# Isolation of Unique Sequence Human X Chromosomal Deoxyribonucleic Acid†

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ABSTRACT: Labeled unique sequence human X chromosomal DNA has been isolated by hybridization of labeled human DNA with DNA from a human–mouse hybrid cell line, A9/HRBC2-A, which contains a single human chromosome, the X. Homopolymer tails of poly(dA) were added to nick-translated unique sequence human DNA to permit separation of the labeled DNA from unlabeled driver DNA by binding to oligo(dT)–cellulose. Human DNA sequences homologous with mouse DNA were removed from this labeled poly(dA)-tailed human probe by hybridization with excess unlabeled mouse DNA. The labeled human DNA which did not hybridize with mouse DNA was then hybridized with excess unlabeled A9/HRBC2-A DNA, with which only X chromosomal human DNA will hybridize. After hybridization, the labeled human X chromosomal DNA was separated from the unlabeled A9/HRBC2-A DNA by binding to oligo(dT)–cellulose. The purity of the final X chromosomal DNA preparation was greater than 90%, and the hybridization with mouse DNA was less than 2%. When carried out under standard conditions for DNA reassociation, this procedure is complicated by the formation of hybrids between the poly(dA) tails of the probe and T-rich sequences in mouse and human DNA. However, these imperfectly paired hybrids are less stable than those of unique sequence DNA and can be eliminated by carrying out the hydroxylapatite chromatography at an elevated temperature of 71 °C.

Labeled DNA probes specific for a single human chromosome would be a useful tool in somatic cell genetics. Such probes could be used to identify the presence of a particular human chromosome in interspecies hybrid cells segregating human chromosomes. In addition, they could be used to determine what fraction of a particular human chromosome is present in cells containing subchromosomal amounts of human DNA. We have been studying the process of chromosome-mediated gene transfer (McBride & Ozer, 1973), in which subchromosomal amounts of donor DNA are transferred to recipient cells of a different species. The use of this method for genetic analysis requires a knowledge of the amount of DNA transferred. To provide this information, we have sought to determine how much human X chromosomal DNA is present in mouse cells which have incorporated the X-linked human gene for hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8). This paper describes the preparation of the unique sequence human X chromosomal DNA probe required for these experiments.

One method of preparing DNA from a single chromosome would require a physical separation of the desired chromosome type followed by isolation of DNA from the purified chromosome preparation. Significant progress has been made in the separation of human chromosomes by flow microfluorometry (Carrano et al., 1979), but the preparation of pure fractions containing a single chromosome type has not yet been achieved. An alternative method for the preparation of chromosome-specific DNA is to use a biochemical approach similar to that employed by Kunkel et al. (1976) for the isolation of repetitive human Y chromosomal DNA and more recently total human X chromosomal DNA (Schmeckpeper et al., 1979). This method requires two sources of DNA which are identical except that one contains the desired chromosome while the other does not. A trace of labeled DNA from cells containing the desired chromosome is hybridized repeatedly with excess DNA from cells lacking it, and the specific DNA is isolated as labeled DNA which remains unhybridized. The disadvantage of this procedure is that it allows only an indirect isolation of the desired DNA and requires repeated extensive hybridizations to remove the large excess of undesired DNA.

The procedure reported here for isolation of unique sequence human X chromosomal DNA is related to that of Kunkel et al. (1976) in that it utilizes DNA from two sources: mouse and a human–mouse hybrid cell line containing only the human X chromosome. However, it permits a direct isolation of the desired DNA, which is made possible by the addition of poly(dA) tails (Lee et al., 1977) to the labeled DNA so that it can be separated from exess unlabeled driver DNA by binding to oligo(dT)–cellulose. We describe the altered conditions necessary to obtain reliable reassociation kinetics with poly(dA)-tailed DNA and demonstrate that unique sequence DNA from a single chromosome can be successfully isolated by this procedure.

## Experimental Procedures

*Cells.* The cell line A9/HRBC2 (Miller et al., 1971; DiCioccio et al., 1975), provided by M. Siniscalco, is a hybrid line produced by Sendai virus-induced fusion of mouse HPRT$^-$ $LA_9$ cells (Littlefield, 1966) and diploid human male erythroblasts. It contains one human X chromosome, a human chromosome fragment, and, in a small percent of the cells, a human–mouse translocation chromosome (Balazs et al., 1978). This cell line expresses the human X-linked markers SA-X surface antigen, hypoxanthine phosphoribosyltransferase, glucose-6-phosphate dehydrogenase, and phosphoglycerate kinase (Balazs et al., 1978). A9/HRBC2-A is a clonal derivative of A9/HRBC2 isolated in this laboratory. Chromosome analysis of this clonal line by G-banding (Kozak et al., 1977) and by Giemsa-11 staining (Friend et al., 1976) reveals a human X chromosome and a possible human–mouse translocation chromosome in all metaphases examined (D. E. Moore and O. W. McBride, unpublished experiments). No

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other human chromosomes or chromosome fragments were detected.

The hybrid cell line 1W1-5, supplied by W. Bodmer, is a hybrid of an 8-azaguanine-resistant mouse L cell derivative, IR (Rowley & Bodmer, 1971), and human peripheral blood lymphocytes (Nabholz et al., 1969). It expresses the human X-linked markers SA-X surface antigen, hypoxanthine phosphoribosyltransferase, glucose-6-phosphate dehydrogenase, and phosphoglycerate kinase but contains only a fragment of the human X chromosome, presumably the long arm (Buck & Bodmer, 1976). Chromosome analysis of 1W1-5 in this laboratory by Giemsa-11 staining indicates only a single acrocentric human chromosome fragment in all cells. No intact human chromosomes or human–mouse translocation chromosomes were detected.

A9/HRBC2-A, 1W1-5, and HeLa $S_3$ (Puck & Marcus, 1955) cells used for DNA isolation were grown in suspension as previously described (Burch & McBride, 1975). HeLa $S_3$ cells were labeled by growth for 4 days in suspension medium containing 0.3 $\mu$Ci/mL [$^3$H]thymidine (sp act. 60 mCi/mmol).

*Preparation of DNA.* DNA was isolated from tissue samples or from cells grown in culture. Mouse DNA was isolated from livers, brains, and kidneys of fasted adult $C_3H$ mice. The source of human DNA was a female placenta. Tissue samples were frozen and then broken into small pieces and pulverized in a Waring blender with dry ice (Britten et al., 1974). The pulverized tissue was suspended in 0.1 M NaCl, 50 mM Tris-HCl, and 50 mM EDTA, pH 8 (500 mL/50 g of tissue), and NaDodSO$_4$[1] was added to a concentration of 1%. Pronase (Calbiochem, nuclease free) was added to a concentration of 100 $\mu$g/mL, and the viscous solution was incubated at 37 °C for 2 to 3 h. Additional 100 $\mu$g/mL Pronase was added, and the incubation was continued for another 2 to 3 h. The solution was deproteinized once with 0.5 volume each of phenol (redistilled, water saturated, pH 8) and chloroform–isoamyl alcohol (24:1) and then once with an equal volume of chloroform–isoamyl alcohol. Sodium acetate was added to a concentration of 0.1 M, 2 volumes of cold 95% ethanol was layered on, and the DNA was spooled out. Excess alcohol was removed from the spooled DNA, which was then dissolved at a concentration of ~0.5 mg/mL in 10 mM NaCl and 5 mM EDTA, pH 8. Heat-treated (30 min at 80 °C in 0.1 M sodium acetate, pH 5) RNase A (Boehringer Mannheim) was added to a concentration of 50 $\mu$g/mL, and the solution was incubated at 37 °C for 2 to 3 h. This was followed by a 2-h incubation at 37 °C with 20 $\mu$g/mL Pronase and then deproteinizations with phenol and chloroform–isoamyl alcohol. The DNA was spooled out twice from 0.2 M sodium acetate following the addition of 2 volumes of ethanol and was then dissolved at a concentration of 1 to 2 mg/mL in 10 mM NaCl and 5 mM EDTA, pH 8. The yield of DNA from 50 g of tissue was ~80 mg.

Cells for DNA isolation were grown in suspension culture and harvested by centrifugation. The cell pellets were washed twice with phosphate-buffered saline and suspended in 0.1 M NaCl, 50 mM Tris-HCl, and 50 mM EDTA, pH 8 ($10^7$ cells/mL). NaDodSO$_4$ was added to a concentration of 1%, and the procedure was continued as described for DNA isolation from tissue. About 15 mg of DNA was recovered from $10^9$ cells.

