

Genetic analysis of the human Y chromosome by chromosome-mediated gene transfer

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Summary

Chromosome-mediated gene transfer (CMGT) can be used to segregate fragments of human chromosomes in human–rodent hybrid cells. As with all somatic cell genetics methods, a selection technique is needed to isolate the hybrid cell lines produced by CMGT. Expression of the *MIC2* gene product on the cell surface (the 12E7 antigen) provides an endogenous selectable marker for the human Y chromosome. Using chromosome transfer followed by separation of

12E7 antigen-positive cells on the fluorescence-activated cell sorter, a series of cell lines containing segregated fragments of the Y chromosome have been derived. The possibility of using these fragments to derive fine structural mapping data for the Y chromosome is considered in this review.

Key words: Y chromosome map, *MIC2*, *TDF*, chromosomal rearrangements, human CMGT, chromosome-mediated gene transfer.

Introduction

In the presence of a gene (or genes) on the Y chromosome, the indifferent gonads of a mammalian embryo are induced to develop as testes. This is the first reaction in a series of events that results in the formation of a functional male. In the absence of the Y chromosome, ovaries are formed and a female individual is the result. Despite this central role in primary sex determination, our understanding of the genetic structure of the Y chromosome has been poor. Molecular analysis has been impeded by the lack of genetic markers and, except for the pseudo-autosomal region, the absence of meiotic recombination.

A classical model, based on cytogenetic observation and the analysis of individuals with chromosome anomalies, has divided the human Y chromosome into four subregions: an X–Y homologous pairing region including the distal part of the short arm, a sex-determining region, a long-arm euchromatic region encoding factors responsible for spermatogenesis and a long-arm heterochromatic region which appears to have no function (reviewed in Davis, 1981; Goodfellow, Darling & Wolfe,

1985a). Further definition of the Y-chromosome subregions has been facilitated by the recent isolation of a number of DNA probes derived from Y-chromosome sequences (Bishop *et al.* 1983; Wolfe, Erickson, Rigby & Goodfellow, 1984; Burk, Ma & Smith, 1985). Hybridization of these probes to the DNA of individuals with sex-chromosome deletions and translocations has provided an approach for determining the relative order of sequences on the Y chromosome (Guellan *et al.* 1984; Vergnaud *et al.* 1986). Although considerable information has been obtained from this type

of mapping, it has the limitation of relying on the chance occurrence and subsequent clinical detection of patients with Y-chromosome aberrations, the complexity of which cannot always be predicted. Variation in Y-chromosome structure between individuals might also confuse the interpretation of mapping information.

In an attempt to develop new methods for genetic analysis of the Y chromosome, we have used chromosome-mediated gene transfer (CMGT) to construct human–rodent somatic cell hybrids containing fragments derived from the Y chromosome. These fragments are being used to extend our knowledge of the arrangement of sequences on the Y chromosome and

as a source of material for obtaining new markers closely linked to the human sex-determining gene.

Method of chromosome transfer

McBride & Ozer (1973) were the first to use purified metaphase chromosomes as vectors for the transfer of genetic information between cultured mammalian cells. In their original experiment, mitotic chromosomes were mixed with a suspension of recipient cells in the presence of poly-L-ornithine to enhance association of cells and chromosomes. The frequency of transfer obtained in this experiment (approximately 10^{-7}) was found to be similar to the rate of spontaneous reversion of mutant mammalian alleles, and thereby precluded the isolation of a large number of transformed cell lines. Miller & Ruddle (1978) achieved a 10- to 100-fold increase in chromosome transfer frequency by using calcium phosphate precipitation and a subsequent treatment of the recipient cells with dimethyl sulphoxide. These conditions had been used previously to enhance the transformation of mammalian cells by viral DNA (Graham & van der Eb, 1973) and are now used routinely in DNA-mediated gene transfer (DMGT) as well as CMGT.

