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Review

Results and Prospects of Chromosomal Gene Transfer between Cultured Mammalian Cells

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Introduction

The fusion of two somatic cells can be considered as the transfer of the total genome from one somatic cell to another somatic cell. During the last 20 years important methodical details of this gene transfer system, such as the isolation and characterization of suitable mutations, selection techniques, and procedures for isozyme and karyotype analyses to follow the segregation of chromosomes from hybrid cells, have been worked out (for reviews see Chu and Powell 1976; Ruddle and Creagan 1975). The knowledge of these basic experimental procedures was used in attempts to fractionate the genome of one cell and to use purified metaphase chromosomes as vectors for the transfer of genetic information into cultured recipient cells. The first convincing demonstration of chromosomal gene transfer was reported by McBride and Ozer (1973a) as the transfer of the gene coding for Chinese hamster hypoxanthine phosphoribosyl-transferase activity (HPRT) via purified chinese hamster chromosomes into established mouse L-A9 cells defective in functional mouse HPRT. Clones which expressed Chinese hamster HPRT were grown in selective HAT medium containing hypoxanthine, aminopterin, and thymidine (Szybalska and Szybalski 1962; Littlefield 1964). The frequency of these gene transfer clones (or *transferent cells* as they will be referred to in this paper, see Degnen et al. (1976) was similar ($1 \cdot 10^{-7}$) to that of spontaneous reversions in the defective mouse HPRT gene. Therefore the HPRT activity of every clone which grew up from chromosomal gene transfer experiments had to be analyzed in order to rule out revertant cell clones. Since this initial report, a number of laboratories have contributed further to the characterization and application of the chromosomal gene transfer system. The general statements to be presented in this review concerning the state of the transferred gene(s) (or *transgenome* as it will be referred to in this paper), appear to be a reasonable extraction of our present knowledge. It should be pointed out that in this review emphasis will be placed on the experimental conclusions of the rewieved papers. The major reports from different laboratories will be discussed under the corresponding heading summarized from the results of several contributions. For more experimental details the reader is referred to the original publications or to a previous review by McBride and Athwal (1976).

1. Fragments of the Donor Chromosomes are Found in Transferent Recipient Cells

The original report on chromosomal gene transfer by McBride and Ozer (1973a) did not approach the question whether or not an intact chinese hamster donor chromosome was expressed in transferent mouse cells. This question could be studied by using purified human metaphase chromosomes for gene transfer. In addition to the gene for human HPRT, several other genes had been regionally assigned to the human X chromosome. After transfer of the gene for human HPRT via human chromosomes (from Hela cells or lymphoblastoid cells) into LA9 mouse cells or Chinese hamster cells, only the expression of human HPRT could be detected in transferent mouse or Chinese hamster clones. The genes for phosphoglycerate kinase (PGK) and glucose 6-phosphate dehydrogenase (G6PD), both genes flanking the HPRT gene, were not expressed. (Willecke and Ruddle 1975; Burch and McBride 1975; Wullems et al. 1975). Later it was found that a newly discovered X-linked human surface antigen, Sax, (Buck et al. 1976) was not present on 2 transferent clones characterized by Burch and McBride (reported by McBride and

Athwal 1976), nor in 3 transferent clones isolated by Willecke and Ruddle (unpublished results, obtained in collaboration with Buck and Bodmer). More recently, by the use of specific anti human α galactosidase (α GAL) antiserum, it was shown (Davies and Willecke 1977) that the human X-linked gene for α Gal was not expressed in the transferent mouse clones described by Willecke and Ruddle (1975). In none of the mentioned transferent clones could a chromosomal fragment be demonstrated by cytological methods. These results suggested that the size of the transgenome could be shorter than the distance on the X-chromosome between the flanking marker genes, α GAL and G6PD, next to the HPRT gene. This distance was computed to be about 1% of the haploid human genome. At present it cannot be strictly ruled out that the genes flanking the HPRT gene may be present but not expressed in transferent cells. This appears unlikely, however, since first a chromosomal fragment of this size should have been detected by cytological methods, and second the expression of the flanking genes together with human HPRT was not inhibited in somatic cell hybrids characterized after fusion of human and mouse parental cells.

