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Genetics

Stable association of the human transgenome and host murine chromosomes demonstrated with trispecific microcell hybrids

(somatic cell genetics/gene transfer/chromosome transfer)

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ABSTRACT **Trispecific microcell hybrids were prepared by transferring limited numbers of chromosomes from a human/mouse gene-transfer cell line to a Chinese hamster recipient line. The donor cells employed were murine L-cells that stably expressed the human form of the enzyme hypoxanthine phosphoribosyltransferase. Karyotypic, zymographic, and back-selection tests of the resulting human/mouse/Chinese hamster microcell hybrids provided strong genetic evidence for a stable association of the human transgenome with host murine chromosomes in stable gene-transfer cell lines. This association, which may represent physical integration of the transgenome into the host cell genome, occurred at multiple chromosomal sites.**

Chromosome-mediated gene transfer is a technique that allows small pieces of genetic material to be transferred from one mammalian cell to another (1–6). This process, which was convincingly demonstrated by McBride and Ozer in 1973 (1), utilizes isolated metaphase chromosomes as vectors for genetic exchange. According to current views (7), chromosomes enter the recipient cells by phagocytosis and are subsequently degraded by lysosomal enzymes. By using appropriate selective conditions, transformant clones that express a particular donor-derived phenotype can be isolated at low frequency. Both intra- and interspecific gene transfer experiments have been reported using mouse, Chinese hamster, and human somatic cells, and a variety of selectable markers have been transferred (1–8).

The transformant cell lines generated by chromosome-mediated gene transfer share the following general properties. First, no donor chromosomes or chromosome fragments are detectable cytologically. Second, isozyme analyses of transformant cell lines unambiguously demonstrate expression of the complementing gene derived from the donor cells (1, 3–6, 8), and, in some cases (9, 10), coexpression of tightly linked loci. Unlinked markers characteristic of the donor cells are not present in the transformant clones (3). Third, expression of the transferred marker may be stable or unstable. Unstable lines lose the transferred genetic element ("transgenome") at different rates (usually 1–10% per cell generation) (1, 3, 10), and can give rise to stably transformed lines upon prolonged cultivation (6, 9). Such stable lines seem to express the transferred marker as an integral genetic element of the host cell.

Little is known concerning the state, physical nature, or location of the transgenome in either stable or unstable gene transfer clones. The properties of unstable versus stable transformants are consistent with the view that the transgenome is an autonomous genetic entity in unstable lines. According to this view such cells could give rise to stably transformed lines by the physical insertion of the transgenome into host cell chromosomes. This working hypothesis predicts a stable association of the transgenome with recipient chromosome(s) in stable transformants. In fact, indirect evidence supporting this view has recently been obtained in serial gene transfer experiments (8, 11). In this report we describe studies employing recently developed techniques for microcell-mediated chromosome transfer (12) which provide direct evidence for a stable association of the transgenome with host cell chromosomes. Furthermore, we show that this association is not a site-specific process and does not occur at the homologous locus of the recipient cell genome. Rather, the transgenome can become associated with a variety of chromosomes of the host cell.

MATERIALS AND METHODS

All cell lines were maintained in monolayer culture at 37° under 10% CO_2/90% air in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (International Biological Laboratories). Parental cell lines and microcell hybrids were found to be free of mycoplasma by the culture method of Hayflick as modified by Barile (13).

CT11C is one of the three independent gene transfer clones originally isolated by Willecke and Ruddle (3). These lines were prepared by transferring the human gene for hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) into HPRT$^-$ murine L-cells (A9) using donor metaphase chromosomes derived from HeLa S3. All three clones expressed only the human form of HPRT as judged by electrophoretic and immunochemical tests. Upon prolonged cultivation one of these unstable lines (CT11C) gave rise to a stable transformant which is designated CT11C$_I$. This stabilized population was used without subcloning in the experiments reported here.

