Investigation of chromosome-mediated gene transfer using the *HPRT* region of the human X chromosome as a model

Catrin A. Pritchard and Peter N. Goodfellow

Laboratory of Human Molecular Genetics, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, UK

A panel of over 50 hybrid cells containing varying portions of the long arm of the human X chromosome have been obtained by chromosome-mediated gene transfer (CMGT) of human chromosomes to mouse cells deficient in *HPRT*. This panel is used to investigate the size and integrity of transfected human chromosome fragments and also to examine the effect of including a selectable DNA plasmid in the transfection mix. Chromosomal rearrangements are found to be generated in the chromosome transfer process, and the human X centromeric region is detected in the transfected cells at an unusually high frequency. Extensive lengths of X chromosome DNA are transferred intact, suggesting potential uses of CMGT in cloning large genes and loci for which only the chromosomal map position is known.

[**Key Words:** Chromosome transfer; human gene mapping; X chromosome; centromere selection]

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Chromosome-mediated gene transfer (CMGT) can be used to construct hybrid cells that retain fragments of the selected donor chromosome (McBride and Ozer 1973). Chromosome breakage is generally assumed to be random and the chromosome fragment size can vary from 50 kb up to a whole chromosome (Wullems et al. 1976; Olsen et al. 1981). The intermediate size of the transgenome produced by CMGT provides a method for linking and ordering genes over longer distances than the high-resolution mapping techniques of DNA-mediated gene transfer or cosmid cloning and at a more sensitive level than can be achieved by somatic cell hybrids or in situ hybridization. In addition, if fragmentation is random, the frequency with which loci are separated by CMGT could be used as a measure of genetic distance (for review, see McBride and Peterson 1980).

Despite these advantages CMGT has been restricted in its applications. One major drawback has been the lack of suitable selective methods for isolating the cells containing the required chromosomal fragment. Until recently, only those regions of the genome encoding endogenous selectable loci were amenable to analysis, the best examples being the *HPRT* locus on the long arm of the human X chromosome (Miller and Ruddle 1978; Murphy et al. 1985) and the *TK* locus on the long arm of human chromosome 17 (Klobutcher and Ruddle 1979).

In previous experiments, new selection methods were used to extend the number of regions of the genome that could be subjected to analysis by CMGT (Pritchard and Goodfellow 1986). By cotransferring the selectable DNA plasmid, pSV2neo, with human chromosomes to mouse *HPRT*⁻ cells, it was shown that the frequency of combined uptake of the *HPRT* chromosomal locus with

pSV2neo was close to the frequency of uptake of *HPRT* alone. This observation demonstrated the feasibility of including an unlinked selectable marker in the transfection mix, followed by preselection for incorporation of that marker as an enrichment step in CMGT experiments. Furthermore, the fact that the *neo* gene and the *HPRT* chromosomal locus remained associated after a second chromosome transfer provided evidence that cotransformation could be used to introduce a selectable marker into a specific region of the human genome. Cell-surface antigen expression was also used as a selective tool. In particular, cotransformation followed by fluorescence-activated cell sorting for expression of the human-specific 12E7 antigen was used to isolate cells containing fragments derived from the human Y chromosome.

To assess the validity of using CMGT as a gene mapping tool we have used the *HPRT* system as a model for investigating the integrity of transgenomic chromosomal fragments. Over 50 CMGT transfectants selected for incorporation of the human *HPRT* locus have been tested for the cotransfer of sequences syntenic with *HPRT* and for their total human chromosomal content. Some of these cell lines were generated by the cotransformation of chromosomes with pSV2neo, as previously reported (Pritchard and Goodfellow 1986), and the effect of the presence of the DNA plasmid on the CMGT process is also investigated. The combined data provide formal proof that interstitial deletions occur during transfection and that multiple fragments of transfected chromatin arise frequently. In addition, we observe the preferential retention of the human X centromeric region. Despite these problems, L1 (formerly *KpnI*) repeat

Figure 1. The human X chromosome. The consensus long-arm map positions of the loci used in this report are indicated.

fingerprinting shows that large portions of the human X chromosome are retained intact.

Results

The long arm of the human X chromosome has been mapped by family linkage studies, by in situ hybridization of metaphase spreads, and by the analysis of panels of somatic cell hybrids (summarized in Goodfellow et al. 1985). The consensus map positions of the loci considered in this report are illustrated in Figure 1.