It is not clear during which step of chromosomal gene transfer the donor chromosomes are fragmented. McBride and Ozer (1973b) had shown that purified morphological intact metaphase chromosomes contained DNA molecules which were smaller – possibly due to endogeneous nucleolytic activity – than predicted for the size of the unsheared DNA molecules of these chromosomes. Thus at least part of the fragmentation of chromosomes might have already occured before uptake of donor chromosomes into recipient cells. Further degradation may occur in phagosomal vesicles after fusion with lysosomes inside the recipient cells. Phagocytized chromosomes have been observed in the cytoplasm of recipient cells (Itterson et al. 1969). Radioactively labelled external chromosomes have been found in mitotically arrested metaphases of recipient cells up to 4 generations after uptake of chromosomes (Ebina et al. 1970; Sekiguchi et al. 1973). At present all available data are consistent with the notion that most of the chromosomes may be degraded in lysosomes of the recipient cells. The amount of radioactivity measured in recipient cells shortly after uptake of chromosomes was about 3 orders of magnitude higher than could be accounted for by the number of transferent clones which were finally isolated in these experiments. About 20% of all recipients cells ingested one DNA equivalent or more of an average size chromosome within 2 hours at 37° (McBride and Ozer 1973b). In this context it is surprising that fragments of metaphase chromosomes could survive at all the endocytotic uptake process and were transcribed and translated in transferent clones. The mechanism by which macromolecules such as fragments of metaphase chromosomes may be internally released functionally intact from phagosomes is not clear and should be studied in more detail. Most likely the condensed and protected state of DNA in chromatin is required for successful transfer of somatic cell genes into cultured mammalian cells. (This review does not cover the literature on attempts to transfer mammalian genes via isolated DNA, for review see Ottolenghi-Nightingale 1974). Transfer of non-viral genes via isolated metaphase chromosomes seems to yield more reproducible results than transfer via purified DNA. The widely applied technique of Graham and van der Erb (1973) for the infection of mammalian cells by isolated viral DNA did not give positive results when used with somatic cell DNA, presumably because the number of selected gene copies was too small in comparison with isolated viral DNA (Willecke, unpublished experiments, cf. Bacchetti and Graham 1977).

Wullems et al. (1975) found a 3 fold increase in the frequency of chromosomal gene transfer when recipient cells enriched for mitotic cells were used. This finding may be explained by the assumption that intracellular degradation of donor chromosomes in recipient cells may be diminished when the cell cycle phase of the recipient cells is the same as that of the donor cells. So far the selection of transferent cell clones in most cases has involved the use of HAT selective medium. Since long term cultivation of cells in HAT medium had been described earlier to cause chromosomal abnormalities (Miller et al. 1971), it seemed possible that part of the observed chromosomal fragmentation was due to the use of HAT medium. In order to rule out this possibility, gene transfer experiments should be performed using selective growth media other than HAT medium. Recently Spandidos and Siminovitch (1977) described the transfer of methotrexate and ouabain resistence via Chinese hamster chromosomes into Chinese hamster CHO cells. No donor chromosomal fragment could be detected by karyotypic analysis of the (aneuploid) transferent clones. Therefore this result supported the notion that observed chromosomal fragmentation was most likely independent of the selection method used.

Wullems et al. (1976b) reported that the gene for human HPRT has been transferred together with an intact human X chromosome via purified metaphase chromosomes from human Hela cells into hybrid cells consisting of a 2S Chinese hamster genome plus about four human chromosomes. Possibly the recipient hybrid cell could have been preselected for low degradation of human genetic material or alternatively the expression of the few remaining human chromosomes might inhibit the chromosomal fragmentation. If this result could be reproduced it would clearly indicate that chromosomal fragmentation found after conventional chromosomal gene transfer may be an artifact which could possibly be experimentally controlled. In addition, the transfer of intact chromosomes

would be of great value for establishing syntenic relationships in somatic cell genetics of mouse and man.