Microcell hybrids were prepared by transferring limited numbers of chromosomes from CT11C$_I$ into HPRT$^-$ Chinese hamster E36 cells (14) and selecting for the expression of (human) HPRT using the hypoxanthine/aminopterin/thymidine (HAT) selective system (15). The generation of such microcell hybrids has been described elsewhere (12). Independent microcell hybrid clones were picked by the ring technique and expanded. Electrophoretic analyses of hybrid cell extracts were performed using published procedures (16, 17). Karyotyping was accomplished using a sequential staining technique (18) involving Giemsa/viokase banding followed by Hoechst 33258 staining. The distribution of introduced murine chromosomes in each clone was determined by scoring Hoechst-stained metaphase spreads and interphase nuclei for the presence of bright chromocenters diagnostic of the centric heterochromatin of murine chromosomes.

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; HAT, hypoxanthine/aminopterin/thymidine.

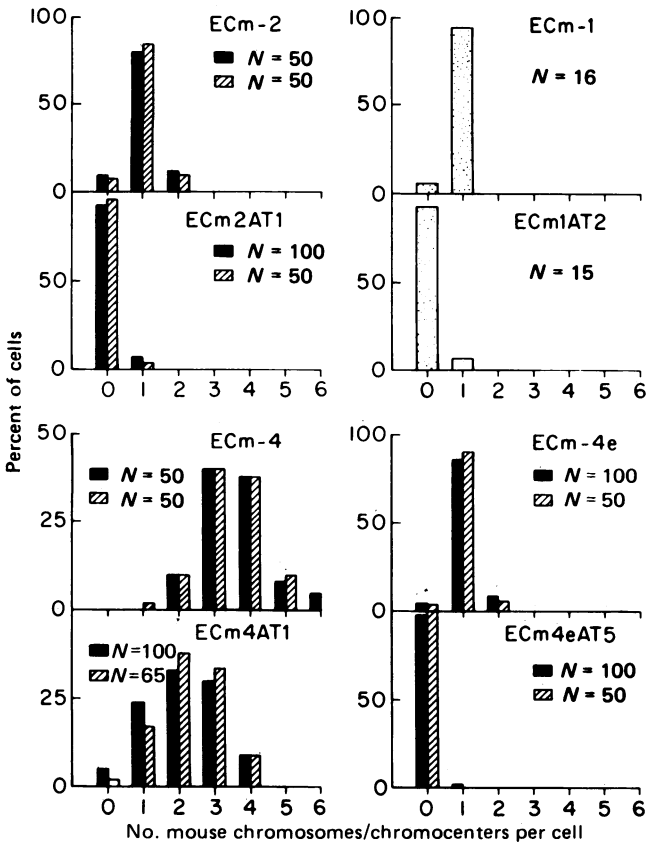

FIG. 1. Distribution of introduced murine chromosomes in trispecific microcell hybrids and their back-selectants (indicated by AT in the name of the cell line). The data were obtained by scoring Hoechst 33258-stained interphase nuclei (solid bars) or metaphase spreads (hatched bars) for the presence of high fluorescence intensity chromocenters or by karyotyping Giemsa/viokase-banded preparations (stippled bars).

All hybrid clones were subject to back-selection in medium containing 6-thioguanine (10 μg/ml) and 8-azaguanine (30 μg/ml) and the resulting HPRT$^-$ populations were characterized as described above without subcloning.

RESULTS

The microcell hybrids generated by fusion of CT11C$_I$ microcells with intact E36 recipients had either a single (1S hybrids) or a double (2S hybrids) input of Chinese hamster chromosomes characteristic of E36 (1S modal chromosome number = 21) and also contained from one to four mouse chromosomes derived from CT11C$_I$. In addition, electrophoretic analyses of extracts prepared from each of the microcell hybrids demonstrated that the cells expressed the human and only the human form of HPRT. We have used the descriptive term "tribrid" to emphasize the trispecific nature of these cells. The characterization of individual tribrid clones is considered in detail below.