In some cases, both RNA (for use in other experiments in this laboratory) and DNA were prepared from the same batch of cells. The cells were centrifuged and washed as described above and then suspended in 8 M guanidine hydrochloride, 25 mM sodium acetate, pH 5.0, and 1 mM dithiothreitol ($3 \times 10^7$ cells/mL) and blended for 0.5 min at top speed in a Waring blender. One-half volume of ethanol was added, and the solution was stored at −20 °C for 4 h. The solution was centrifuged for 15 min at 4000*g*, and the pellet was used for isolation of RNA (Cox, 1968; Deeley et al., 1977). The supernatant, containing the DNA, was adjusted to 0.2 M sodium acetate, and an additional 1.5 volumes of ethanol was added to precipitate the DNA. The resulting suspension was stored for 16 h at −20 °C and then centrifuged at 3000*g* for 30 min. The pellet was suspended in 0.1 M NaCl, 50 mM Tris-HCl, and 50 mM EDTA, pH 8 (1 mL/8 $\times 10^7$ cells), and NaDodSO$_4$ was added to a concentration of 1%. The procedure was continued as described for DNA isolation from tissue, except that DNA was precipitated with ethanol and collected by centrifugation rather than by spooling. This was necessary for good recovery because the blending used at the beginning of this procedure reduces the size of the DNA so that it does not spool well. The yield of DNA from $10^9$ cells was ~7 mg.

*Shearing and Molecular Weight Determination of DNA.* DNA samples were sheared by high-speed blending in a Virtis 45 or 60 homogenizer (Britten et al., 1974). The samples were adjusted to 10 mM sodium acetate, pH 6, and 66% glycerol and sheared at 45 000 rpm for 20 min in a dry ice–ethanol bath. The sheared DNA was concentrated by ethanol precipitation, dissolved in 10 mM NaCl and 5 mM EDTA, pH 8 (2–5 mg/mL), and deproteinized with phenol and chloroform–isoamyl alcohol. The DNA was again precipitated with ethanol and dissolved in 10 mM NaCl and 5 mM EDTA, pH 8, at a concentration of 10–15 mg/mL. The solution was dialyzed against 0.4 M PB at room temperature for at least 24 h and then stored at −20 °C.

The molecular weights of sheared DNA samples were determined by sedimentation in isokinetic alkaline sucrose gradients (Noll, 1967). Six gradients were prepared for the Beckman SW 40 rotor by using a mixing chamber containing 59 mL of 15% sucrose in 0.1 N NaOH and a reservoir containing 34.6% sucrose in 0.1 N NaOH (McCarty et al., 1974). DNA samples, denatured in 0.5 N NaOH, were layered over the gradients, which were then centrifuged at 39 000 rpm for 44–48 h at 20 °C. The size of the DNA was calculated (Studier, 1965) by comparison with the sedimentation of a sheared $^{14}$C-labeled DNA standard, 530 nucleotides in length, calibrated by sedimentation in isokinetic alkaline sucrose gradients by using DNA restriction fragments of known length as standards.

*Isolation of Unique Sequence DNA.* Twenty milligrams of sheared female human placental DNA (mean length 570 nucleotides) at a concentration of 13.3 mg/mL in 0.4 M PB was denatured at 97 to 98 °C for 1.5 min and then incubated at 68 °C for 1.5 h to an E$C_0t$ of 1000. The sample was diluted to 0.12 M PB and applied to a column containing 16 g of hydroxylapatite (Bio-Rad HTP) equilibrated with 0.12 M PB and 0.1% NaDodSO$_4$ and maintained at 60 °C. The column was washed with 160 mL of 0.12 M PB and 0.1% NaDodSO$_4$ to elute single-stranded DNA, followed by 150 mL of 0.4 M PB and 0.1% NaDodSO$_4$ to elute double-stranded DNA. Measurements of the $OD_{260}$ of the single- and double-stranded DNA fractions indicated that 65% of the DNA was hybridized. Recovery of DNA from the column was 96%. To assure complete removal of double-stranded DNA, we applied the

[1] Abbreviations used: NaDodSO$_4$, sodium dodecyl sulfate; PB, equimolar mono- and disodium phosphate buffer, pH 6.8; $C_0t$, product of DNA concentration in moles of nucleotide per liter and incubation time in seconds; E$C_0t$, the equivalent $C_0t$ in 0.12 M PB; HAP, hydroxylapatite.

fractions (50 mL) containing the single-stranded DNA to a second column containing 3 g of hydroxylapatite and eluted them with 0.12 M PB and 0.1% $NaDodSO_4$. No detectable double-stranded DNA was eluted from this column with 0.4 M PB and 0.1% $NaDodSO_4$. The single-stranded DNA eluted from the second column (60 mL) was dialyzed against 0.2 M sodium acetate, pH 6, and 5 mM EDTA and precipitated with ethanol. The DNA was then dissolved in 1 mL of 10 mM NaCl and 5 mM EDTA, pH 8, and dialyzed at room temperature against 0.4 M PB.

*Labeling of Unique Sequence DNA.* Unique sequence human DNA at a concentration of 4 mg/mL in 0.4 M PB was reassociated by incubation at 68 °C for 96 h to an E$C_0t$ of $1.9 \times 10^4$ and was then labeled by nick translation (Kelly et al., 1970; Maniatis et al., 1975). The nick translation reaction mixture contained 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM $\beta$-mercaptoethanol, 50 $\mu$g/mL bovine serum albumin, 39 $\mu$g of reassociated unique sequence DNA, 3 mCi of [$^3$H]TTP (New England Nuclear, 53.1 Ci/mmol), 100 nmol each of dATP, dGTP, and dCTP (P-L Biochemicals), and 250 units of *Escherichia coli* DNA polymerase I (Boehringer Mannheim) in a final volume of 0.4 mL. The reaction mixture was incubated at 13 °C for 4–6 h until the amount of [$^3$H]TTP incorporated, assayed by trichloroacetic acid precipitation, reached a plateau, typically at ~10% incorporation. The reaction was stopped by extracting twice with an equal volume of phenol. The aqueous phase was then applied to a column (0.7 × 12.5 cm) of Sephadex G-50 Fine (Pharmacia) in 0.12 M PB to remove phenol and unreacted deoxynucleoside triphosphates. The DNA, with a specific activity of $\sim 6 \times 10^6$ cpm/$\mu$g, was recovered in a volume of 1 mL. Foldback sequences generated in the course of the nick translation reaction (~14%) were removed by denaturing the nick-translated DNA in 0.12 M PB for 1.5 min at 97 to 98 °C, incubating to a $C_0t$ of $10^{-2}$, and removing double-stranded DNA on a 0.2-g column of hydroxylapatite.

*Addition of Poly(dA) Tails.* Poly(dA) tails were added to the 3′-OH termini of nick-translated unique sequence DNA by incubating with terminal transferase under conditions similar to those described by Eden et al. (1978). The reaction mixture contained 26 $\mu$g of denatured nick-translated DNA (mean length 220 nucleotides), 6 $\mu$Ci of [$^{14}$C]dATP (New England Nuclear, 52.2 mCi/mmol), 1.25 mM $CoCl_2$, 0.1 M sodium cacodylate, pH 7.2, 0.1 mM dithiothreitol, and 300 units of terminal deoxynucleotidyltransferase (Bethesda Research Labs) in a final volume of 0.4 mL. Incubation was at 37 °C for 4 h. The incorporation of [$^{14}$C]dATP, assayed by trichloroacetic acid precipitation, was 27%, indicating that an average of ~80 dA residues were added per DNA fragment. The reaction was stopped by phenol extraction, and the sample was passed through a column of Sephadex G-50 Fine in 0.12 M PB and 0.1% $NaDodSO_4$ to remove unreacted dATP. The DNA recovered from the Sephadex column was applied to a column containing 2 mL of oligo(dT)–cellulose (see below) to isolate DNA capable of binding to oligo(dT).