2. The Size of the Transgenome is at most 0.2% of the Haploid Donor Genome or about $7 \cdot 10^6$ Nucleotide Base Pairs long

In order to estimate the size of the transgenome in transferent clones, the indirect method of cotransfering two linked genes had to be applied. Direct measurements of the size of the transgenome have not been possible since so far no purified mRNA of the transferred genes has been available for molecular hybridization. Cotransfer of the human genes for cytosol thymidine kinase (TK_s) and galactokinase (GALK) into mouse L-cells and Chinese hamster cells has been reported (Willecke et al. 1976a; Wullems et al. 1977). No chromosomal fragments could be cytologically detected in the transferent clones. The genes for TK_s and GALK had been previously assigned to a relatively narrow region (q21-q22) on human chromosome 17. (Elsevier et al. 1974). Since this region comprises about 0.2% of the haploid human genome, it was concluded that the size of the transgenome in the mentioned transferent clones was at most about 0.2% of the human haploid genome. No estimate of the minimal size of the transgenome was deduced from these data.

About 25% of the isolated transferent mouse clones expressed both human genes; the remaining clones expressed only human TK_s, for which thay had been selected in HAT medium. The percentage of cotransfer of these closely linked genes appears to be reproducible: 33% cotransfer of human TK_s and GALK was reported in sequential gene transfer experiments (Willecke et al. 1976b, see next paragraph). These results suggested that it might, in turn, be possible to estimate from the relative frequencies of cotransfer the relative distance between closely linked genes (Willecke et. 1976b). Evidently more systems have to be studied in order to clarify this point. McBride and Athwal (1976) and Ruddle and McBride (1977) reported cotransfer of TK_s and GALK via Chinese hamster chromosomes into mouse cells. In order to explain the results, the authors suggested by comparison with the close linkage of both genes in the human karyotype that the same genes may be similarly linked in the Chinese hamster genome. It is not known why this gene linkage is higly conserved in the evolution of mammals.

Very recently Spandidos and Siminovitch (1977b) reported the cotransfer via purified chromosomes from the Chinese hamster CHO cell line of 3 genes controlling consecutive biochemical steps in folate metabolism. The authors suggested that their cotransfer data indicate close linkage of the corresponding genes which had as yet not been mapped by any other method. At present no other general method of somatic cell genetics is available for mapping closely linked genes. Thus cotransfer on chromosomal fragments may be the method of choice for mapping closely linked genes if one of the genes is selectable under cell culture conditions.

Since the size of the transgenome is presumably smaller than the smallest chromosome of the transferent cells, it may be possible to purify the transgenome by size fractionation of chromosomes from the recipient genome. So far no attempts have been reported to re-isolate the transgenome from transferent cells. Such an experimental approach may eventually be useful for enrichment of structural genes together with their presumed adjacent regulatory gene sequences.

3. Multiple Copies of Transgenomes May Exist in Transferent Clones

In order to determine the number of viral or somatic gene copies per cell, usually molecular hybridization of mRNA or of complementary DNA with cellular DNA is used (see for example: Tonegawa 1976). Since no mRNA of transferred genes is available, however, the number of transgenome copies in transferent cells has to be indirectly estimated. It was assumed that the frequency of chromosomal gene transfer was proportional to the number of gene copies in the donor cells. The validity of this assumption has not been rigorously proven. Different frequencies of chromosomal gene transfer, depending on the experimental system which was studied, were reported from different laboratories. For one type of experimental system and working conditions, however, remarkably constant frequencies of chromosomal gene transfer were found. The groups of McBride and of Willecke used similar experimental conditions and found, in independent experiments, average frequencies of chromosomal gene transfer between $1.0 \cdot 10^{-1}$ and $2.6 \cdot 10^{-7}$ (summarized by McBridge and Athwal 1976). When metaphase chromosomes were isolated from transferent mouse clones containing human TK_s (Willecke et al. 1976b) or human HPRT (Athwal and McBride 1976, 1977) and used for a second chromosomal gene transfer into appropriate mouse or Chinese hamster cells, new transferent cell clones were isolated at about the samen frequency (i.e. about $1 \cdot 10^{-7}$) as in the first chromosomal gene transfer experiment. The results of these sequential or serial gene transfer experiments could be expected if there were about the same number of transgenome copies in transferent cells as in the original donor cells. Since the donor metaphase chromosomes had been isolated from near diploid human lymphoblastoid cells, they should have contained about 4 genes copies per cell.