Fig. 1 shows the distribution of introduced mouse chromosomes in several tribrid lines and their 8-azaguanine-, 6-thioguanine-resistant back-selectants. ECm-2 was a typical 1S tribrid that contained 21 Chinese hamster chromosomes. In addition, the majority (>80%) of cells in this population contained a single mouse chromocenter. Detailed karyotyping of Giemsa/viokase-banded preparations (25 metaphase spreads examined) showed that a particular small telocentric murine chromosome derived from CT11C$_I$ was present in >90% of the cells (Fig. 2).

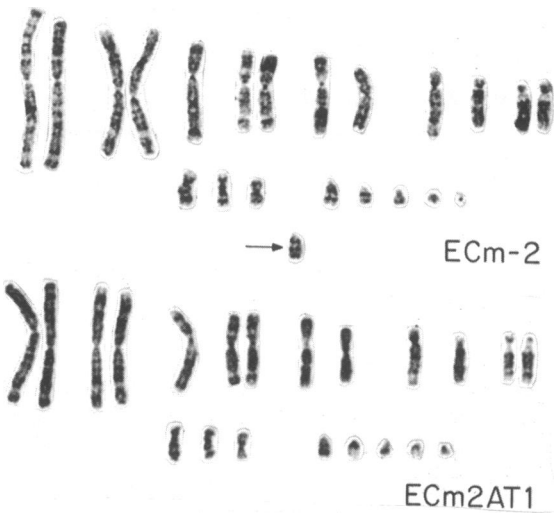

FIG. 2. Representative Giemsa/viokase-banded karyotypes of ECm-2 and ECm2AT1. The arrow indicates the single murine chromosome present in the primary tribrid, which segregated concordantly with human HPRT activity.

Electrophoretic analyses were performed for twenty isozymes representing at least eight murine linkage groups (Table 1). ECm-2 clearly expressed the human and only the human form of HPRT (Fig. 3). Therefore, the transgenome-encoded selectable marker had been successfully transferred from a stable gene-transfer line to a hamster recipient using a chromosome transfer methodology. Despite the presence at high frequency of a particular mouse chromosome in ECm-2, no murine isozymes were detected in the clone. This was not unexpected because the presence of about half of the mouse linkage groups would not have been revealed by our isozyme analyses. Significantly, murine α-galactosidase, an X-linked marker, was not present in ECm-2.

ECm-2 was subjected to back selection in medium containing azaguanine and thioguanine and three independently derived subpopulations were produced (designated ECm2AT1, -2, and -3). These subpopulations had lost expression of human HPRT. Concordant with the loss of human HPRT activity, the telocentric murine chromosome characteristic of ECm-2 was also lost. Fig. 1 shows that >90% of the cells of the ECm2AT1 back-selectant population contained no murine chromosomes. A representative karyotype of this population is shown in Fig. 2. A small fraction of the population (<10%) contained a single mouse chromosome, but this chromosome was distinct from that present at high frequency in ECm-2. Such cells probably arose from ECm-2 cells initially containing two different mouse chromosomes.

The HPRT$^-$ back-selectants were subjected to selection in HAT-containing medium. No HAT-resistant, HPRT$^+$ colonies could be isolated though as many as 10^7 cells were plated in HAT selective medium.

These data show concordant segregation of *human* HPRT activity and a particular *murine* chromosome and provide strong genetic evidence for the hypothesis that the transgenome had become stably associated with a host chromosome in CT11C$_I$. Because neither murine X chromosomes nor X-linked markers were present in ECm-2, this association seems not to have occurred at the homologous locus of the murine genome. These conclusions were strengthened and amplified by characterizing other independent tribrid clones.