Chromosome uptake probably occurs when donor chromosomes enter the recipient cell by phagocytosis. Within the host cell cytoplasm, the chromosomes are degraded to fragments and these are either lost from the cell or destroyed by the action of nucleases. Occasionally, a chromosomal fragment may be incorporated into the host cell nucleus, generating a transfected cell (reviewed in de Jonge & Bootsma, 1984). Current evidence indicates that breakage of the incorporated chromosomes occurs at random to produce fragments ranging in size from 50 kb up to a whole chromosome (Klobutcher, Miller & Ruddle, 1980; Olsen, McBride & Moore, 1981). The stability of the transferred phenotype also varies; chromosomal fragments without a centromere are lost from the transfected cells at a rapid rate in the absence of selection, although relative stability can be achieved by the acquisition of host centromeric activity (Klobutcher & Ruddle, 1979).

A strategy for isolating human-rodent somatic cell hybrids containing fragments of the Y chromosome

Antibodies defining cell surface antigens, in combination with the fluorescence-activated cell sorter (FACS), have been used to manipulate and characterize panels of somatic cell hybrids for gene mapping (Tunnacliffe & Goodfellow, 1984). This form of selection has also been applied to the analysis of

DNA transfectants and has provided a rational approach for cloning the human DNA sequences responsible for cell surface antigen expression (Stan-ners *et al.* 1981; Kuhn, McClelland & Ruddle, 1984). Following this example, we have combined CMGT with FACS selection to recover cells containing fragments of human chromosomes that code for cell surface antigens.

The first structural gene to be assigned to the Y chromosome was the *MIC2* gene which encodes expression of a cell surface antigen, defined by the 12E7 monoclonal antibody (Levy, Dilley, Fox & Warnke, 1979; Goodfellow *et al.* 1983). This antigen is also encoded by a gene on the short arm of the X chromosome (Goodfellow *et al.* 1980). The location of both loci in the sex-chromosome pairing region and the observation that the X- and Y-encoded gene products of *MIC2* are identical led to the suggestion that they are a pair of pseudoautosomally inherited genes (Goodfellow, 1983; Banting, Pym & Goodfellow, 1985). Recent genetic investigations have confirmed this suggestion (Goodfellow *et al.* 1986). Moreover, family studies have indicated that, of the human pseudoautosomal loci described to date, *MIC2* has the lowest frequency of recombination with the male-determining gene (Goodfellow, Darling, Thomas & Goodfellow, 1986). Consequently, the *MIC2* gene can be used as a selectable marker to manipulate the Y-chromosome subregions involved in sex determination and X-Y meiotic pairing.

In CMGT, approximately 1 recipient cell in every 10^4 will incorporate and maintain a chromosomal fragment. Of these, only 1 cell in 100 will contain the particular chromosomal fragment of interest so that the transfer frequency for a single locus in CMGT is about 10^{-6} . As this low frequency precludes the use of cell surface antigens as selectable markers, we explored the feasibility of using cotransformation methods to enrich for CMGT-competent cells. Wigler and colleagues (1979) first demonstrated cotransformation with DMGT. We showed that chromosomes may also be cotransfected with an added selectable DNA plasmid (Pritchard & Goodfellow, 1986). Our calculations indicate that, using cotransformation, one tenth of the cells selected for incorporation of the DNA plasmid also contain a chromosomal fragment. This achieves a 1000-fold enrichment for CMGT transfectant cells and improves the transfer frequency for a single chromosomal locus from 10^{-6} to 10^{-3} . We have exploited this strategy to isolate CMGT transfectant cells expressing the 12E7 antigen. An outline of the experimental approach is illustrated in Fig. 1A.

Secondary chromosome transfer experiments have revealed an additional advantage of cotransformation (Pritchard & Goodfellow, 1986). When the chromosomes of a primary CMGT transfectant are used as

