
Isolation and characterization of cloned human DNA fragments carrying reiterated sequences common to both autosomes and the X chromosome

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ABSTRACT

Several recombinants were identified and purified from a cloned library of human DNA by virtue of their homology to DNA from a mouse-human hybrid cell line containing a single human chromosome, the X, and their lack of homology to mouse DNA. Three recombinants were characterized in detail, and all were homologous to reiterated DNA from the human X chromosome. These recombinants also were homologous to reiterated sequences on one or more human autosomes and, therefore, were not X chromosome specific. The recombinant DNA fragments homologous to human reiterated X DNA were the same fragments homologous to human reiterated autosomal DNA. Digestion of genomic DNAs with several restriction enzymes revealed that the pattern of fragments homologous to one recombinant, λ Hb2, was the same on autosomes as on the X chromosome, suggesting that the molecular organization of these elements on the X is not distinct from their organization on autosomes.

INTRODUCTION

A better understanding of chromosome structure, function and evolution can be obtained by analyzing the DNA sequences carried by individual chromosomes. Mammalian X chromosomes are of particular interest because they are subject to the phenomenon known as X inactivation as a form of dosage compensation (1), they are genetically well characterized (2) and their cytogenetic morphology and gene content appear to have been strongly conserved during evolution (3). We have previously described some of the properties of human X chromosome DNA isolated by solution hybridization experiments (4). Olsen *et al.* (5) have used similar tactics to isolate human single copy X chromosome DNA sequences. Recently Wolf *et al.* (6) described two X chromosome DNA fragments isolated from a partial library of human DNA by sampling recombinants for their X chromosome homology and specificity.

To date, most of the human DNA sequences mapped to specific chromosomes have been those for specific structural genes (*e.g.*, ref. 7-10). These DNA sequences were all identified by strategies dependent upon isolation of the transcribed RNA for use as a molecular probe directly (7) or indirectly via cDNA sequences copied from the mRNA (8-10). However, much of the genomic DNA, including reiterated as well as single copy sequences, is thought to be

non-coding (11). Furthermore, reiterated sequences, including some found on mammalian X chromosomes (4,12), can be either chromosome-specific (13) or distributed on several chromosomes (14,15). Thus, different strategies must be used to isolate and study non-coding sequences carried by individual chromosomes.

We describe here an approach to isolating cloned human DNA fragments with homology to the human X chromosome, some of which fragments should also have homology to additional human chromosomes. Specifically, a library of human DNA cloned in the λ vector Charon 4A was screened with radiolabeled DNA from a mouse-human hybrid with a single human chromosome, the X, and with radiolabeled DNA from the parental mouse line. Hybridization conditions used for the screen were quite stringent, estimated to allow no more than 10% mispairing (16,17). We chose to study further those recombinants which hybridized with DNA from the hybrid cell line, but not with mouse DNA. Recombinants chosen by our screening method contain human-specific DNA sequences homologous to human X chromosome DNA, although the method does not guarantee that all DNA in such recombinants will be homologous to X DNA.

We show here that three recombinants chosen by our screening method, all homologous to reiterated X chromosome DNA, are also homologous to reiterated autosomal DNA, as expected from our previous study (4) in which we demonstrated by solution hybridization (C_0t analysis) that the most highly reiterated human-specific DNA sequences on the X chromosome are not confined to the X, but are also on one or more autosomes. These kinds of X DNA sequences collectively account for about 6% of all human X chromosome DNA (4). These sequences are of particular interest because of their abundance on the X chromosome and since analysis of specific sets of dispersed reiterated sequences may help elucidate their genomic functions. The reiterated elements of the recombinants described below can be used to study the molecular organization of particular human chromosomes, as long as that chromosome is isolated in a hybrid cell context. Indeed, using this approach we have found that one recombinant carries representatives of genomic reiterated elements whose organization on the X chromosome is indistinguishable from their organization on autosomes.