ECm-1 was a 2S tribrid clone that contained about 40 Chinese hamster chromosomes. A single murine chromosome was

Table 1. Isozyme expression in trispecific microcell hybrids and their derivatives

Isozyme	Murine chromosome assignment	Hybrids and derivatives							
		ECm-1	ECm1AT1, ECm1AT2, ECm1AT3	ECm-2	ECm2AT1, ECm2AT2, ECm2AT3	ECm-4	ECm4AT1, ECm4AT2, ECm4AT3	ECm-4e	ECm4eAT1, ECm4eAT2, ECm4eAT3
Dipeptidase-1	1	C		C		C	C	C	C
Phosphoglucomutase-2 (EC 2.7.5.1)	4	C		C		C	C	C	C
6-Phosphogluconate dehydrogenase (EC 1.1.1.43)	4	C		C		C			
Glucosephosphate isomerase (EC 5.3.1.9)	7	C		C		C	C	C	C
Glutathione reductase [NAD(P)H] (EC 1.6.4.2)	8	C		C		C	C		
Adenine phosphoribosyltransferase (EC 2.4.2.7)	8	C		C		C			
Malate dehydrogenase (decarboxylating)-1 (EC 1.1.1.40)	9	C		C		C			
Mannosephosphate isomerase (EC 5.3.1.8)	9	C		C		C	C		
Galactokinase (EC 2.7.1.6)	11	C		C		C			
Purine-nucleoside phosphorylase (EC 2.4.2.1)	14	CM	C	C	C	CM	C	CM	C
Esterase-10	14	CM	C	C	C	CM	C	CM	C
α-Galactosidase (EC 3.2.1.22)	X	C		C		C	C		
Hypoxanthine phosphoribosyltransferase (EC 2.4.2.8)	X	H	—	H	—	H	—	H	—
Adenosine kinase (EC 2.7.1.20)	na	C	C	C	C	C	C		
Ribokinase (EC 2.7.1.15)	na	C		C		C			
Uridine phosphorylase (EC 2.4.2.3)	na	C	C	C	C	C	C		
Triosephosphate isomerase (EC 5.3.1.1)	na			C		C			
Dipeptidase-2	na	C		C		C	C		
Peptidase-S	na	C		C		C	C	C	C
Tripeptidase-1	na	CM	C	C	C	C	C		

Extracts of the hybrid cell lines were analyzed according to published procedures (16, 17). The data for the three HPRT$^-$ back-selectant populations produced from each primary tribrid are presented in a single column. The murine chromosome assignment refers to the normal diploid karyotype. C, Chinese hamster; M, mouse; H, human; —, isozyme activity not detected; na, not assigned.

present in >90% (15/16 karyotypes) of the cells (Fig. 1). This large telocentric chromosome was one of several present in CT11C$_I$ (and in its A9 parent) that did not exhibit a brightly fluorescent centromere when stained with Hoechst 33258 (19). The banding characteristics of this chromosome and the isozyme data (see below) suggested that it was a rearranged murine chromosome 14.

ECm-1 expressed the human form of HPRT (Fig. 3). Murine isozymes were also present in this tribrid clone (Table 1). These included purine-nucleoside phosphorylase and esterase-10, syntenic markers assigned to mouse chromosome 14, as well as tripeptidase-1, an isozyme that has not yet been mapped in the murine genome. No other mouse isozymes were detected in ECm-1, including the X-linked marker α-galactosidase.

The azaguanine and thioguanine-resistant back-selectants of ECm-1 (ECm1AT1, -2, and -3) no longer expressed human HPRT. Moreover, none of the three back-selectants expressed the murine forms of purine-nucleoside phosphorylase, esterase-10, and tripeptidase-1 (Table 1). Karyotypes showed that the large telocentric chromosome present in the ECm-1 primary tribrid had been uniformly segregated from the back-selectants. The data for ECm1AT2 are illustrated in Fig. 1. Fourteen of fifteen karyotypes consisted only of hamster chromosomes. In one karyotype, one murine chromosome was present but was distinct from the mouse chromosome-14-analogue present in ECm-1. As was observed for the ECm-2 back-selectants, no HAT-resistant colonies could be recovered from ECm1AT1, -2, or -3, using inocula as large as 10^7 cells.