*Oligo(dT)–Cellulose Chromatography of Poly(dA)-Tailed DNA.* Nick-translated DNA with poly(dA) tails was recovered after hybridization with unlabeled driver DNA by binding to oligo(dT)–cellulose (Collaborative Research type T3 or P-L Biochemicals type 7). Binding was accomplished either by passage through a column or by incubation in suspension. DNA samples in 0.4 M PB and 0.1% $NaDodSO_4$ or 0.12 M PB and 0.1% $NaDodSO_4$ were denatured at 98 °C, cooled to room temperature, and applied to a column at a maximum flow rate of 1 mL/(min cm$^2$). For binding in suspension, denatured DNA samples and washed oligo(dT)–cellulose were shaken for 16 h at room temperature and then poured into a column. Columns were washed with a minimum of 10 volumes of the application buffer before bound DNA was eluted with 2–10 mM Tris-HCl, pH 7.5, and 0.1% $NaDodSO_4$. When DNA samples were bound by passage through a column, the bound DNA was eluted at room temperature. With samples bound in suspension, however, elution at an elevated temperature (usually 50 °C) was required.

*DNA Reassociation and Hydroxylapatite Chromatography.* DNA samples were reassociated in 0.12 M PB and 2 mM EDTA at 60 °C or in 0.4 M PB and 2 mM EDTA at 68 °C. Samples were sealed in glass capillaries, denatured for 1.5 min in boiling water, and then incubated at 60 or 68 °C. Incubations were terminated by freezing the samples in acetone–dry ice. Since the rate of reassociation in 0.4 M PB is 4.9 times the rate in 0.12 M PB (Britten et al., 1974), the $C_0t$ values for incubations in 0.4 M PB have been multiplied by 4.9 to give the equivalent $C_0t$ (E$C_0t$) in 0.12 M PB.

The extent of DNA reassociation was determined by hydroxylapatite chromatography (Britten et al., 1974). Samples were thawed and diluted to 2.9 mL of 0.12 M PB and 0.1% $NaDodSO_4$. A 0.2-mL aliquot was counted to determine recovery, and 2.5 mL, containing a maximum of 250 $\mu$g of DNA, was applied to a 0.2-g column of hydroxylapatite equilibrated with 0.12 M PB and 0.1% $NaDodSO_4$. The column temperature was maintained at 60 °C for reassociated DNA without poly(dA) tails and at 71 °C for reassociated DNA samples containing poly(dA)-tailed DNA. Single-stranded DNA was eluted with four additional applications of 2.5 mL of 0.12 M PB and 0.1% $NaDodSO_4$ at 60 °C (or 71 °C). Double-stranded DNA was eluted either by washing the column with four applications of 2.5 mL of 0.4 M PB and 0.1% $NaDodSO_4$ at 60 °C (or 71 °C) or by raising the column temperature to 98 °C and eluting with four applications of 2.5 mL of 0.12 M PB and 0.1% $NaDodSO_4$ at 98 °C. When double-stranded DNA was eluted with 0.4 M PB, all fractions were counted in 12.5 mL of NE-260 scintillation fluid (Nuclear Enterprises) and a correction was made for the slightly decreased counting efficiency in 0.4 M PB (96–98% of the counting efficiency in 0.12 M PB). When double-stranded DNA was eluted with 0.12 M PB at 98 °C, all fractions were counted in 12.5 mL of a scintillation fluid composed of 312 mL of Spectrafluor (Amersham), 1890 mL of toluene, and 1060 mL of Triton X-100. Recovery of counts per minute from the columns in a typical experiment (see Figure 2) was 101 ± 4%.

*Preparation of Human X Chromosomal DNA.* Poly(dA)-tailed, nick-translated unique sequence human DNA (15.7 $\mu$g, $5.8 \times 10^6$ cpm/$\mu$g) was reassociated with 25.6 mg of mouse DNA in 0.4 M PB and 2 mM EDTA at 68 °C to E$C_0t$ = $8.3 \times 10^4$. Double-stranded DNA was removed on a 20-g column of hydroxylapatite in 0.12 M PB and 0.1% $NaDodSO_4$ at 60 °C. The single-stranded fraction, containing $5.7 \times 10^7$ cpm of human DNA and ~400 $\mu$g of unlabeled mouse DNA, was then reassociated with 25.6 mg of unlabeled A9/HRBC2-A DNA in 0.4 M PB and 2 mM EDTA at 68 °C to E$C_0t$ = $3.6 \times 10^4$. Double-stranded DNA was bound to a 17-g column of hydroxylapatite in 0.12 M PB and 0.1% $NaDodSO_4$ at 71 °C and was eluted with 0.4 M PB and 0.1% $NaDodSO_4$. The double-stranded fractions (60 mL) were diluted to 0.12 M PB and 0.1% $NaDodSO_4$, denatured at 98 °C, added to 2 g of oligo(dT)–cellulose, and shaken with the cellulose for 16 h at room temperature. The cellulose was poured into a column and washed with 200 mL of 0.12 M PB

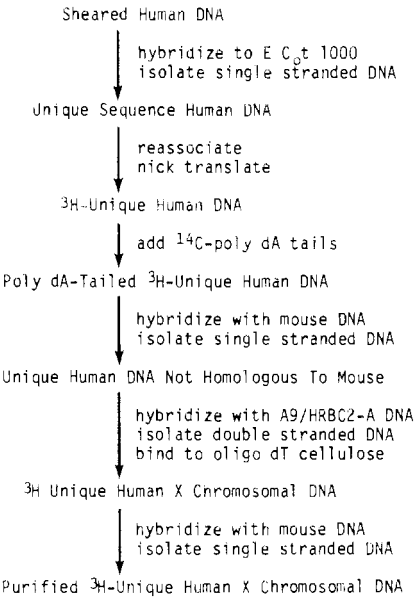


FIGURE 1: Outline of experimental procedure used to isolate unique sequence human X chromosomal DNA. See text for details.

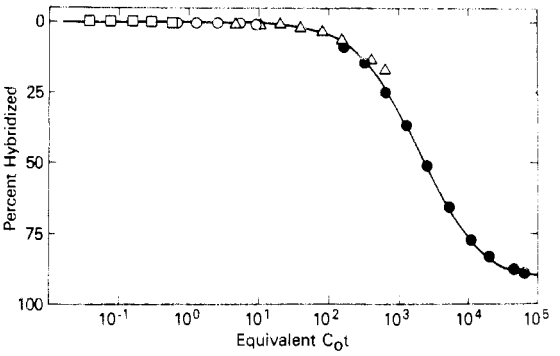


FIGURE 2: Reassociation kinetics of nick-translated unique sequence human DNA with unlabeled total human DNA. Nick-translated unique sequence human DNA (mean length 410 nucleotides, sp act. $5.8 \times 10^6$ cpm/$\mu$g) was reassociated with excess unlabeled human DNA (mean length 570 nucleotides) in 0.12 M PB at 60 °C or the equivalent criterion of 0.4 M PB at 68 °C. The DNA concentrations used were the following: (□) 0.026 mg/mL DNA, 0.12 M PB, ($2.5 \times 10^3$)-fold driver excess; (○) 0.024 mg/mL DNA, 0.4 M PB, ($2.5 \times 10^3$)-fold driver excess; (△) 0.52 mg/mL DNA, 0.4 M PB, ($2.3 \times 10^4$)-fold driver excess; (●) 13.5 mg/mL DNA, 0.4 M PB, ($4.7 \times 10^4$)-fold driver excess. The fraction of the cpm bound to hydroxylapatite at 60 °C is plotted as a function of E $C_0t$.

and 0.1% $NaDodSO_4$. Bound counts per minute were eluted with 2 mM Tris-HCl, pH 7.5, 0.02% $NaDodSO_4$, and 0.1 mM EDTA at 50 °C. The unbound fractions in 0.12 M PB and 0.1% $NaDodSO_4$ were again denatured and incubated with 2 g of oligo(dT)–cellulose as described above to increase the recovery of poly(dA)-tailed labeled DNA. The fractions eluted with Tris buffer from both columns were pooled, adjusted to 0.4 M PB, and applied to a small (0.5 mL) column of oligo(dT)–cellulose to concentrate the labeled poly(dA)-tailed DNA. The bound DNA was eluted in 0.8 mL of 2 mM Tris, 0.1% $NaDodSO_4$, and 0.1 mM EDTA at 50 °C. A portion ($3.8 \times 10^5$ cpm) of the labeled DNA recovered from oligo(dT)–cellulose was reassociated with 3.9 mg of unlabeled mouse DNA in 0.4 M PB and 2 mM EDTA at 68 °C to E $C_0t = 8.5 \times 10^4$. Double-stranded DNA was removed on 3 g of hydroxylapatite in 0.12 M PB and 0.1% $NaDodSO_4$ at 60 °C. The single-stranded DNA, containing $3.2 \times 10^5$ cpm and ~60 $\mu$g of unlabeled mouse DNA, was concentrated by binding to 80 mg of hydroxylapatite in 0.02 M PB and 0.017% $NaDodSO_4$. Bound counts per minute, representing purified labeled human X chromosomal DNA, were eluted with 0.12 M PB and 0.1% $NaDodSO_4$ at 80 °C.

