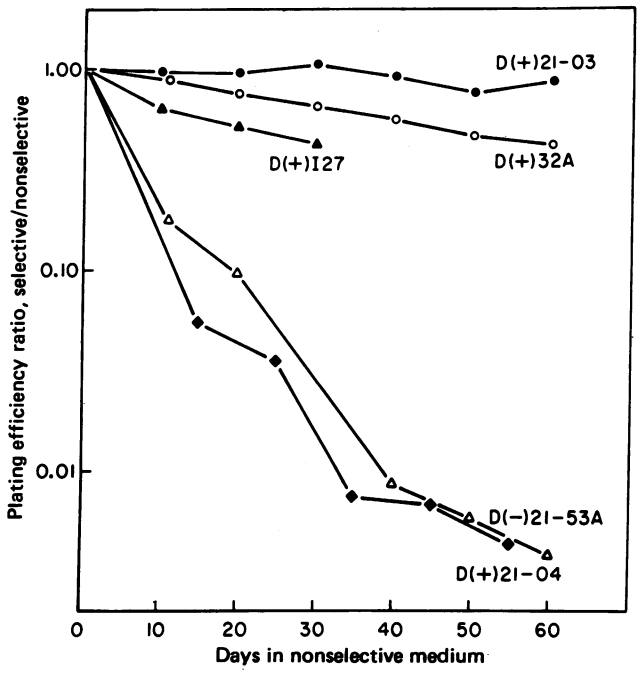

FIG. 3. Stability of HPRT marker under nonselective conditions in five transformed clones. At time 0, all five cell lines that had been maintained in αMEM/HAT selective medium were transferred to αMEM/HT nonselective medium. Samples of the cell lines were removed at the indicated times and tested for efficiency of colony formation in selective and nonselective media.

DISCUSSION

This study demonstrates that the choice of facilitators can alter the frequency of transfer of genetic material transferred via isolated chromosomes. A broad range of transgenome sizes is possible. The largest transgenomes consist of cytologically detectable donor chromosome fragments and contain syntenic markers that are not closely linked to the selected marker.

In our interspecific human HeLa S3-into-mouse A9 transfer system, the calcium phosphate transfer technique produces a 1-order-of-magnitude increase in the frequency of chromosome-mediated gene transfer, compared to earlier studies utilizing the traditional transfer techniques (1, 3). This finding confirms and extends the applicability of a viral DNA transfection technique (28) which was effective in the intraspecific metaphase chromosome transfer system of Spandidos and Siminovitch (15, 21, 29, 30). Our modified method, prior to Me_2SO enhancement, produces an interspecific HPRT transfer frequency of 2.9×10^{-6} event per cell, which is similar to the reported intraspecific Chinese hamster transfer frequency for the methotrexate-resistance marker (15, 29). Therefore, species specificity does not impose functional limitations on the increased frequency of single-locus transfer produced by this technique.

The mechanism by which a Me_2SO treatment after chromosome adsorption creates an additional 10-fold increase in the transfer frequency is not known. In the related viral transfection system, Me_2SO post-treatment facilitates the transfer of purified herpes virus DNA (31). Me_2SO may influence the properties of the cell membranes. Me_2SO enhances polyethylene glycol-induced fusion of cellular membranes (32), and Wullems *et al.* (13) have found that an unrelated fusogen, inactivated Sendai virus, increases chromosome-mediated gene transfer into nonmitotic recipient cells. Me_2SO may also affect DNA metabolism. For example, in the Friend erythroleukemia system, Me_2SO treatment appears to induce an endodeoxyribonuclease and single-stranded nicking of the cellular DNA

(33, 34). This could stimulate an error-prone UV type of DNA repair mechanism.

Me_2SO post-treatment not only increased the transfer of the selected marker HPRT but it also facilitated the cotransfer of syntenic markers. Limited cotransfer was detected even without the use of Me_2SO. Previous studies utilizing the original McBride and Ozer (10) technique never resulted in the detection of transferred markers syntenic to human HPRT or detectable chromosome fragments in a fully interspecific system. Because of the low frequency of events, only seven mouse recipient clones (11, 12) and a few Chinese hamster recipient clones (13) have been analyzed. Intraspecific systems lack the biochemical and cytological markers required to examine this type of event (13, 35, 36). The only reported case of the transfer of cytologically detectable material and the flanking X chromosome markers made use of a hybrid recipient population (17). In this instance, the transferred genetic material was an intact X chromosome in each of 16 transferrents analyzed.

By contrast, we observe a range of subchromosomal transgenome sizes. Some of our transgenotes express HPRT without the flanking markers and contain no visible chromosomal fragments, as was found in the earlier interspecific studies. Other transgenotes contain cytologically detectable donor chromosome fragments and co-express both linked markers, and still others represent intermediate situations. It is possible that some of our chromosomes were not simply broken but were rearranged in a more complex manner. Our initial cytologic and isozyme data imply that HPRT lies between PGK and G6PD, but closer to the latter marker. This is in accord with the regional mapping of the X chromosome (37) and the irradiation-induced gene segregation data of Goss and Harris (38, 39).

The interpretation of intraspecific chromosome-mediated cotransfer in the Chinese hamster system has been based on the assumption that a transgenome always represents less than 0.2% of the donor genome (5, 21, 29). The observation of larger chromosome fragments in some of our interspecific transfer events, which have been generated by using similar techniques, raises other possibilities. A full assessment of the properties of the intraspecific transfer system should help clarify the situation.

The enhanced frequency of transfer events plus the wide range of possible transgenome sizes, which can be readily obtained with Me_2SO post-treatment, broadens the range of potential applications for chromosome-mediated gene transfer. It is now feasible to map entire chromosomes in both quantitative and cytological terms. Moreover, transgenomes that are smaller than recipient chromosomes, especially "dot chromosomes," offer good starting material for gene isolation. Differential centrifugation or flow sorting could provide a purified transgenome, resulting in substantial enrichment of the transferred gene. The ability to transfer biologically active fragments of isolated chromosomes should facilitate the structural and functional analysis of the chromosomal unit.

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