These data have shown that in both ECm-1 and ECm-2 the human transgenome was associated with a murine chromosome with which it segregated concordantly. However, the particular mouse chromosome involved was clearly distinct in these two tribrid clones. We conclude that the stable association of the

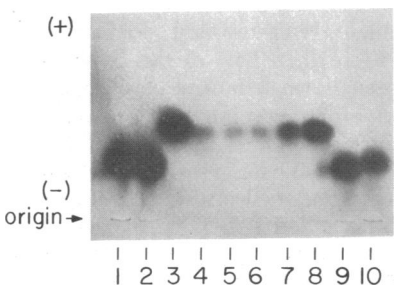

FIG. 3. Autoradiograph of HPRT activity in extracts after electrophoretic separation (16, 17). Channels 1 and 10, mouse (kidney) control; 2 and 9, Chinese hamster (kidney) control; 3 and 8, human (HeLa S3 cells) control. Only human HPRT activity was present in the trispecific microcell hybrids ECm-1, ECm-4, ECm-2, and ECm-4e (channels 4, 5, 6, and 7, respectively).

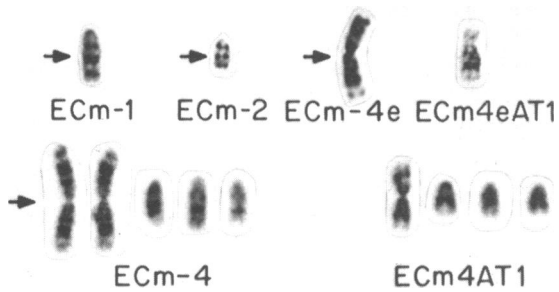

FIG. 4. Representative Giemsa/viokase-banded partial karyotypes showing the murine chromosomes in trispecific microcell hybrids and their HPRT$^-$ back-selectants. Arrows indicate mouse chromosomes that segregated concordantly with human HPRT activity in the different clonal lines.

transgenome and host cell chromosomes can occur at multiple chromosomal sites.

ECm-4 was a 2S tribrid clone which was karyotypically heterogeneous. Individual cells of this clonal population contained two to five introduced mouse chromosomes (Fig. 1). Among these were several different telocentric chromosomes, none of which was present in the population at high frequency. All cells karyotyped also contained at least one metacentric murine chromosome. Upon selection for the HPRT$^-$ phenotype, a single mouse chromosome disappeared from the population (Fig. 1). The particular chromosome lost seemed to be one of the murine metacentrics.

The metacentric mouse chromosomes present in ECm-4 were very similar in size and in banding pattern. However, in very well-banded preparations it became clear that there were two distinct metacentric chromosomes derived from CT11C$_I$ present in ECm-4. One of these chromosomes was found in about 50% of the karyotypes of both ECm-4 and its ECm4AT1 back-selectant. This was a murine isochromosome 15 (Fig. 4). The other metacentric was present at high frequency in ECm-4 but absent from its back-selectants. One arm of this chromosome had a banding pattern identical to that of a normal mouse chromosome 15, while the other arm seemed to be derived from murine chromosome 14. These data indicated that the human transgenome had become stably associated with a particular metacentric murine chromosome (designated 14/15) composed of elements of mouse chromosomes 14 and 15. This interpretation was strengthened by isozyme analyses which showed concordant segregation of human HPRT activity with the phenotypic markers of murine chromosome 14, purine-nucleoside phosphorylase and esterase-10 (Table 1). In order to verify this conclusion, we performed subcloning experiments.

Ten independent, HAT-resistant subclones were prepared from ECm-4. More than 85% of the cells of one subclone (ECm-4e) contained a single murine chromosome (Fig. 1). The particular murine chromosome present in the cells was the 14/15 metacentric of ECm-4 and was found in all 28 cells karyotyped (Fig. 4). Like ECm-4, ECm-4e expressed murine esterase-10 and purine-nucleoside phosphorylase in addition to human HPRT (Table 1).