## Results

*Experimental Design.* The procedure used to isolate unique sequence human X chromosomal DNA requires a reduced interspecific hybrid cell line which has retained only a single human chromosome, the X. The hybrid cell line we have used, A9/HRBC2-A, is a mouse–human hybrid containing only the human X chromosome and a possible small human–mouse translocation. An outline of the experimental procedure used is illustrated in Figure 1. Unique sequence DNA was isolated from sheared total human DNA and was labeled by nick translation. Tails of poly(dA) were added to the nick-translated DNA so that it could be separated from unlabeled driver DNA by binding to oligo(dT)–cellulose. The tailed, labeled human DNA was first hybridized with excess unlabeled mouse DNA to remove those sequences homologous with mouse DNA. The labeled DNA remaining after this step was reassociated with excess unlabeled A9/HRBC2-A DNA, with which only the human X chromosomal DNA will hybridize. The labeled X chromosomal DNA in the hybrid fraction was separated from the unlabeled A9/HRBC2-A DNA by binding to oligo(dT)–cellulose. Finally, a small amount of remaining labeled DNA homologous with mouse DNA was removed by a second hybridization with excess unlabeled mouse DNA.

The critical part of this procedure is the use of the poly(dA) tails to separate the labeled human DNA from the hybrid cell driver DNA. For successful results it is necessary to establish conditions such that (1) the poly(dA) tails do not affect the kinetics or fidelity of hybridization of the nick-translated DNA and (2) the poly(dA)-tailed labeled DNA can be recovered on oligo(dT)–cellulose in the presence of a large excess of unlabeled DNA. The details of our procedure, designed to meet these requirements, will now be described.

*Purity of Unique Sequence DNA.* Unique sequence human DNA was isolated from female placental DNA by reassociating the DNA to E $C_0t = 1000$ and taking the single-stranded fraction from hydroxylapatite. A portion of this single-stranded DNA was then reassociated and nick translated with [$^3$H]TTP to give a labeled probe of specific activity $5.8 \times 10^6$ cpm/$\mu$g. Trace amounts of the probe were reassociated with excess unlabeled total human DNA to assess the purity of the unique sequence DNA preparation. The kinetics of reassociation are illustrated in Figure 2. The solid line represents a theoretical curve for reassociation of a single frequency class of labeled DNA with an E $C_0t_{1/2}$ of 2000 and a nonhybridizable fraction of 8%. The data points fall directly on this curve, indicating a pure unique sequence DNA preparation free of detectable repetitive DNA. The small nonhybridizable fraction of 8% probably represents DNA fragments which are too short to form stable hybrids under the conditions used.

*Reassociation Kinetics of Poly(dA)-Tailed DNA.* $^{14}$C-Labeled poly(dA) tails, ~80 residues long, were added to the $^3$H-labeled nick-translated DNA with terminal transferase. The effect of poly(dA) tails on the reassociation kinetics of nick-translated DNA was determined by reassociating trace amounts of the poly(dA)-tailed probe with excess human DNA in a procedure similar to that described in Figure 2 for nick-translated DNA. The results are illustrated in Figure 3A. There was considerable decrease in the size of the probe (from 410 to 220 nucleotides) during the 2-month interval between nick translation and poly(dA) addition, which is reflected in a decreased hybridizability (from 89% to ~67%) of the

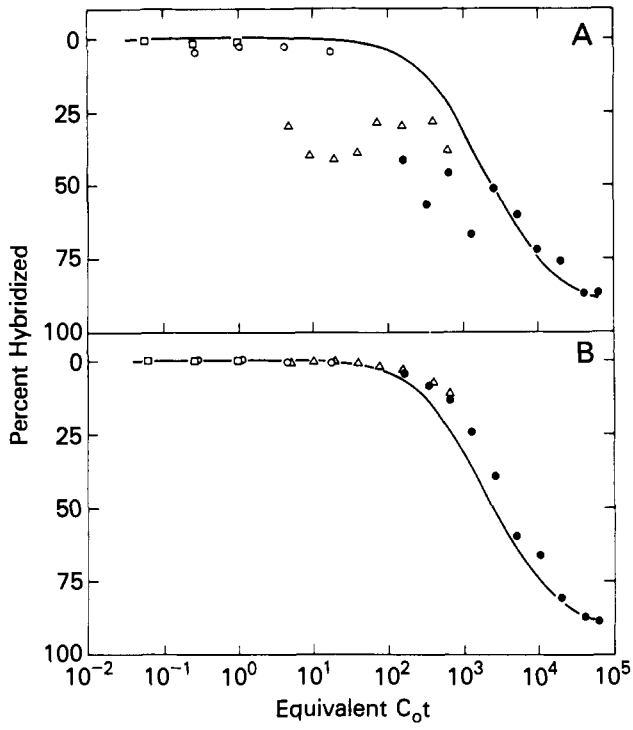


FIGURE 3: Reassociation kinetics of poly(dA)-tailed nick-translated unique sequence human DNA with unlabeled total human DNA. Poly(dA)-tailed nick-translated unique sequence human DNA was reassociated with excess unlabeled human DNA (mean length 570 nucleotides) in 0.12 M PB at 60 °C or the equivalent criterion of 0.4 M PB at 68 °C. The DNA concentrations used were the following: (□) 0.024 mg/mL DNA, 0.12 M PB, ($1.7 \times 10^3$)-fold driver excess; (○) 0.022 mg/mL DNA, 0.4 M PB, ($1.6 \times 10^3$)-fold driver excess; (△) 0.51 mg/mL DNA, 0.4 M PB, ($1.8 \times 10^4$)-fold driver excess; (●) 13.3 mg/mL DNA, 0.4 M PB, ($3.7 \times 10^4$)-fold driver excess. The fraction of the $^3H$ cpm bound to hydroxylapatite is plotted as a function of E $C_0t$. The solid line represents the reassociation of nick-translated unique sequence human DNA without poly(dA) tails from Figure 2. (A) Hydroxylapatite columns at 60 °C. (B) Hydroxylapatite columns at 71 °C.

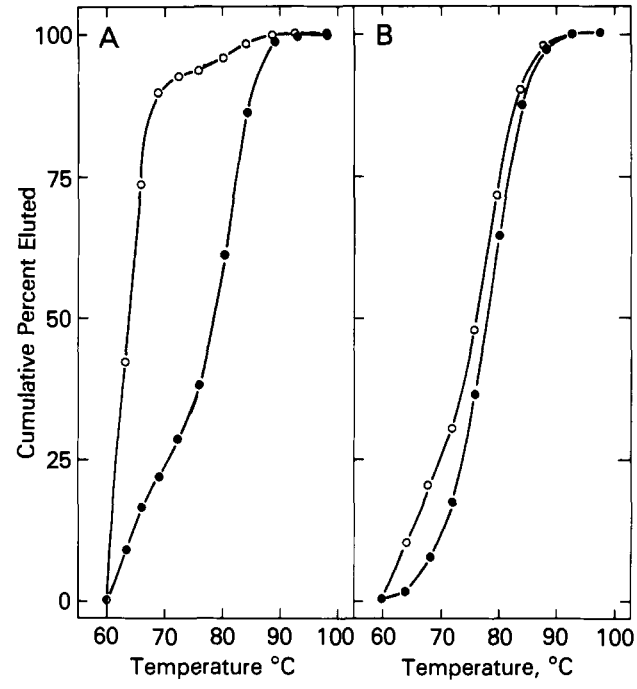


FIGURE 4: Thermal stability of hybrids formed between human DNA and nick-translated unique sequence human DNA with and without poly(dA) tails. Samples containing ~10000 cpm of the nick-translated unique sequence human DNA probe with (○) or without (●) poly(dA) tails and a ($1.5 \times 10^5$)-fold excess of unlabeled human DNA were prepared in duplicate, denatured, and incubated to E $C_0t$ = 300 or 60000. Reassociated samples were applied to 0.2 g of hydroxylapatite in 0.12 M PB and 0.1% $NaDodSO_4$ at 60 °C, and single-stranded DNA was eluted with four applications of 2.5 mL of 0.12 M PB and 0.1% $NaDodSO_4$. The temperature of the water-jacketed columns was raised in 3–5 °C increments up to 98 °C, and the hybridized DNA dissociated at each temperature was eluted with three applications of 2.5 mL of 0.12 M PB and 0.1% $NaDodSO_4$. The cumulative percent of the bound cpm eluted is plotted as a function of temperature. (A) Samples incubated to E $C_0t$ = 300. The percent bound at 60 °C was 9% of the probe without poly(dA) tails and 58% of the poly(dA)-tailed probe. (B) Samples incubated to E $C_0t$ = 60000. The percent bound at 60 °C was 63% of the probe without poly(dA) tails and 59% of the poly(dA)-tailed probe.

poly(dA)-tailed probe. The data have been adjusted to correct for this difference so that the reassociation kinetics of the probe before and after poly(dA) addition can be compared on the same scale.