Isoenzyme analysis

The mouse and human forms of the housekeeping enzymes G6PD and PGK can be easily distinguished. The genes encoding these two enzymes map to Xq28 and Xq13, respectively (Fig. 1). Therefore, we tested the panel of CMGT transfectants for the cotransfer of *G6PD* and *PGK* with *HPRT* (Table 1). The cell lines Reta F1-14 and G1-16 were generated by cotransferring human chromosomes with pSV2neo into mouse cells deficient in *HPRT* and cells incorporating the human *HPRT* chromosomal locus were selected with HAT medium. The Reta neo A1-16 series was obtained in a similar way but, in this case, G418 and HAT medium were used to select for uptake of the *neo* gene along with the human *HPRT* locus. Reta H1-8 resulted from the transfection of *HPRT*⁻ mouse cells by human chromosomes alone.

Table 1. Human X-linked isoenzymes analyzed

Cell line	Selection	Human isoenzymes		
		PGK	HPRT	G6PD
Reta F1-14	HAT	2/14	14/14	0/14
Reta G1-16	HAT	5/16	16/16	3/16
Reta H1-8	HAT	2/8	8/8	3/8
Reta neoA1-16	G418 HAT	3/16	16/16	4/16

All the transfectants express the human form of *HPRT*. In 10 of 54 cases *G6PD* is cotransferred and in 12 of 54 cases *PGK* is cotransferred. In two cell lines both *G6PD* and *PGK* segregated with the selected *HPRT* gene. As *G6PD* maps closer to *HPRT* on the X chromosome long arm, it was unexpected that *PGK* enzyme activity is transferred with *HPRT* at a higher frequency (see below).

Presence of the human X centromere

The sequence *DXZ1* has been assigned to Xp11-Xq12, the pericentromeric region of the human X chromosome (Fig. 1). The probe for *DXZ1*, pBamX5, recognizes tandem clusters of an alphoid repeat on the X chromosome, represented as a prominent 2.0-kb band in *Bam*HI digests (Yang et al. 1982; Willard et al. 1983). Additional minor bands of 1.65 kb and 3.0 kb and multimers of the 2.0-kb and 3.0-kb monomers are also detected. Dosage experiments indicate that there are approximately 5000 copies of the 2.0-kb repeat and that it constitutes the major component of the human X centromere. The minor bands, although associated with the centromere, are not interspersed with the 2.0-kb repeat (Willard et al. 1983).

The cell bank of CMGT transfectants was tested for the presence of the human X centromeric repeat by DNA hybridization to pBamX5. Figure 2 shows a Southern blot of DNA from 34 CMGT transfectants that has been hybridized with insert prepared from pBamX5. The centromeric region is transferred and retained at an unusually high frequency and considerably more often than *PGK*; the 2.0-kb *Bam*HI repeat band is observed in 23 out of 34 cell lines shown in the figure and, of the 52 tested in total, 32 react positively with pBamX5. This is also the case when pSV2neo is included in the chromosome-calcium phosphate transfection mix and selected for incorporation with G418 medium (see Fig. 2, tracks 31–36).

We also observe a wide variation in the number of copies of *DXZ1* in the transfected cells. Some cell lines have five times as many centromeric repeats as the X-only hybrid HORL9X (Fig. 2, tracks 4, 17, 18, 26, 27) whereas others have less than one time as many copies (Fig. 2, tracks 12, 19, 28, 29, 34). The hybridization signals of the 3.0-kb band and the 2.0-kb and 3.0-kb multimers coincide with the amplification of the 2.0-kb monomer. However, all CMGT transfectants except three (Fig. 2, tracks 4, 18, 26) appear to lose the 1.65-kb band.

Figure 2. Presence of the X centromeric repeat, *DXZ1*. (Lane 1) 1RE3; (lane 2) HORL9X; (lanes 3–16) Reta F1–14; (lanes 17–30) Reta G1–16; (lanes 31–36) Reta neo A2, A5, A9, A11, A14, and A17.

The tandem array of *DXZ1* repeats is not only transferred as a block but is selectively retained in CMGT. On the basis of this result, we expect a gradient to exist in the frequency of transfer of Xq sequences in CMGT which will depend on chromosomal distance from the centromere and/or *HPRT*. The proximity of *PGK* to the centromere is a probable reason why this locus is detected in more CMGT transfectants than expected (Table 1); all transfectants retaining *PGK* also retain centromeric sequences.