The published experiments of sequential chromosomal

gene transfer gave no indication that the transgenome might be significantly amplified in transferent cells as reported, for example, for relaxed plasmids in bacteria (see Falkow 1975). This point should be re-investigated, however, since both the results from our laboratory (Willecke et al. 1976) and from the laboratory of Athwal and McBride (1976, 1977) were obtained with transferent cells which were shown to be phenotypically stable for expression of the transgenome (for discussion of phenotypic stability, see next paragraph). Recently we isolated chromosomes from a transferent mouse clone (CT 11D) harbouring human HPRT (Willecke and Ruddle 1975) which was phenotypically unstable at the time of chromosome isolation. When these chromosomes were used for sequential gene transfer into mouse L-A9 recipient cells ($HPRT^-$), we isolated up to 5 times more transferent clones than in parallel experiments with chromosomes purified from the cell clone CT 11A which had expressed reverted mouse HPRT activity as a stable phenotype (Willecke and Davies, unpublished observations). These results suggested but did not prove that phenotypically instable transferent clones may contain an amplified number of transgenome copies, due to the selection pressure, when compared with the donor cells.

In this context it should be noted that data from several laboratories (McBride and Ozer 1973; Wullems et al. 1975; Willecke, not published) indicated that transferent mouse clones contained at most about the same amount of transgenome-coded specific HPRT activity as the donor cells. Degnen et al. (1976) found, however, in extracts from mouse cells of unstable phenotype, 5-7 fold higher specific activities of transgenome-coded mouse HPRT when compared with donor cells or phenotypically stable transferent clones. This higher specific activity of a transgenome-coded enzyme is consistent with the notion of moderately amplified transgenome copies. As pointed out above, a convincing answer to this question can only be obtained when the number of transgenome copies can be directly determined by molecular hybridization.

4. Growth of Transferent Cells under Selective Conditions Leads to Phenotypic Stabilization of the Transgenome

McBride and Ozer (1973) noted, in their first report on chromosomal gene transfer, the appearance of phenotypically stable and unstable transferent clones. Phenotypically unstable transferent clones lose the transgenome at a frequency of about 2-3% per mean cell generation when shifted to non-selective growth conditions. This original observation was confirmed by two other laboratories (Willecke and Ruddle 1975, and Wullems et al. 1976a). When it became clear that the human chromosomal fragment, which was expressed as a transgenome, was smaller than the distance between the PGK gene and GPDH gene on the long arm of the human X chromosome, it was concluded that it might have lost the centromeric region of its donor chromosome. Under these conditions the transgenome should be distributed randomly during mitosis of the transferent cells. Those cells which did not receive at least one copy of the transgenome should not survive under selective (HAT) conditions. If the transgenome was covalently integrated or closely associated with a recipient chromosome, the corresponding centromere of the chromosome could provide for equal distribution of new transgenome copies between daughter cells during mitosis. Thus the progeny of transferent cells harbouring transgenomes covalently integrated into recipient chromosomes should preferentially survive under selective growth conditions. This was first observed (Willecke et al. 1976a) with mouse transferent cell clones containing human TK_s or human HPRT. These clones, which were phenotypically unstable for about 30 generations after gene transfer, were kept in continuous culture under selective growth conditions (HAT medium) for about 100 generations. Afterwards the clones were re-analyzed and found to be phenotypically stable. This observation has been confirmed for all phenotypically unstable transferent clones which have been analyzed so far (Degnen et al. 1976; Davies and Willecke 1977; Athwal and McBride 1977, Spandidos and Siminovitch 1977a, 1977c). Degnen et al. (1976) reported the same effect for an intraspecies chromosomal gene transfer of mouse HPRT into mouse cells. They calculated the frequency of stabilization of the transgenome and that of its loss from instable transferent clones as 10^{-5} and 10^{-1}, respectively. As pointed out by Degnen et al. (1976), one can maintain a transferent cell population in its unstable phenotype by subculturing the cells under conditions which act against preferential growth of the rare phenotypically stable cells in the population.