The solid curve in Figure 3A, which represents the reassociation kinetics of the probe without poly(dA) tails, is the result expected if the poly(dA) tails have no effect on the reassociation kinetics of the probe. The data points, however, indicate that there is a significant effect. Although there is considerable scatter in the data, at E $C_0t$ values below $\sim 10^4$ there is clearly a pattern of hybridization in excess of that expected for any given $C_0t$ value, while beyond E $C_0t = 10^4$ the points fall on the expected curve. At a particular $C_0t$ value the amount of excess hybridization appears to depend on the ratio of driver to probe. Compare, for example, at E $C_0t$ = 4 the value of 3% hybridization using a driver/probe ratio of $1.6 \times 10^3$ with that of 30% hybridization using a 10-fold greater driver/probe ratio of $1.8 \times 10^4$. In contrast, in the reassociation of the nick-translated probe without poly(dA) tails (see Figure 2), the hybridization at a given $C_0t$ does not vary with the driver/probe ratio and overlapping $C_0t$ points at different DNA concentrations agree very well. This pattern of excess hybridization observed when the poly(dA)-tailed probe is reassociated with human DNA suggests the presence in human DNA of T-rich sequences capable of forming hybrids with poly(dA). Poly(dA) tails are apparently present in excess over T-rich sequences because at a given $C_0t$ value the fraction of the poly(dA)-tailed probe appearing in hybrids increases as the ratio of human DNA to the probe is increased. These T-rich sequences are not limited to human DNA but are found in mouse DNA as well (data not shown).

*Thermal Stability of Hybrids Formed with Poly(dA)-Tailed DNA.* Hybrids formed between poly(dA) and T-rich sequences in human DNA would presumably not be perfectly paired and should therefore have a lower thermal stability than the well paired unique sequence DNA hybrids. In addition, the lower thermal stability of the A-T base pair would make the poly(dA)-containing hybrids inherently less stable than those of unique sequence DNA. Thus, it should be possible to distinguish between hybrids of unique sequence DNA and those involving only pairing between poly(dA) tails and T-rich driver DNA sequences. The thermal stability of hybrids formed between human driver DNA and the probe with and without poly(dA) tails was compared at two different E $C_0t$ values to test this assumption: E $C_0t$ = 300, where the poly(dA)-tailed probe exhibited significant excess hybridization, and E $C_0t$ = 60000, where the probe with and without poly(dA) tails hybridized to the same extent (see Figure 3A).

Duplicate samples of the probe with and without poly(dA) tails were reassociated with a large excess [($1.5 \times 10^5$)-fold] of human DNA to E $C_0t$ = 300 and 60000. The thermal stability of the hybrids formed in each case was determined

by stepwise elution of the hybrids from hydroxylapatite with increasing temperatures. The elution profiles of the probe with and without poly(dA) tails at E $C_0t$ = 300 are shown in Figure 4A. At this E $C_0t$ and driver excess, 58% of the nick-translated probe with poly(dA) tails was bound to hydroxylapatite at 60 °C, while only 9% of the probe without poly(dA) tails was bound. There is, however, a dramatic difference in the thermal stability of the hybrids bound in each case. The $T_m$ of the hybridized probe without poly(dA) tails is 78.5 °C. About 25% of the bound counts per minute are eluted by 70 °C, indicating a small fraction of low melting hybrids. In contrast, at least 90% of the hybrids formed with the poly(dA)-tailed probe are eluted by 70 °C. The elution profile of the hybridized poly(dA)-tailed probe is biphasic, indicating two components of differing thermal stability. The larger, low melting component presumably represents hybrids between the poly(dA) tails and T-rich sequences in the driver, while the more stable component represents hybrids between the unique sequence DNA of the poly(dA)-tailed probe and complementary driver sequences. When the hybridization due only to poly(dA) tails is eliminated by considering only hybrids bound to hydroxylapatite above 70 °C, the hybridization of the two probes is similar [6.8 and 5.5% for the probe without and with poly(dA) tails, respectively].

The results are quite different at the high E $C_0t$ of 60 000, where the hybridization of the poly(dA)-tailed probe fell on the expected curve (see Figure 3A). The elution profiles of the hybrids formed between human DNA and the probe with and without poly(dA) tails at E $C_0t$ = 60 000 are shown in Figure 4B. Here the binding to hydroxylapatite at 60 °C of the two probes is similar [63 and 59% for the probe without and with poly(dA) tails, respectively], and the poly(dA)-tailed hybrids are only slightly less stable. With increasing $C_0t$, the T-rich sequences in the human driver DNA apparently form stable hybrids with their complementary sequences so that they are no longer able to form those less stable hybrids with the poly(dA) tails which are observed at lower $C_0t$. Thus, the poly(dA) tails should have little or no effect on the final hybridization of the unique DNA sequences of the probe.

*Hydroxylapatite Chromatography of Reassociated Poly(dA)-Tailed DNA at Elevated Temperature.* Since the excess hybridization due to pairing of poly(dA) tails with T-rich sequences in driver DNA can be eliminated by increasing the temperature, it seemed possible to obtain reliable reassociation kinetics by analyzing reassociated poly(dA)-tailed DNA samples at a temperature above 70 °C. The result obtained with hydroxylapatite chromatography at 71 °C is illustrated in Figure 3B, in which the reassociated DNA samples analyzed are duplicates of those analyzed at 60 °C in Figure 3A. As in Figure 3A, the solid curve represents the reassociation of nick-translated DNA without poly(dA) tails, and the data points, adjusted to correct for decreased hybridizability, indicate the reassociation of the poly(dA)-tailed probe. With hydroxylapatite chromatography at 71 °C, the excess hybridization seen at 60 °C disappears, the agreement between overlapping $C_0t$ points at different DNA concentrations is good, and the data points fall close to the expected curve. The apparent $C_0t_{1/2}$ is increased slightly compared to that of the original nick-translated probe, presumably as a result of the decreased size of the DNA portion of the poly(dA)-tailed probe. In addition, there is a slight sharpening of the curve at 71 °C. This may result from a greater elution from hydroxylapatite at 71 °C of unique sequence hybrids formed at lower $C_0t$ (26%) than of those formed at higher $C_0t$ (15%) (see Figure 4). We chose to maintain the lower stringency (0.12 M PB at 60 °C or 0.4 M PB at 68 °C) during reassociation of the poly(dA)-tailed samples and to use the higher stringency (0.12 M PB at 71 °C) only for hydroxylapatite chromatography in order to avoid increased breakdown of the probe caused by a higher temperature during incubation. Analysis of the thermal stability of hybrids formed with the poly(dA)-tailed probe (see Figure 4) indicates that the pairing of the poly(dA) tails with T-rich driver sequences which can occur at the lower stringency does not prevent the formatin of the desired unique sequence DNA hybrids.

*Recovery of Poly(dA)-Tailed DNA on Oligo(dT)–Cellulose.* Before use, the poly(dA)-tailed DNA (26 μg) was chromatographed on oligo(dT)–cellulose (2-mL column) to select for DNA capable of binding to oligo(dT). While 99% of the $^{14}C$ counts per minute were bound to the oligo(dT)–cellulose, only 86% of the $^{3}H$ counts per minute were bound, indicating that 14% of the nick-translated DNA either failed to react with the terminal transferase or had too few dA residues added to permit binding to oligo(dT) under the conditions used (0.12 M PB and 0.1% $NaDodSO_4$). When the DNA which did bind to oligo(dT) was eluted and reapplied to an oligo(dT)–cellulose column, nearly 100% of the $^{3}H$ counts per minute were bound. During a period of 4 months, the binding of $^{3}H$ counts per minute to oligo(dT)–cellulose gradually decreased to ~85%. This decreased binding is probably caused by DNA strand scission, leaving some DNA fragments without poly(dA) tails.