As the frequency of cotransfer of sequences proximal to *HPRT* will be distorted by an additional selection for the centromeric region, we have concentrated on looking for the transfer of sequences distal to *HPRT* on the long arm of the X chromosome.

Transgenome size

DNA from 40 *HPRT*-selected CMGT transfectants was analyzed by hybridization to five unique Xq sequences

Figure 3. Dot blot analysis of five CMGT transfectants containing microtransgenomes. (See Materials and methods for a description of the cell extraction and dot blot procedures.) In each case, increasing amounts of extract from 10^6 cells (10, 20, 40, and 80 μ l) were spotted onto the filter. (Lane 1) HORL9X; (lane 2) 1RE3; (lanes 3–7) CMGT transfectants.

and also by probing quantitative dot blots with human repetitive DNA (Materials and methods). From these results we are able to divide the transfectants into two classes, depending on their transgenomic chromosomal content. Sixteen transfectants, representing one-third of all those examined, contain cytologically undetectable transgenomes. They cotransfer none of the tested Xq loci syntenic with *HPRT* and are estimated to contain less than 1000 kb of donor DNA. The dot blot analysis of five independent transfectants belonging to this class is illustrated in Figure 3.

The remaining 24 transfectants can be characterized as possessing macrotransgenomes: They frequently transfer other loci with *HPRT*, almost always retain donor centromeric sequences, and give strong hybridization signals with human repetitive DNA. In Table 2 we summarize the results of the Xq sequence analysis of 17 transfectants belonging to this class. The estimates of transgenome size by dot blot hybridization are also included. This data shows that Xq fragments of varying size are transferred with the selected *HPRT* gene. The estimated fragment size is usually consistent with the Xq sequences present. However, some cell lines possess very large amounts of human DNA (Table 2, neoA14 and neoA17). In these cases, the donor chromosomes were cotransfected with pSV2neo and the transfected cells selected with G418 and HAT medium. Association of the *neo* gene with donor chromosomes other than

Table 2. Presence of DNA markers syntenic with *HPRT* in 17 CMGT transfectants containing macrotransgenomes

	F10	F12	G1	G2	G4	G5	G11	G12	G13	H2	H3	H4	H5	A5	A9	A14	A17
<i>HPRT</i>	++	++	++	++	+	++	++	++	++	+	+	+	+	++	++	++	++
52A	–	+	–	+	+	–	–	–	–	+	+	+	+	–	–	+	+
F9	+	–	–	+	+	+	–	+	–	+	+	–	–	–	–	+	+
DX13	–	+	+	–	+	+	+	–	–	–	–	–	+	–	+	–	–
F8	–	–	+	–	–	+	–	–	–	–	–	+	+	+	–	–	+
St14	–	–	+	–	+	–	–	–	–	–	–	–	+	–	–	+	–
G6PD	–	–	+	–	–	–	–	–	+	–	–	–	+	+	–	–	–
Fragment size ($\times 10^6$ bp)	60	76	78	64	4	20	40	70	35	30	33	43	44	40	50	319	100

The presence (+) or absence (–) of five Xq DNA markers and human *HPRT* and *G6PD* isoenzymes are shown. Fragment size was calculated by dot blotting (see Materials and methods).

Figure 4. Presence of Factor IX, DX13, and 52A in the transgenomes. *Eco*RI-restricted DNA from 10 CMGT transfectants was transferred to a nylon filter and hybridized with insert prepared from Factor IX genomic probe and pDX13. The 52A hybridization results for these cell lines are indicated at the top of each lane. (Lane 1) IRE3; (lane 2) HORL9X; (lane 3) OX; (lanes 4–9) CMGT transfectants H1–6; (lanes 10–13) CMGT transfectants neoA2, A11, A14, and A17. Differences in intensity of the bands reflect differences in the amount of DNA loaded in each case.

those encoding *HPRT* is thought to be the most likely reason why these cell lines retain more human material.

Chromosomal rearrangements occur in CMGT

The data presented in Table 2 illustrate that rearrangements are present in many of the transferred X chromosomal fragments. This is found to be true whether or not a DNA plasmid is included in the transfection mix, though the generation of additional microdeletions by plasmid integration cannot be excluded.