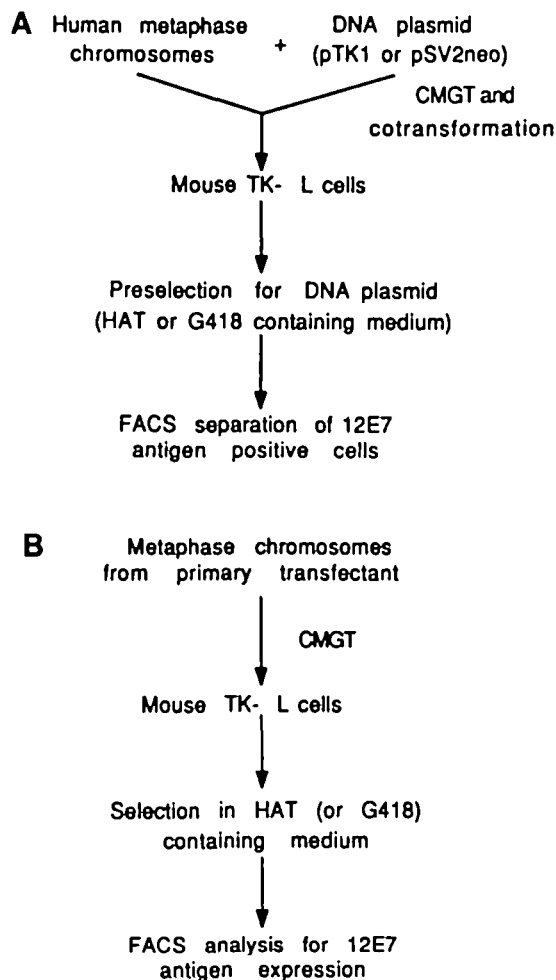


Fig. 1. Derivation of CMGT transfectants for the Y chromosome using the human male cell line OXEN (49:XXXXY) as donor and the mouse L cell, LMTK- as recipient. (A) Primary transfection. After FACS separation, single-cell clones were isolated and tested for the cotransfer of X- or Y-derived DNA sequences with 12E7 antigen expression. (B) Secondary transfection. In this case, the primary transfectants were used as chromosome donors. Clones selected with HAT- or G418-containing medium were analysed for the presence of *MIC2* and other Y chromosome loci (see text).

donors for a second round of transfer, the chromosome-encoded 12E7 antigen expression and the plasmid-encoded HAT (or G418) resistance are often transferred together (Table 1). Mammalian cells must therefore possess the ability to recombine and ligate exogenously added DNA and chromatin so that they become associated in cotransfection. We have selected for the integrated DNA plasmid after secondary chromosome transfer to generate a different set of breaks within the Y chromosome, as depicted in Fig. 1B. It has also been possible to use the integrated thymidine kinase (*tk*) gene as a reference point for pulsed-field gel electrophoresis restriction mapping. We have yet to investigate the feasibility of employing back-selection methods to construct a

Table 1. Secondary chromosome transfer

Primary CMGT transfectant	Secondary chromosome transfer	
	HAT (or G418)-resistant clones	Clones expressing 12E7 antigen
E2P3	15	0/15
E1P4	8	0/8
IP2.2	10	9/10
IP2.6	19	10/19
K3P2	18	10/18

The chromosomes of five primary CMGT transfectants were used as donors for a second chromosome transfer to mouse LMTK- cells (see Fig. 1). After HAT (or G418) selection, the surviving clones were tested for their ability to express the 12E7 cell surface antigen. In three out of five cases, the *tk* (or *neo* resistance) gene is associated with the transferred *MIC2* chromosomal fragment

deletion map between the exogenous and endogenous markers.

Mapping the Y chromosome by CMGT

Cloned unique sequence DNA probes have been used to analyse the molecular properties of the Y chromosome. Not all Y-derived sequences have been assigned subchromosomal locations and the order of loci in some regions has been difficult to determine. Studies of the sex-determining region, however, have been facilitated by the availability of individuals with abnormal sexual phenotypes. For example, Vergnaud *et al.* (1986) have constructed a deletion map of the region around the human sex-determining gene by assessing the presence or absence of Y chromosome-derived sequences in the DNA of XX males. The physical order of loci proximal to *TDF*, as they suggest, is shown in Fig. 2. This model is based on the assumption that XX males inherit *TDF* by an abnormal X-Y recombination event (Ferguson-Smith, 1966; de la Chapelle, 1981). For several XX males, this supposition has been confirmed by the demonstration that the paternal Y-chromosome pseudo-autosomal region is translocated with *TDF* to the paternal X chromosome at meiosis (Weissenbach *et al.*, this symposium). The use of CMGT for increasing our knowledge of the molecular structure of the Y chromosome and for defining the precise location of the male-determining gene is evaluated here.