Thus, the poly(dA)-tailed probe alone binds well to oligo(dT)–cellulose, but for recovery after hybridization it must bind in the presence of a large excess of unlabeled driver DNA. In preliminary experiments on the isolation of human X chromosomal DNA, we used a poly(dA)-tailed nick-translated probe of unique sequence A9/HRBC2-A DNA and hybridized it with unlabeled human DNA rather than hybridizing a human probe with unlabeled A9/HRBC2-A DNA as described in this paper. Since the most extensive studies on binding to oligo(dT)–cellulose were done with this poly(dA)-tailed A9/HRBC2-A probe, they will be described here. The results are entirely applicable to the poly(dA)-tailed human probe.

The effect of various unlabeled DNA samples on binding of the poly(dA)-tailed probe to oligo(dT)–cellulose is presented in Table I. All DNA samples were denatured before application to oligo(dT)–cellulose because the reassociated poly(dA)-tailed probe does not bind to oligo(dT)–cellulose. The configuration of extensively reassociated DNA apparently prevents access of the oligo(dT) chains to the poly(dA) tails. As indicated in Table I, the binding of human DNA to oligo(dT)–cellulose, determined with $^{3}H$-labeled HeLa DNA, is negligible. However, when the poly(dA)-tailed probe is applied to oligo(dT)–cellulose in the presence of excess unlabeled human DNA, the binding of the probe is greatly inhibited. A sample of human DNA was first applied to the column followed by the probe to confirm that this decreased binding was not caused by competitive binding of the human DNA to oligo(dT). This caused no decrease in binding (Table I). The decreased binding may thus be due to an interaction between T-rich human DNA sequences and the poly(dA) tails which occurs rapidly while the denatured DNA is being cooled to room temperature. Although there are a limited number of T-rich human DNA sequences capable of hybridization with the poly(dA) tails at 60 °C in 0.12 M PB, there are apparently many more T-rich regions in human DNA which can interact with the poly(A) tails under the less stringent conditions used for binding to oligo(dT)–cellulose. These interfering driver sequences appear to be present in unique as well as repetitive

Table I: Effect of Excess Unlabeled DNA on Binding of Poly(dA)-Tailed Nick-Translated Probe to Oligo(dT)–Cellulose[a]

| sample | total $^3$H cpm | % $^3$H cpm bound |
|---|---|---|
| human DNA ($^3$H-labeled HeLa DNA)[b] | 523 000 | 0.02 |
| probe[c] alone | 19 000 | 96.1 |
| probe + human DNA | 17 500 | 13.5 |
| probe alone | 31 400 | 87.5 |
| probe + human DNA | 29 700 | 21.3 |
| human DNA, then probe[d] | 28 700 | 84.1 |
| probe alone | 19 000 | 96.1 |
| probe + unique[e] human DNA | 18 000 | 21.9 |
| probe + repetitive[f] human DNA | 18 500 | 6.1 |
| probe alone | 8 500 | 93.3 |
| probe + mouse DNA | 7 600 | 11.5 |
| probe alone | 18 700 | 91.3 |
| probe + *E. coli* DNA | 19 100 | 90.8 |

[a] Indicated amounts of labeled DNA ± 100–200 μg of unlabeled DNA, in a volume of 1 mL, were denatured for 1 min at 97 to 98 °C in 0.12 M PB and 0.1% $NaDodSO_4$, quickly cooled to room temperature, and applied to 0.2-mL columns of oligo(dT)–cellulose. Columns were washed with 3 mL of 0.12 M PB and 0.1% $NaDodSO_4$, and bound DNA was eluted with 4 mL of 10 mM Tris-HCl and 0.1% $NaDodSO_4$, pH 7.5. All fractions (1 mL) were counted with 12.5 mL of NE-260. [b] $^3$H-Labeled HeLa DNA was in vivo labeled; specific activity $5.2 \times 10^6$ cpm/mg. The column was washed with 14 mL of 0.12 M PB and 0.1% $NaDodSO_4$ before eluting bound cpm. [c] Probe was poly(dA)-tailed, nick-translated unique sequence A9/HRBC2-A DNA; specific activity $1.5 \times 10^7$ cpm/μg. [d] A sample containing 200 μg of human DNA in 1 mL of 0.12 M PB and 0.1% $NaDodSO_4$ was denatured for 1 min at 97 to 98 °C, cooled to room temperature, and applied to a 0.2-mL column of oligo(dT)–cellulose. The denatured probe in 1 mL of 0.12 M PB and 0.1% $NaDodSO_4$ was then applied to the column, and the procedure was continued as described above. [e] Unique DNA was isolated as DNA that remains single stranded after reassociation to E $C_0t = 1000$. [f] Repetitive DNA was isolated as DNA that is hybridized after reassociation to E $C_0t = 1000$.

human DNA. They are present also in mouse DNA but apparently not in *E. coli* DNA, which caused no decrease in binding of the probe (Table I).

Attempts to remove the presumed interfering T-rich sequences from the driver DNA preparations by hybridization to poly(rA) or by binding to poly(A)–Sepharose were unsuccessful. We therefore tried to establish conditions that would favor binding of the poly(dA) tails to oligo(dT) over interaction of driver DNA sequences with the poly(dA) tails. Increasing the salt concentration above 0.12 M PB decreased the binding of the probe in the presence of driver DNA, while decreasing the salt concentration much below 0.12 M PB decreased the binding of the probe alone to oligo(dT)–cellulose columns (data not shown). A greater binding of probe to oligo(dT)–cellulose in the presence of driver DNA was achieved by adding the heat-denatured DNA solution to the oligo(dT)–cellulose before cooling. The mixture was then stirred for 16 h at room temperature. This procedure, which increased the binding of the probe in the presence of driver DNA to ~45%, was used in the isolation of X chromosomal DNA described in this paper.

*Preparation of Human X Chromosomal DNA.* The results of each hybridization step in the procedure used to isolate human X chromosomal DNA are given in Figure 5. Human DNA sequences homologous with mouse DNA were removed by a preparative hybridization with unlabeled mouse DNA. A temperature of 60 °C, rather than 71 °C, was used for the hydroxylapatite chromatography after this step to permit maximum removal of homologous sequences. At this high $C_0t$ and relatively low driver/probe ratio there should be little or

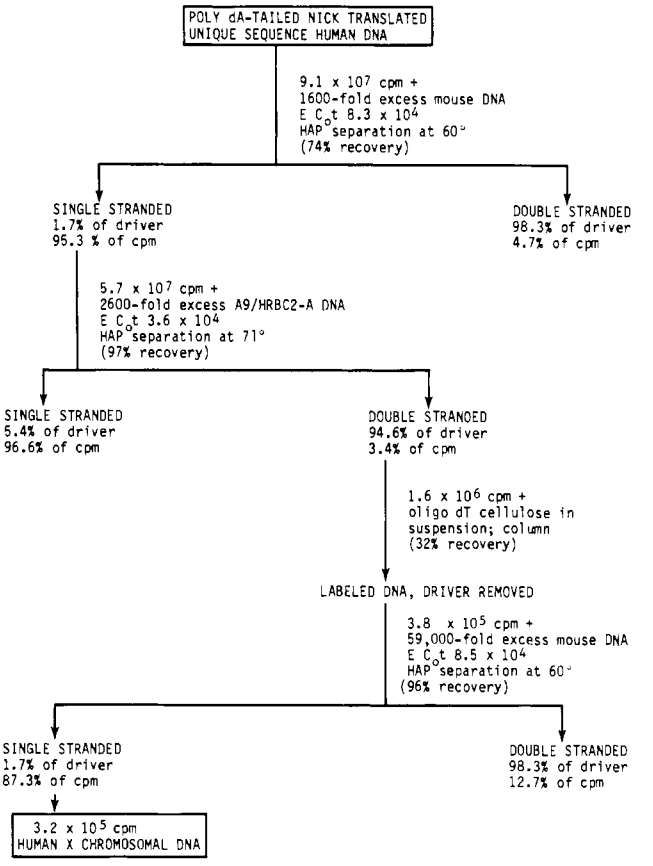


FIGURE 5: Diagram of preparative hybridization steps in the isolation of unique sequence human X chromosomal DNA. The total recovery of $^3$H cpm at each step as well as the $^3$H cpm carried on to the next step is indicated.

no labeled DNA lost to the double-stranded fraction because of hybridization of the poly(dA) tails.