5. Several Lines of Experimental Results Suggest Stable Association or Covalent Integration of the Transgenome at Non-Homologous Cites on Chromosomes of Recipient Cells

The demonstration of phenotypic stabilization with transferent cell clones is in accordance with the notion of covalent integration of the transgenome in the recipient genome. Furthermore, the results of the mentioned studies of sequential chromosomal gene transfer were also suggestive of a close association or covalent integration of the transferred chromosomal fragment with a recipient chromosome (Willecke et al. 1976b; Athwal and McBride 1976, 1977). Since purification of metaphase chromo-

somes include several steps of differential centrifugation of a chromosomal fragment of significantly smaller size than the smallest metaphase chromosome, the transgenome might have been lost during the purification procedure. Recently Spandidos and Siminovitch (1977a) combined studies of sequential chromosomal gene transfer with fractionation of purified metaphase chromosomes into 3 size classes. They found that methotrexate and ouabain resistance could be transferred via Chinese hamster chromosomes of the middle and large size class, respectively, into Chinese hamster cells. When chromosomes from transferent clones were fractionated and used for sequential gene transfer, it became evident that the corresponding transgenome had been present in all 3 different size classes of donor chromosomes. Thus the authors concluded that integration was not restricted to a particular chromosome in the transferent cell. It should be pointed out, that in this and all other experiments described so far, it was not possible to distinguish between covalent integration and strong association of the transgenome with recipient chromosomes. In order to get more insights into the molecular mechanisms of association or integration it is highly desirable to determine the site of this event on the recipient chromosome as exactly as possible.

We asked whether preferential association or integration of the human transgenome in the homologous chromosomal region could be demonstrated in mouse transferent cells. For this purpose transferent mouse clones were fused with appropriate Chinese hamster cells and the segregation of the transferred human HPRT gene with the gene for α GAL was analyzed (Davies and Willecke 1977). The genes for mouse HPRT and α GAL had been mapped on the mouse X chromosome (Kozak et al. 1975). No co-segregation of human HPRT and mouse α GAL was found in appropriate somatic cell hybrids. Thus human HPRT could not been preferentially integrated at the position of the defective mouse HPRT region on the homolous mouse X chromosome. This rules out a mechanism of integration similar to general (legitimate) bacterial recombination. Since our studies were carried out with transferent cells isolated after *interspecies* chromosomal gene transfer, the action of unknown restriction enzymes might have prevented a legitimate recombination. The mentioned results of Spandidos and Siminovitch (1977a) indicated, however, that in transferent cells harbouring transgenomes derived from donor chromosomes of the *same species* the homologous region on the recipient chromosome is not a preferential site of integration for the transgenome.

Fournier and Ruddle (1977a, 1977b) prepared microcells (Ege and Ringertz 1974) from transferent mouse cells harbouring human HPRT. These microcells, containing between 1 to 5 total chromosomes, were fused with Chinese hamster cells. The enzyme activity of human HPRT segregated in several of the isolated somatic cell hybrids together with two abnormal mouse chromosomes containing elements of mouse chromosomes 14 and 15 (Fournier and Ruddle 1977b). This result is consistent with the notion that the gene for human HPRT could be associated or integrated in this abnormal mouse chromosome. It is not clear whether the integration event could have caused the structural abnormality of the recipient chromosome. A similar analysis has been carried out in this laboratory for the mouse transferent cells harbouring human TK_s. In this case, the enzyme activity of human TK_s seemed to segregate in somatic cell hybrids of one transferent clone together with mouse chromosome 9, which was present in free and translocated copies in these hybrid cells (Willecke et al., submitted for publication). The mouse genes for TK_s and GALK had been mapped on mouse chromosome 11 (Kozak and Ruddle 1977; McBreen et al.; 1977). Thus mouse chromosome 9 of the analyzed transferent clone presumably carried the integrated transgenome, although it did not contain the TK_s-GALK region homologous to the transgenome. Thus it appears possible to construct by chromosomal gene transfer mammalian cell clones which contain a selectable gene rearranged and firmly associated with a non-homologous recipient chromosome. For example, mouse chromosome 9 cannot as yet be selected for in somatic cell hybrids. Most recently we were able, by subcloning the mentioned mouse microcell and the Chinese hamster somatic cell hybrid, to isolate a clone in which 94% of the cells contained an acrocentric mouse chromosome an as the only mouse chromosome in the Chinese hamster genome (R. Mierau, K. Willecke, unpublished experiments). These characterized hybrids of transferent clones may eventually be very valuable for the assignment of new genes or as donor material for studying the regulation of gene expression in cultured mammalian cells (Willecke et al. 1977b).