An illustration of rearrangement is presented in the Southern blot of Figure 4. DNAs from 10 independent transfectants were hybridized with a genomic probe for the Factor IX gene and with the probe DX13 (locus designation *DXS15*). These sequences recognize *Eco*RI fragments of 4.8 kb and 2.1 kb, respectively. The 52A (locus designation *DXS51*) hybridization data are also included

in the figure. All the cell lines reacting positively with Factor IX also hybridize with 52A (Fig. 4, tracks 5, 6, 12, 13). However, Factor IX is missing from H5, which is the only transfectant containing DX13 (Fig. 4, track 8). As Factor IX is known to be located between 52A and DX13 on the X chromosome long arm (Fig. 1), a rearrangement must be present in the transfected chromosomal fragment of this cell line.

To test the extent of chromosomal rearrangements, human L1 (formerly *KpnI*) repeat analysis was carried out. The L1 repeats are dispersed throughout the human genome and are estimated to occur approximately once every 300 kb (Singer and Skowronski 1985). Differences in the genomic sequences surrounding an L1 repeat family member will generate a restriction fragment length variant (RFLV) pattern characteristic of that region of the genome. Therefore, L1 hybridization patterns can be used to study the portions of the human genome segregated by CMGT and will sensitively detect any sequence rearrangements.

Fingerprints of *Hind*III-digested DNAs from 10 transfectants are shown in Figure 5. For comparison, the L1 hybridization patterns of the mouse–human hybrids HORL9X (human X only) and 1W15 (human Xq only) are also included. This Southern blot was probed with the pBK1.5-cloned member of the L1 repeat family, so that all cell lines show the presence of the prominent 1.9-kb *Hind*III consensus band.

Every RFLV present in 1W15 is also found in HORL9X, suggesting that the fingerprint of 1W15 is diagnostic of the X chromosome long arm. The additional bands of HORL9X may be attributed to the presence of the short arm of the X chromosome in this hybrid. The fact that the RFLV patterns of these hybrids can be superimposed indicates that only low levels of polymorphism of the L1 repeats can exist for individual X chromosomes and supports similar observations for other human chromosomes (Porteous et al. 1986; Scambler et al. 1986). The discernible Xq-specific bands are illustrated diagrammatically in the figure.

Figure 5. L1 repeat fingerprints of human X chromosome–mouse hybrids and of 10 CMGT transfectants. (Lane 1) HORL9X; (lane 2) 1W15; (lane 3) IRE3; (lanes 4–9) CMGT transfectants H1–H6; (lanes 10–13) CMGT transfectants neoA2, A5, A9, and A11. A diagram of Xq-specific bands is shown adjacent to lane 1.

The CMGT transfectants H1 and neoA11 possess microtransgenomes and show faint hybridizations with pBK1.5 on longer exposures of the autoradiogram (Fig. 5, tracks 4 and 13). The cell line H6 shows stronger hybridization of the 1.9-kb consensus band and contains several distinct RFLVs (Fig. 5, track 9). The fact that most of these (Fig. 5, bands a–h) can be detected in the fingerprints of 1W15 and HORL9X strongly suggests that a large proportion of the 9000 kb of human DNA in this transfectant, as quantitated by dot blot analysis, has been transferred intact.

The other transfectants shown in Figure 5 contain considerably more human chromosomal DNA. There is some overlap in the L1 fingerprints of these cell lines and many of the RFLV bands can be found in the DNAs of the X chromosome hybrids. The CMGT transfectant H5 was previously shown to be deleted for the Factor IX gene (Fig. 4) and the cell lines H4, neoA5 and neoA9 are all known to possess deletions of Xq sequences (Table 2). As expected, novel L1 fragments are present in the DNAs of these transfectants (arrows, Fig. 5).

Cotransfer of loci syntenic with HPRT

The DNA of 40 transfectants was screened for the presence of six Xq markers. For reasons discussed previously, only those loci between *HPRT* and the centromere on the X chromosome long arm were investigated in detail. The cotransfer data for several of these cell lines is presented in Table 2 and shows that, although deletions are often formed, sequences syntenic with *HPRT* are transferred at a high frequency. The cotransfer frequencies of the six Xq markers are summarized in Table 3. It is evident that, as with the x-irradiation hybrids of Goss and Harris (1975), the frequency with which linked genes are lost or retained is related to their physical distance from the selected *HPRT* gene (see Fig. 1). Unlike the centromere, a selective force to retain telomeric-associated sequences is not observed but as yet we are unable to test for the presence of the Xq telomere directly.