MATERIALS AND METHODS

Cultured Cell Lines. A human 45,XO fibroblast cell line, a human 47,XXX fibroblast cell line (GM-254), the mouse A9 cell line, and the mouse-human hybrid cell lines AHA-11a, AHA-16e and AHA-16eAz (originally AHA-16eAGThG)

were maintained as described (4). AHA-11a contains a single human chromosome, the X. A back-selected line lacking the human X chromosome, AHA-11aAz, was derived from AHA-11a at the time of these experiments as described (4). AHA-11aAz lacked human X-linked glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49) activity and had no cytologically-detectable human chromosomal material. AHA-16e contains thirteen human chromosomes (1, 2p⁻, 3, 4, 10, 11, 13, 14, 15, 18, 21, 22, X) at frequencies greater than 40% as revealed by trypsin-Giemsa banding at the time of these experiments. AHA-16eAz was derived from AHA-16e by back-selection in medium containing 8-azaguanine and 6-thioguanine (4). When AHA-16eAz was first described (4), the line lacked a cytologically detectable human X chromosome and was negative for three X chromosome-linked human isozymes and an X-associated cell surface marker. These cells die in medium containing hypoxanthine/aminopterin/thymidine (HAT) (18). Analysis of AHA-16eAz at the time of these experiments confirmed the lack of a cytologically identifiable human X chromosome and the absence of human G6PD activity and indicated the presence of the following human chromosomes at frequencies greater than 40%: 2p⁻, 10, 11, 13, 14, 18, 21.

Four additional cell lines were used in a few experiments. The mouse-human somatic cell hybrid XVI - 18A (19) contains 7 human autosomes (1p/14q, 2, 5, 7, 8, 17, 21) at frequencies greater than 40% as revealed by trypsin-Giemsa banding at the time of these experiments. The mouse-human somatic cell hybrid VII - 2 HAT (10,20) contains a single human chromosome, an 11p/17q translocation chromosome, in all cells. The Chinese hamster-human somatic cell hybrid, cl 2D, (see Fig. 1 in ref. 21), containing a single human chromosome, the X, and the Chinese hamster parent Wg3-h have been described (21) and were generously provided by S. Goss.

Purification and Radiolabeling of DNA. Nuclear DNA from cultured cells or from human male (46,XY) or female (46,XX) placentae was prepared as described (4). Recombinant bacteriophage DNA was prepared from CsCl-banded phage by sequential extractions with phenol and chloroform, followed by two precipitations from ethanol.

DNA was labeled with [α -³²P]dATP and [α -³²P]dCTP by nick translation with E. coli DNA polymerase I as described (22), except that no DNase I was used and the sample was incubated at 13-14^o for 48 hr. The specific activities of the nick translated DNAs were 1-3 x 10⁸ cpm/ μ g.

Library Screening. A human genomic library of fetal liver DNA cloned in Charon 4A was generously provided by T. Maniatis (23). Recombinant phage were plated (24) using NZYDT agar (25) on the EK2 host E. coli DP50supF at

2500-3000 plaques per 15 cm Petri plate. The plaques were screened for homology to the human X chromosome using a modification of the procedure described by Benton and Davis (26). Three replicate filters were prepared sequentially from each plate (26) and baked overnight at 70°. The filters were prehybridized in sealed bags for 18 hr at 37° in a solution of 3 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50% deionized formamide, 250 µg/ml salmon sperm DNA (previously fragmented to 0.3 kb by boiling 20 min in 0.2 N NaOH, and then neutralized before use), 1% glycine, 50 mM Na-PO₄ buffer (pH 6.8) and 0.2% each Ficoll, polyvinylpyrrolidone and bovine serum albumin, (10 x Denhardt's solution, ref. 27). Filters were then transferred to fresh bags. Hybridization solution was added and the filters were rotated at 37° for 90 hr. The hybridization solution was the same as the prehybridization solution except that 1) 3 x SSC was reduced to 2 x SSC, 2) the formamide concentration was increased to 70% and 3) ³²P-labeled AHA-11a or ³²P-labeled A9 DNA was added to a final concentration of 8 x 10⁶ cpm/ml. Two replicate filters from each plate were hybridized with ³²P-labeled hybrid cell AHA-11a DNA and one filter from each plate was hybridized with ³²P-labeled mouse A9 DNA. Stringent hybridization conditions (16) were chosen in an attempt to reduce the cross-reaction between human X chromosome DNA and mouse DNA (4). After hybridization, filters were rinsed three times at room temperature in 2 x SSC and then subjected to a series of 65° washes in buffered 1 M NaCl and buffered 0.5 M NaCl as suggested by Dr. Keith Peden (Peden, K., Mounts, P. and Hayward, G.W., manuscript in preparation). After the 65° washes, the filters were rinsed in 2 x SSC at room temperature. The filters were dried and exposed to X-ray film (Kodak X-Omat) with intensifying screens (Dupont Cronex Lightning Plus 2L) at -80° for 5 d. After developing films, the set of triplicate autoradiograms was aligned with the plate (26). Individual plaques were selected and the phage eluted by storing the agar plug for 16-18 hr at 4° in a solution of 0.25 M NaCl, 10 mM MgCl₂, 10 mM Tris HCl, pH 7.4, 0.1 mM EDTA.