It would be interesting to know whether the transgenome can be integrated or associated at virtually any site in the recipient genome or whether there exists a number of preferred sites for integration. The mechanism of integration or association is not at all clear and should be studied in more detail when molecular hybridization studies of transgenomes become possible. So far the results of integration of transgenomes are reminiscent to integration of simian virus 40 in the rat genome where several integration sites have been analyzed with restriction enzymes and molecular hybridization (Botchan et al. 1976). Fox et al. (1971) concluded from the studies of Drosophila cells genetically transformed with purified DNA from *Drosophila melanogaster*, that the transferred DNA is not integrated, but closely associated with the homologous region on the recipient chromosome. This exosome model cannot be valid for the description of phenotypically stable mammalian transferent clones since so far no integration or

association or mammalian transgenomes with homologous regions on the recipient chromosomes has been described. It is tempting to speculate that regions of (moderately) repetitive DNA may be necessary for integration of the transgenome. Due to the low frequency of chromosomal gene transfer events it has not been possible to study whether or not integration of the transgenome is mutagenic for the transferent cell. Furthermore we do not know whether the phenotypically stable (integrated?) mammalian transgenome can become unstable (excised?) again.

Recently we found in somatic cell hybrids isolated after fusion of microcells and derived from a phenotypically stable mouse transferent clone with Chinese hamster cells, that the human transgenome was present but no mouse chromosome could be detected (Willecke et al., Mol. gen. Genetics, in press (1978)). This result is consistent with the notion that the integrated transgenome might have become exised and reintegrated into the Chinese hamster genome. In this context, studies by Shani et al. (1976) have to be mentioned which showed that integrated Simian virus 40 can be reactivated by the transfer of purified chromosomes from transformed Chinese hamster cells to monkey cells which were permissive for SV40 virus replication.

Conclusions and Prospects

After reviewing the progress that has been made since the original demonstration of chromosomal gene transfer, it becomes evident that most of the published results, with regard to the length of the transgenome, the number of transgenome copies and the site and mechanism of integration into the recipient genome, are rather indirect. In order to study the molecular events of chromosomal gene transfer it appears necessary to isolate mRNA of a transfered gene of non-viral origin and use it for molecular hybridisation with recipient cell DNA in the test tube and in situ on recipient chromosomes. Only then can one expect to get a direct answer to such important questions as why integration at non-homologous sites seems to be more likely than legitimate mitotic recombination in transferent cells and whether transgenomes can "jump" from one genetic site to another like transposons in bacteria (Nevers and Saedler 1977). As previously predicted (Willecke et al. 1976b) and recently confirmed (Spandidos and Siminovitch 1977b), chromosomal gene transfer can serve as a method to prove and study close linkage of mammalian genes, an area of somatic cell genetics which is still very much underdeveloped. A new exciting aspect of chromosomal gene transfer has been just opened up by the reports of Spandidos and Siminovitch (1977c) and Cassingena et al. (1977), who showed that the ability to grow in semisolid agar can be transferred from oncogenically transformed Chinese hamster or human cells on purified metaphase chromosomes into Chinese hamster or human recipient cells, respectively. Interestingly, the transferent cells in these intraspecies gene transfer systems could not be distinguished from revertants by physical differences in the gene products but only by transition from phenotypic instability to stability. It appears that the transformed phenotype can be experimentally dissected by chromosomal gene transfer in effects caused by different genes. Chromosomal gene transfer of mammalian genes between cultured cells is no longer only being studied in order to analyze its own rules; it has been developed into a fairly well defined genetic method which is being used to approach important problems of cell biology and somatic cell genetics. Possibly plant genetics will profit in the future from these studies (Day 1977).

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