Discussion

Little is known about the mechanisms by which cells incorporate and process calcium phosphate-precipitated DNA and chromatin. It is currently believed that a large amount of material is initially incorporated and de-

graded in the host cell cytoplasm and that the resulting products are either lost from the cells, destroyed by nucleases, or retained in the host cell nucleus (for review, see de Jonge and Bootsma 1984). In the process of DNA-mediated gene transfer (DMGT) rarely is more than 50 kb of genomic DNA retained intact; gross rearrangements of transfected DNA are known to take place (Perucho et al. 1980). We have here looked at the quantitative and qualitative properties of chromosomal fragments generated by the analogous process of CMGT.

Quantitative dot blot analysis shows that the amount of human DNA transferred in CMGT ranges from about 100 kb up to a whole chromosome, the transgenome size being one of the two classes originally reported (Klobutcher et al. 1980). By looking at the cotransfer of unique sequences syntenic with the selected gene, however, it is shown that many of these segregated fragments are rearranged physically. This seems to be a general property of the CMGT process and occurs whether or not the donor chromosomes are cotransferred with a DNA plasmid. Dispersed L1 repeats were used to check the fidelity of transfer and to estimate the size of DNA transferred intact. Although novel band patterns are observed frequently, extensive sets of Xq-specific L1 repeats, indicative of faithful transfer, appear to be present in many of our HPRT-selected CMGT transfectants. On the basis of these observations, we are able to suggest potential uses of CMGT; if the map position of a gene or disease locus is known and flanking markers are available, then CMGT provides a useful means of segregating the defined region of the genome for subsequent cDNA or genomic saturation cloning experiments. The use of CMGT transfectants as a cloning resource has been described previously (Murphy and Ruddle 1985; Weis et al. 1986). We also envisage using CMGT for transferring the genetic factors responsible for phenotypes refractory to analysis by DMGT. These include loci larger than 50 kb, gene clusters, and the factors necessary for X chromosome activation.

The human X centromeric repeat *DXZ1* is retained in 32 out of 52 HPRT-selected CMGT transfectants. As yet, it is not known whether the transferred 2.0-kb *Bam*HI repeats act as a functional centromere at mitosis or if the 1.65-kb and 3.0-kb repeat bands are essential functional components. If the centromere is active, then it is likely to exist autonomously; attachment to a host chromosome would generate a potentially unstable dicentric chromosome and might result in the deletion or suppression of either host or donor centromeric activity (Mann and Davies 1983). Multiple copies of the human X centromeric repeat arise in several CMGT transfectants, suggesting either that the replication of the donor chromosomal fragments or their segregation at mitosis is not controlled by the host cell machinery. Nondisjunction events occurring at cell division are the most likely cause of amplification in this situation, as experiments on yeast have shown that the centromere plays an active role in copy number control (for review, see Blackburn and Szostak 1984). The fact that a force to retain donor telomeric sequences is not observed in CMGT raises the interesting question of how the broken

Table 3. *Cotransfer frequencies of Xq syntenic markers*

Marker	Frequency of transfer
52A	9/40
Factor IX	9/40
DX13	7/40
Factor VIII	6/40
St14	4/40
G6PD	4/40

The cotransfer frequency represents the number of cell lines showing the presence of the Xq marker divided by the number of cell lines tested (in this case 40).

chromosome ends are healed and replicated. Using CMGT we can begin to answer such questions and start to study the behavior and functional components of the chromosomes of higher eukaryotes.

The observations that the chromosomal fragments generated by CMGT are variable in size and contain different Xq sequences strongly support the suggestion that chromosomal breakage occurs randomly (McBride and Peterson 1980). Despite this, the centromere selection means the fragments are not retained in the transfected cells with equal probabilities and presents a major disadvantage in using the technique as a gene mapping tool. We are currently investigating a number of approaches designed to avoid the problem of the centromere selection. Loci on the distal side of the selected *HPRT* gene, however, are observed to be present in the transfectants at a frequency that is related to their physical distance from *HPRT*. Further assessment of the correlation between cotransfer frequency and intergenic distance will require the analyses of much larger panels of CMGT transfectants.

Materials and methods

Cells and culture conditions

The human cell line used as chromosome donor was OX (Sirota et al. 1981), an Epstein-Barr virus (EBV)-transformed lymphoid cell line obtained from Dr. M. Fellous (Pasteur Institute). IRE3 is a clone of the mouse L cell 1R (Nabholz et al. 1969) and was used as the *HPRT*-deficient recipient cell. HORL9X is a mouse 1R-human somatic cell hybrid which contains only the human X chromosome (Goodfellow 1975), and the hybrid 1W15 possesses only the human X chromosome long arm in a mouse background (Nabholz et al. 1969).