The labeled human DNA not homologous with mouse DNA was hybridized with A9/HRBC2-A DNA, to which human X chromosomal DNA sequences will hybridize. Incubation at this step was to a lower E $C_0t$ than that in the hybridization with mouse DNA to decrease the amount of labeled DNA in the double-stranded fraction due to self-hybridization. The higher criterion of 71 °C was used for the hydroxylapatite chromatography after this step in order to exclude from the double-stranded DNA fraction any labeled human DNA in which only the poly(dA) tails were hybridized. The hybridized labeled human DNA was recovered from the unlabeled A9/HRBC2-A DNA by binding to oligo(dT)–cellulose in suspension (45% recovery). A further purification from the unlabeled driver as well as a concentration of the labeled DNA was achieved by then binding to an oligo(dT)–cellulose column (71% recovery).

Analytical hybridizations (hydroxylapatite chromatography at 71 °C) after these two steps indicated that ~6% of the hybridizable labeled DNA could still hybridize with mouse DNA. This was removed by a second hybridization with unlabeled mouse DNA. In order to achieve a total DNA ratio of driver to probe of ~3000, we used a weight ratio of mouse DNA 20-fold greater than this since the purified X chromosomal DNA at this step represents only ~5% of the total DNA. As with the first preparative hybridization with mouse DNA, hydroxylapatite chromatography after this step was at 60 °C. The percent of the labeled DNA in the double-stranded fraction at this step was larger than expected from the results of the analytical hybridizations. This could be due to the lower temperature of 60 °C used for the preparative hybridization.

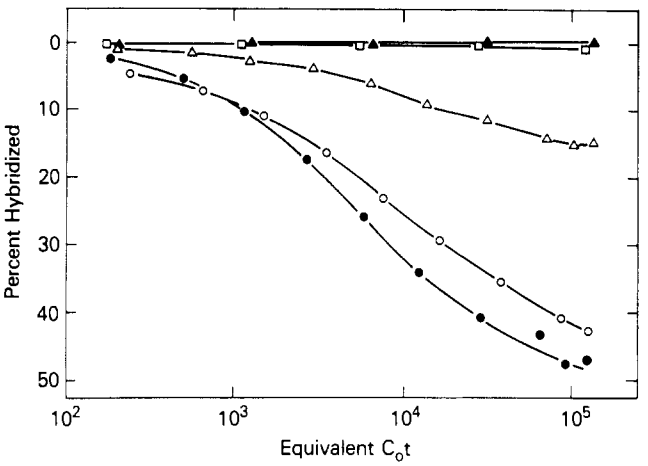


FIGURE 6: Analytical hybridizations of purified unique sequence human X chromosomal DNA probe with various driver DNA samples. Samples of poly(dA)-tailed nick-translated human X chromosomal DNA were reassociated with excess unlabeled DNA (mean length 500–570 nucleotides) in 0.4 M PB at 68 °C. The driver DNA samples were the following: (▲) *E. coli*, ($7.7 \times 10^5$)-fold excess; (□) mouse, ($6.5 \times 10^5$)-fold excess; (△) 1W1-5, ($7.5 \times 10^5$)-fold excess; (○) A9/HRBC2-A, ($8.9 \times 10^5$)-fold excess; (●) human, ($6.8 \times 10^5$)-fold excess. The fraction of the $^3$H cpm bound to hydroxylapatite at 71 °C is plotted as a function of E $C_0t$.

The input counts per minute and percent recovery at each step in this procedure are indicated in Figure 5. When the fraction of the total counts per minute recovered from one step that was carried on to the next step is taken into account, the recovery of labeled DNA in the final X chromosomal DNA preparation is 0.6%. Since the human X chromosome represents ~5.4% of the haploid human genome (Bergsma, 1972), this figure is ~11% of the theoretical maximum recovery.

*Analysis of Purified Human X Chromosomal DNA.* The reassociation kinetics of the purified human X chromosomal DNA probe with various unlabeled DNA preparations is illustrated in Figure 6. The complete lack of hybridization of the probe with *E. coli* DNA indicates that at this driver excess and E $C_0t$ range there is no self-hybridization of the probe. The absence of hybridization with *E. coli* DNA here, as well as after the previous preparative hybridization step (data not shown), also indicates that the driver A9/HRBC2-A DNA used in the preparative hybridization was effectively removed from the probe on oligo(dT)–cellulose. The hybridization with mouse DNA is only slightly greater than that with *E. coli*, reaching a maximum of 0.9% at the highest $C_0t$ point. The maximum hybridizability of the probe with human DNA is 48%. Two factors contribute to this low hybridizability: a reduced size of the probe and a decreased binding of hybrids to hydroxylapatite at the elevated temperature of 71 °C. The increased E $C_0t_{1/2}$ value of 5000 for hybridization with human DNA, compared to 2000 for the original nick-translated unique sequence human DNA (see Figure 2), indicates that the probe had decreased to ~40% of its original size.

The probe appears to hybridize to a slightly lesser extent (43%) with A9/HRBC2-A DNA than with human DNA. However, the extent of hybridization with A9/HRBC2-A DNA is underestimated relative to that with human DNA because the rate of hybridization with A9/HRBC2-A DNA is slower and hybridization is not quite complete at the highest E $C_0t$ ($1.2 \times 10^5$). Thus, more than 90% (i.e., 43/48) of the DNA sequences of the probe are human DNA sequences present in A9/HRBC2-A. The remaining few percent represent contaminating human DNA sequences which are not in A9/HRBC2-A. These could result from a small amount of self-hybridization of the labeled human DNA in the preparative hybridization with A9/HRBC2-A and could presumably be decreased by a second preparative hybridization with a large excess of A9/HRBC2-A DNA. At the lowest two $C_0t$ points in Figure 6 the probe appears to be hybridizing slightly faster with A9/HRBC2-A DNA than with human DNA, suggesting that there may be multiple copies of some human X chromosomal DNA sequences in A9/HRBC2-A. Although this has been observed in two separate reassociation experiments, there are not sufficient data to analyze the shape of the curve. Considering the E $C_0t$ points above 1000, the E $C_0t_{1/2}$ of the probe with A9/HRBC2-A DNA is about twofold greater than that with human DNA, indicating a roughly twofold greater concentration of human X chromosomal DNA sequences in human DNA than in A9/HRBC2-A DNA. At least this much difference is expected because the human DNA was isolated from a female and thus contains two X chromosomes per diploid genome, while the A9/HRBC2-A line contains only one human X chromosome per cell. Since this cell line has greater than the diploid number of mouse chromosomes (D. E. Moore and O. W. McBride, unpublished experiments), we would expect the concentration of human X chromosomal DNA in A9/HRBC2-A to be even less than half that of the female human DNA.

The labeled X chromosomal DNA was reassociated with DNA from another mouse–human hybrid cell line, 1W1-5, which contains only a fragment of the human X chromosome. As shown in Figure 6, 15.5% of the probe was hybridized with 1W1-5 DNA, suggesting that 1W1-5 contains at least 36% (i.e., 15.5/43) of the human X chromosome. Enzyme analyses indicate that 1W1-5 could contain as much as the whole long arm of the human X chromosome (Buck & Bodmer, 1976), representing ~60% of that chromosome. Our finding of ~36% of the human X chromosome suggests that 1W1-5 may contain less than the entire long arm of the human X, or it may indicate the magnitude of non X chromosomal human DNA sequences present in the A9/HRBC2-A cell line. As with A9/HRBC2-A, the E $C_0t_{1/2}$ of the probe with 1W1-5 DNA is twice that with human DNA, reflecting the presence of only one human X chromosome fragment per cell.

## Discussion

We have described a procedure for direct isolation of labeled unique sequence human X chromosomal DNA using a human–mouse hybrid cell line, A9/HRBC2-A, which contains only the human X chromosome. A positive selection for human X chromosomal DNA is made possible by the use of a unique sequence human DNA probe to which tails of poly(dA) have been added. The labeled human DNA which hybridizes with A9/HRBC2-A DNA can then be isolated on oligo(dT)–cellulose.