Concordant segregation of human HPRT activity and the two isozymes of mouse chromosome 14 was observed in three azaguanine- and thioguanine-resistant back-selectants (ECm4eAT1, -3, and -5) prepared from ECm-4e (Table 1). Fig. 1 shows that nearly 100% of the cells of ECm4eAT5 did not contain any mouse chromosomes. Analysis of another back-selectant, ECm4eAT1, revealed a murine chromocenter distribution identical to that of ECm-4, i.e., nearly 90% of the cells contained a single mouse chromosome. This chromosome arose from the 14/15 metacentric by a deletion of the terminal two-thirds of one of its arms (Fig. 4). Cells containing such a rearranged chromosome expressed neither murine nucleoside phosphorylase nor esterase-10. Therefore, it is clear that in both ECm-4 and ECm-4e the human transgenome had become stably associated with a 14/15 metacentric murine chromosome, and that the site of this association was that portion of the chromosome derived from mouse chromosome 14. In the ECm4eAT3 back-selectant population, one-third of the cells contained no murine chromosomes, while two-thirds of them contained a terminally deleted 14/15 metacentric identical to that found in ECm4eAT1.

DISCUSSION

The studies described in this report provide direct evidence that in stable gene-transfer cell lines the (human) transgenome had formed a stable association with host (murine) chromosomes. This association was not a site-specific process and did not occur at the homologous locus of the mouse genome. Rather, the particular murine chromosome that segregated concordantly with the transgenome-encoded selectable marker varied from one microcell hybrid clone to another. This finding indicated that multiple associations between the transgenome and host chromosomes had formed during conversion of the CT11C cell line from the unstable to the stable phenotype.

While it is clear that the association of the human transgenome with host murine chromosomes can occur at multiple chromosomal sites, the specificity of this process remains to be elucidated. Such an analysis would require that the site of association of the transgenome be determined in a number of independent, stable gene-transfer clones. The specific transgenome involved might also play a role in the determination of association sites.

The precise nature of the association between the transgenome and host chromosomes cannot be determined from genetic studies. However, the association was extremely stable, such that dissociation of the human transgenome from the mouse chromosome with which it associated was never observed. This association did not generally result in chromosomal rearrangements that were detectable cytologically, i.e., the chromosomes of CT11C$_I$ that carried the transgenome were indistinguishable from their counterparts present in A9. However, fragmentation of murine chromosomes associated with the human transgenome has occasionally been observed. For example, two independent tribrid clones produced in the same hybridization experiment as the microcell hybrids described in this report contained small, centromere-containing fragments derived from murine chromosomes. The human transgenome was stably associated with these fragments. In addition, two of the three HPRT$^-$ back-selectant populations derived from ECm-4e and described here contained a 14/15 metacentric murine chromosome that had undergone a terminal deletion. It is tempting to speculate that the site of association of the transgenome with such chromosomes was adjacent to the deletion break point. The genetic instability of the region might be caused by such an association. Alternately, such instability at discreet loci might increase the probability of association of the transgenome with these sites.

It seems likely that the stable association between the transgenome and host chromosomes corresponds to an actual physical integration of the transgenome into host cell genetic material. Such a model is consistent with all known properties of stable gene transfer cell lines. In contrast, no evidence has been obtained for an association of the transgenome with host

chromosomes in unstable lines. Using microcells derived from various unstable gene-transfer clones in chromosome transfer studies similar to those reported here, we have been unable to introduce the transgenome-encoded selectable marker into HPRT$^-$ hamster cells despite repeated attempts (six hybridization experiments).

Irrespective of the exact mode of interaction of the transgenome with host chromosomes, our results indicate that this association is sufficiently stable to allow the construction of new genetic tools. Specifically, it is now possible to introduce a transgenome-encoded selectable marker into a variety of chromosomes of any mammalian species. Using a chromosome transfer approach, these marked chromosomes can be transferred to a third species which would serve as a carrier. Thus, a series of hamster cell lines could be constructed, each containing a single and unique murine chromosome carrying the human transgenome and maintained in the population at high frequency by selection. Such novel gene assignment panels would be precisely defined and would eliminate the karyotypic instability that has been encountered in more conventional gene assignment panels. By appropriate experimental design, such panels could be constructed for any mammalian species of choice.

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