The cells were cultured in either Dulbecco's MEM (IRE3 and transfectants) at 10% CO₂ or RPMI (HORL9X, 1W15 and OX) at 5% CO₂ and supplemented with 10% fetal calf serum, penicillin, and streptomycin. The transfectants, HORL9X and 1W15, were maintained routinely in HAT medium (100 μ M hypoxanthine, 10 μ M methotrexate, and 16 μ M thymidine). G418 antibiotic was initially used at a concentration of 400 μ g/ml, but cells were grown in 200 μ g/ml after the initial selection.

Chromosomal transfer

The isolation of mitotic chromosomes and transfer by the calcium phosphate coprecipitation procedure were carried out as described by Weis (Weis et al. 1984). The conditions for the cotransfection of chromosomes with pSV2neo have been described previously (Pritchard and Goodfellow 1986). After selection, surviving colonies were isolated by ring cloning and then expanded for isoenzyme testing, dot blotting, and DNA extraction.

Enzyme assays

The mouse and human forms of *HPRT* and *G6PD* enzymes were distinguished by starch gel electrophoresis and staining as reported by Harris and Hopkinson (1978). The *PGK* isoenzyme assay has been described by Meera-Khan (1971).

Dot blotting of cell extracts

A rapid quantitative dot blotting method using cell extracts

from 10⁶ cells has been described in detail by Porteous (1985). Dot blots were hybridized to nick-translated total human DNA and washed the next day in 0.1 $\times$ SSC at 65°C. They were then exposed to Kodak XAR-5 film at room temperature for 4–16 hr. Hybridization signals were scanned using a Joyce-Loebl densitometer and for each transfectant, the total human DNA content was calculated using the signals for HORL9X and 1RE3 as relative controls. The dot blot filters were then stripped of probe by boiling for 30 min in 0.1 $\times$ SSC and rehybridized with nick-translated total repetitive mouse DNA. Quantitation of these signals enabled correction for the amount of DNA loaded.

Probes

pBK1.5 is a cloned probe of the L1 repeat family (Shafit-Zagardo et al. 1982) and was supplied by Dr. J. Maio (Albert Einstein School of Medicine). pBamX5 was provided by Dr. H. Willard (University of Toronto) and detects tandem copies of an alphoid-like repeat at the human X centromere (Willard et al. 1983). The unique sequence genomic probes for the long arm of the X chromosome have been reported elsewhere (see Goodfellow et al. 1985). In all cases, inserts were excised from the cloning vectors by the appropriate restriction enzymes and electrophoresed onto NA45 nitrocellulose paper (Schleicher and Schuell). The DNA was then eluted by placing the paper at 65°C for 20 min in 1 M NaCl. Inserts were radiolabeled by the random priming method (Feinberg and Vogelstein 1984).

Southern analysis

DNA was isolated from approximately 2 $\times$ 10⁸ trypsinized cells by the isopropanol precipitation method described elsewhere (Hofker et al. 1985) and dissolved in 10 mM Tris, 1 mM EDTA (pH 8) to a final concentration of 300 μ g/ml. The DNA was then restricted with *EcoRI* or, in the case of the L1 repeat analysis, with *HindIII*, and the fragments were separated according to size by electrophoresis through a 0.8% agarose gel. Southern blotting was carried out to nitrocellulose under standard conditions (Maniatis et al. 1982) or to Gene Screen Plus using the manufacturer's recommended conditions (NEN Research Products). Nitrocellulose filters for probing with pBamX5 or pBK1.5 were hybridized in 50% formamide at 42°C and washed the following day to 0.1 $\times$ SSC (pBamX5) or 0.5 $\times$ SSC (pBK1.5) at 65°C. For the unique sequence probes, hybridizations to nylon filters were carried out under aqueous conditions at 65°C as reported previously (Goodfellow et al. 1986). Filters were washed with 0.2 $\times$ SSC at 65°C and exposed to Kodak XAR-5 film at -70°C with intensification. Probe was removed by washing the filters in 0.4 M NaOH at 42°C for 30 min and then neutralizing them in 0.2 M Tris-HCl (pH 8) before rehybridization.

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C A Pritchard and P N Goodfellow

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