When carried out under standard conditions for DNA reassociation, this procedure is complicated by the formation of hybrids between the poly(dA) tails of the probe and the T-rich sequences in mouse and human DNA. These poly(dA) tails are present in about a $10^7$-fold excess [i.e., 1 poly(dA) tail/200-base fragment compared to 1 unique sequence/$3 \times 10^9$ base pair genomic complexity] over any particular unique sequence in the $^3$H-labeled probe population. Poly(dA) tails are apparently present in excess over T-rich sequences in the total hybridization mixture since, at any given $C_0t$ value, the fraction of the poly(dA)-tailed probe appearing in hybrids increases as the ratio of human DNA to probe is increased. In the experiment described in Figure 3, the ratio of poly(dA) tails to unique sequences varies from ~6000 (i.e., $10^7/1600$) with a 1600-fold excess of unlabeled DNA to ~300 [i.e.,

$10^7/(3.7 \times 10^4)$] with a $(3.7 \times 10^4)$-fold excess of unlabeled DNA. The observation of ~30% excess hybridization of the poly(dA)-tailed probe with an unlabeled DNA excess of $3.7 \times 10^4$ suggests a minimum of ~100 (i.e., $300 \times 0.3$) T-rich sequences per haploid genome.

Although the location of these T-rich sequences is not known, they appear to be present in both repetitive and unique DNA. The presence of T-rich regions in the introns of several mammalian genes, especially at the 3′ end, has recently been demonstrated (Crick, 1979). Since the hybrids formed between these T-rich sequences and the poly(dA) tails are less stable than those of unique sequence DNA, they can be eliminated by carrying out the hydroxylapatite chromatography at an elevated temperature of 71 °C. At this temperature there is only a 15–26% decrease in the recovery of unique sequence hybrids on hydroxylapatite, and the reassociation kinetics of the poly(dA)-tailed probe, analyzed at 71 °C, are nearly indistinguishable from those of a similar probe without poly(dA) tails, analyzed at 60 °C. Since the imperfect hybrids formed between the poly(dA) tails and T-rich sequences do not inhibit hybridization of the unique DNA sequences of the probe, an elevated temperature during reassociation of the poly(dA)-tailed probe is not necessary. Thus, hybridization can be carried out at a temperature range which allows the maximum rate of reassociation, i.e., ~25 °C below the $T_m$ (Britten et al., 1974).

The net recovery of X chromosomal DNA in this procedure was ~11% of the theoretical maximum recovery. This low recovery was partly due to degradation of the probe which resulted in a decreased hybridizability and thus a lower recovery in the positive selection step. In addition, the recovery of hybrids on hydroxylapatite is decreased at the elevated temperature of 71 °C. However, the major factor in the low recovery was the poor binding of the poly(dA)-tailed probe to oligo(dT)–cellulose in the presence of unlabeled A9/HRBC2-A DNA. The best conditions for binding, that is, addition of the denatured DNA in a solution at high temperature to oligo(dT)–cellulose followed by overnight incubation at room temperature in suspension, resulted in a recovery of only 30–45% of the probe. Since the dT oligomers in the oligo(dT)–cellulose are only ~15 residues long compared to the 80–100-residue poly(dA) tails, it is unlikely that conditions could be established that would clearly discriminate against interaction of the poly(dA) tails with T-rich regions in the driver DNA while allowing binding of the poly(dA) tails to oligo(dT). The use of poly(U)–Sepharose (data not presented in this paper), with chains ~100 residues long, resulted in a greatly improved binding of the probe in the presence of driver DNA. However, the bound probe was not quantitatively eluted, even with 90% formamide, and the instability of the Sepharose matrix precluded the use of high temperatures for elution. Preliminary experiments with poly(U)–Sephadex containing poly(U) chains 100 residues and longer, synthesized according to Coffin et al. (1974), suggest that this material will permit both good binding of the probe in the presence of driver and quantitative recovery of the bound probe.

It is not possible to isolate total X chromosomal DNA by a hybridization procedure of the type described here because of the wide range of $C_0t$ values over which mammalian DNA hybridizes (Britten & Kohne, 1968). If the probe consisted of total DNA, the high $C_0t$ values required for hybridization of the unique DNA sequences would result in self-hybridization of the repetitive DNA sequences of the probe. We chose to isolate unique sequence DNA, rather than repetitive DNA, for several reasons: (1) since it is present in a single frequency class, unique sequence DNA will hybridize over a narrow $C_0t$ range; (2) hybrids formed between complementary unique DNA sequences will be perfectly paired, while repetitive DNA hybrids will not, due to the differing degress of homology within repetitive DNA families; (3) unique sequence DNA from the X chromosome will be found only on that chromosome, whereas repetitive DNA from the X chromosome may be present on other chromosomes as well. The procedure we have described for isolation of unique sequence X chromosomal DNA using a poly(dA)-tailed probe might not be applicable to the isolation of repetitive DNA. The higher stringency required to discriminate against hybrids between T-rich driver DNA sequences and the poly(dA) tails might also discriminate against many of the imperfectly paired repetitive DNA hybrids.

Since this probe is designed to detect small amounts of human X chromosomal DNA in mouse cells, it is especially important that the background hybridization of the probe with mouse DNA be low. Although the probe consists only of human DNA, we would expect considerable hybridization with mouse DNA if the positive selection step of hybridization with A9/HRBC2-A DNA were the only step used to prepare the purified X chromosomal probe. While X chromosomal DNA represents ~5.4% of the haploid human genome, about 3 to 4% of unique sequence human DNA is homologous with mouse DNA (A. S. Olsen and O. W. McBride, unpublished experiments). Thus, ~40% of the human DNA which hybridizes with A9/HRBC2-A DNA would also hybridize with mouse DNA. Analytical hybridizations of the purified X chromosomal probe with mouse DNA indicate that the two preparative hybridization steps with mouse DNA removed all but a trace of this human DNA that is homologous with mouse DNA.

We have also prepared a human X chromosomal DNA probe from poly(dA)-tailed nick-translated unique sequence A9/HRBC2-A DNA rather than human DNA. The advantage of this method is that the only human DNA sequences present in the probe are X chromosomal. Therefore, the probe will not be contaminated with any non X chromosomal human DNA sequences resulting from self-hybridization of the labeled DNA in the positive selection step. However, since the A9/HRBC2-A probe is originally ~98% mouse DNA, it is more difficult to remove all the mouse DNA from this probe than it is to remove the 3 to 4% of the human DNA probe which is homologous with mouse DNA. Since we require an extremely low mouse background in the purified probe, the use of a probe prepared from human DNA, as described in this paper, is the preferred procedure.

Analytical hybridizations of the purified unique sequence X chromosomal DNA probe with A9/HRBC2-A and human DNA indicate that the probe is at least 90% pure; that is, at least 90% of the DNA sequences of the probe represent human DNA sequences present in A9/HRBC2-A. The maximum purity of a chromosome-specific probe prepared by this procedure ultimately depends on the chromosomal purity of the hybrid cell line used to prepare the probe. In the case of A9/HRBC2-A there appears to be a small amount of non X chromosomal DNA present in the form of a human–mouse translocation chromosome. Thus, the absolute purity of this probe with respect to X chromosomal DNA will be reduced by a factor which depends on the amount of this non X chromosomal human DNA present in A9/HRBC2-A. An estimate of this could be obtained by determining the extent of hybridization of the probe with DNA from A9/HRBC2-A cells which have lost the human X chromosome. It should be

possible to select for these rare cells by growth in medium containing 6-thioguanine. However, in four separate experiments with a total of $1.1 \times 10^8$ cells we were unable to isolate any A9/HRBC2-A cells resistant to 6-thioguanine. In parallel experiments with other hybrids derived from human and mouse $LA_9$ cells, we were able to isolate cells which had lost the human X chromosome, indicating that there was no problem with the selection system. M. Siniscalco, who provided the parental A9/HRBC2 line, has also been unable to isolate any 6-thioguanine-resistant cells from this line (M. Siniscalco, personal communication). Allderdice et al. (1973) have reported that the cell line $LA_9$ does not contain a normal mouse X chromosome. Since virus-induced cell fusion may be accompanied by chromosome breaks (Miller et al., 1971), it is possible that a portion of the mouse X chromosome has been lost from A9/HRBC2 and that the deleted mouse genetic functions are supplied by the homologous region of the human X chromosome. It is also possible that an essential piece of mouse DNA has been translocated onto the human X chromosome in A9/HRBC2 cells, although there is no karyological evidence for this. In either case, the viability of the cells would require the continued presence of the human X chromosome.

The procedure described here for isolation of unique sequence human X chromosomal DNA can be applied to the isolation of unique sequence DNA from any human chromosome, provided an interspecies hybrid cell can be obtained containing only that particular human chromosome. The development of microcell-mediated chromosome transfer to introduce one or very few chromosomes into recipient cells (Ege & Ringertz, 1974; Fournier & Ruddle, 1977) should aid in the production of these desired hybrids.

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