We derived 9 primary and 29 secondary CMGT transfectants by the methods described in the previous section. The primary transfectants were then analysed for the cotransfer of Y-derived loci with the selected *MIC2* gene and the integrated *tk* (or *neo* resistance) gene. The portions of the Y-chromosome short arm contained within each cell line are illustrated in Fig. 2. Varying but overlapping fragments of

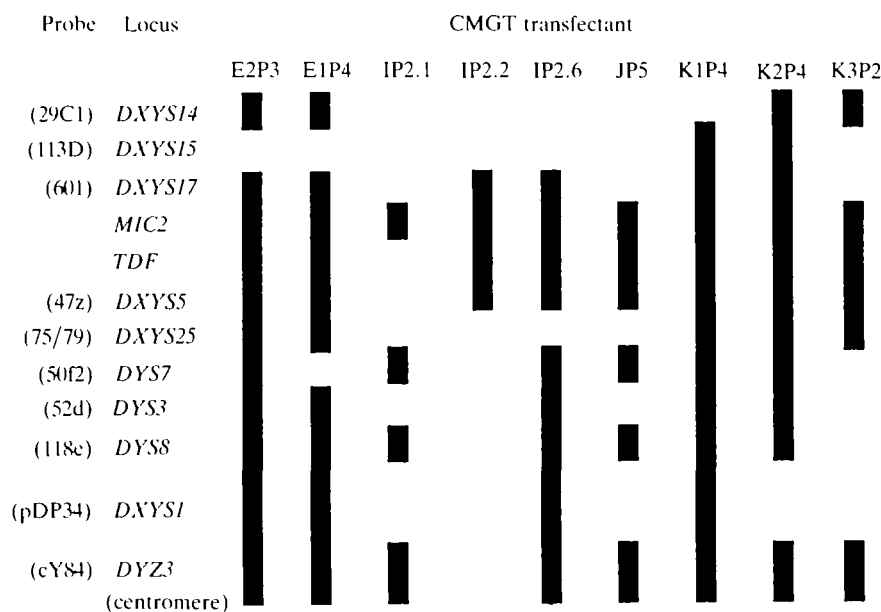


Fig. 2. Schematic representation of the transgenomic fragments contained within nine primary CMGT transfectants. The probes are referenced in Goodfellow, Davies & Roper (1985b).

the Y chromosome are transferred and retained in CMGT, thus supporting the suggestion that chromosome fragmentation occurs at random. A more striking feature, however, is the occurrence of chromosomal rearrangements: deletions are present in the transgenomic fragments of all cell lines except IP2.2 and K1P4 and the formation of smaller, though as yet undetected deletions in these two cases cannot be ruled out. We have recently detected rearrangements in fragments of the X chromosome generated by CMGT and *HPRT* selection (Pritchard & Goodfellow, 1987) and similar reports have been made for many other regions of the human genome after CMGT (Klobutcher & Ruddle, 1979; Porteous *et al.* 1986; Scambler *et al.* 1986). Hence, this is not a special feature of the Y chromosome, but instead reflects the mechanism by which cells incorporate and process calcium-phosphate-precipitated chromosomes.

Deletions appear to form at random in CMGT, indicating that site-specific breaks on the Y chromosome do not occur. By contrast, fragments containing the Y centromeric region, as characterized by the presence of *DYZ3* repeats, are found in eight out of the nine CMGT transfectants shown in Fig. 2 (the exception is IP2.2). This follows similar observations for the centromeric region of the X chromosome (Pritchard & Goodfellow, 1987). The significance of centromeric selection is not clear and it is not known whether the transferred blocks of *DYZ3* repeats contribute to the formation of an active centromere. If the transferred repeats are active at mitosis, then CMGT provides the opportunity to investigate their role in maintaining a functional Y centromere.

The whole-cell dot-blot method can be used to estimate the size of chromosomal fragments generated by CMGT. The dot-blot quantification of nine

primary cell lines containing Y-chromosome-derived fragments is illustrated in Fig. 3. Some cell lines possess very large amounts of human chromosomal DNA (for example, cell lines, K1P4, K2P2 and K3P2). Contaminating material from other chromosomes may be the cause of this. However, nondisjunction events occurring at mitosis might also increase the apparent size of the transgenome. By using the dispersed midcopy L1 (formerly *Kpn1*) family of repeats to fingerprint the individual transfectants, the chromosomal origin of the human material in each cell line can be checked (Porteous *et al.* 1986). In our analysis of the *HPRT*-selected transfectants, we observed the occasional presence of non-X-derived L1 repeat bands. Nevertheless, extensive portions of the X-chromosomal region around *HRPT* remained intact. Similar observations have been made for the L1 repeat fingerprints of the Y-derived CMGT transfectants presented in Fig. 2 (data not shown).

In this section, we have shown that chromosomal rearrangements occur in CMGT, that multiple fragments of transfected chromatin often arise and that the Y centromeric region is retained in transfected cells at an unusually high frequency. These problems raise severe doubts as to the reliability of CMGT for constructing genetic maps of the Y chromosome. The results do, however, point to an alternative application of this technique: by removing fragments of the Y chromosome from the human genome and placing them in a mouse cell background, a highly enriched (over 200-fold) source of new probes from defined subregions of the Y chromosome is provided. The use of CMGT transfectants as cloning resources is illustrated below.

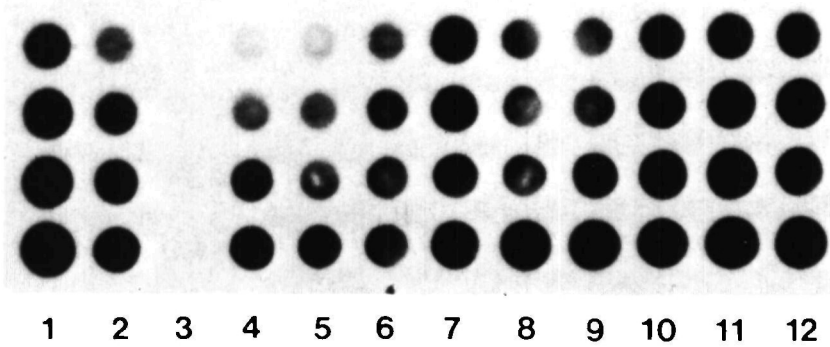


Fig. 3. Quantitative dot-blot analysis of nine primary CMGT transfectants containing Y-derived chromosomal fragments. Extracts from 10^6 cells were prepared by the method of Porteous (1985) and applied to a nitrocellulose filter in increasing dilutions (10, 20, 40 and $80 \mu\text{l}$). The filter was then hybridized with nick-translated total human DNA and exposed to X-ray film after washing. (1) HORL9X (an X-only hybrid); (2) 3E7 (a Y-only hybrid); (3) LMTK- (mouse recipient cell); (4–12) CMGT transfectants E2P3, E1P4, IP2.1, IP2.2, IP2.6, JP5, K1P4, K2P2 and K3P2.

Segregation of the sex-determining region of the Y chromosome by CMGT

The consensus map position of the male determinant on the Y-chromosome short arm is illustrated in Fig. 2: *TDF* has been placed proximal to *MIC2* and distal to sequences defined by cosmid 47 (*DXYS5*). This region probably represents less than 10% of the Y chromosome, comprising approximately 5000 kb of DNA (unpublished observations). Using CMGT, we have aimed specifically to generate a chromosomal fragment spanning the region from *MIC2* to *DXYS5*. The data presented in Fig. 2 show that, of the nine primary CMGT transfectants analysed in detail, the cell line IP2.2 has achieved this objective; its Y-derived chromosomal fragment contains *DXYS17*, *MIC2* and *DXYS5* but none of the other Y-derived sequences tested so far. Despite segregating the sex-determining region, whole-cell dot-blot quantitations show that the size of the transgenome in IP2.2 is considerably higher than expected (Fig. 3). L1 repeat fingerprinting has confirmed that this is because of the presence of donor material derived from other human chromosomes. In an attempt to reduce the proportion of contaminating sequences, prior to construction of a genomic library, a second round of chromosome transfer was undertaken (Fig. 1B). Of ten secondary transfectants analysed, six cotransferred *DXYS5*, nine cotransferred *MIC2* and all ten cotransferred *DXYS17* with the integrated *tk* gene. If, as has been proposed previously, CMGT cotransfer frequency is related to the physical distance between genetic loci (Klobutcher & Ruddle, 1979; Pritchard & Goodfellow, 1987), then these results demonstrate the close association of the *tk* gene with *DXYS17* in the transgenomic fragment of IP2.2.

Although no novel L1 fragments arose in the derivation of the secondary transfectants, varying

subsets of L1 repeats characteristic of IP2.2 were lost. On the basis of this observation, we have deciphered the L1 repeat pattern specific for the chromosomal region around *TDF*. These data have provided the opportunity to identify the secondary transfectant derived from IP2.2 that contains *DXYS5*, *MIC2* and the least amount of contaminating human chromosomal DNA.

Isolation of new DNA markers for the region around *TDF*

A lambda genomic library was constructed from the secondary CMGT transfectant described above. The library was screened with nick-translated total human DNA to identify lambda recombinants containing at least one member of the human *Alu* repeat family. After phage isolation, subfragments devoid of repetitive elements were recovered. These were then used as hybridization probes so that their chromosomal origin could be determined.

Several technical problems have hindered the rapid isolation and characterization of a large number of Y-specific recombinant phage. First, DNA sequences derived from autosomal chromosomes have been isolated at a high frequency. This is a consequence of the presence of asyntenic chromosomal fragments in the CMGT transfectant from which the library was made. Clearly, the rate of recovery of autosomal sequences depends on the relative size of the target chromosomal fragment as compared to the size of the contaminating fragment. It is also important to consider the possible deficiency of *Alu* repeat family members on the Y chromosome (Willard, 1985). A further hindrance is the cross-homology of murine and human repeat sequences. Although most mouse and human repeat elements are species-specific, the human *Alu* family is homologous to a low-copy

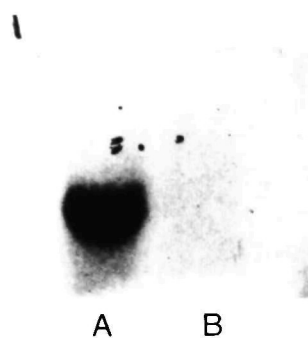


Fig. 4. Hybridization of probe 27a to *Eco*RI-restricted DNA from (A) 3E7 (a Y-only hybrid) and (B) LMTK- (mouse recipient cell).

murine repeat family (Bennett *et al.* 1984). The existence of this family constitutes a problem to library screens that depend on characteristic differences between species. In our analysis, a proportion of the human-repeat selected recombinant phage are of mouse origin.

Despite these drawbacks, new markers for the Y chromosome have been obtained. An example is shown in Fig. 4. This probe hybridizes specifically to the Y chromosome; it reacts with the somatic cell hybrid, 3E7, that contains the Y chromosome as the only cytogenetically detectable human contribution, but fails to react with mouse DNA. By virtue of its mode of derivation, the genomic locus for this probe must be closely associated with the human sex-determining gene. In support of this, preliminary results show that it is present in a high proportion of XX males. With this marker, and others similarly isolated, it should be possible to produce a long-range restriction map of the sex-determining region. These new probes also facilitate saturation cloning experiments aimed at cloning the testis-determining gene.

Conclusion

Chromosome-mediated gene transfer cannot be used alone to construct hybrid mapping panels of human chromosomes because it generates extensive chromosomal rearrangements and retains nonrandomly chromosomal fragments possessing or acquiring donor centromeric activity. At present, the maximum size of the chromosomal fragment transferred faithfully in CMGT is not known. L1 repeat fingerprinting has shown that continuous DNA segments of 1000 kb in length are consistently transferred intact. The most promising attribute of CMGT, therefore, is its ability to remove, from the rest of the genome, large genes, multigene clusters and defined subchromosomal regions. In analogy to DNA-mediated gene transfer, the CMGT transgenome can be used as a source of

cloning material. This directed cloning strategy is facilitating the analysis of the Y chromosomal subregions involved in X–Y meiotic pairing, sex determination and spermatogenesis.

We thank all our colleagues in the Laboratory of Human Molecular Genetics at ICRF for stimulating discussions and help in preparing this manuscript.

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