Purification and Amplification of Recombinants. Each plaque was purified through two or three additional rounds of plating and screening as described above. In some experiments ³²P-labeled AHA-11aAz DNA was substituted for ³²P-labeled A9 DNA. Each of the original isolates was considered pure when, upon subsequent plating, at least 90% of the plaques hybridized to labeled AHA-11a DNA and none hybridized to labeled A9 or AHA-11aAz DNA.

A plaque from each final plating was used to prepare a primary phage stock

(28) in NZYDT broth (25), and the primary stock was used to prepare a large-scale lysate essentially as described (25), except that we used twice the concentration of NZY components (25) in our broth (i.e., 2 x NZY, 1 x DT). Phage were recovered by polyethylene glycol precipitation (29) and resuspended in 0.25 M NaCl, 10 mM Tris HCl, pH 7.4, 0.1 mM EDTA. The phage were partially purified by centrifugation on a three-step CsCl gradient (30) and then banded by equilibrium sedimentation in CsCl as described (31). In both cases, the CsCl was dissolved in 0.25 M NaCl, 10 mM MgCl₂, 10 mM Tris HCl, pH 7.4, 0.1 mM EDTA. The phage band was removed and dialyzed against 0.1 M NaCl, 10 mM Tris HCl, pH 8.0, 1 mM EDTA. The recombinant phage H β G1 (23), a gift of T. Maniatis, was amplified and purified as described above.

All experiments involving growth and cloning of recombinants were performed using P2 EK2 containment procedures in accordance with NIH regulations in effect when the clones were isolated.

Enzyme Digestions and Gel Electrophoresis. Restriction enzymes were obtained from Bethesda Research Laboratories (Rockville, MD) or New England Biolabs (Beverly, MA). DNAs were digested with 1-46 units of restriction endonuclease per μ g DNA, using the buffers recommended by the supplier for each enzyme (Eco RI, Kpn I, Hind III, Bam HI, Xba I, Hae III and Alu I). Typically, each digest received one portion of enzyme, and then a second portion several hours later. Completeness of digestion was indicated by reproducible electrophoretic and hybridization patterns when different enzyme to DNA ratios were used.

Digests, containing up to 10 μ g DNA in 20-50 μ l, were loaded into wells (3mm x 8mm) of agarose gels (0.8-1.5%) made in 40 mM Tris-acetate, pH 8.0, 13 mM sodium acetate, 20 mM EDTA. Gels were run in this buffer at room temperature with constant current for 110 mAmp-hr. Gels were then stained with ethidium bromide (2 μ g/ml for 15-30 min) and photographed, and the DNA was transferred to nitrocellulose filters as described by Southern (32). Molecular weights (in kilobase pairs, kb) of fragments were determined by comparison with migration of fragments of known size run on the same gel. The molecular weight standards were Hind III digests of λ DNA (fragments (in kb) of 23.7, 9.5, 6.7, 4.3, 2.2, 2.0, and 0.5; information from BRL, supplier of DNA), and Hae III digests of ϕ X-174 RF DNA (fragments (in kb) of 1.3, 1.1, 0.9, 0.6 and 0.3 plus others not resolved on our gels; ref. 33).

Filters were hybridized as described above for screening filters using 5 ml of hybridization mix per 10 x 14 cm filter. The hybridization time was 68-72 hr unless noted otherwise. The radioactivity in the hybridization mix

ranged from 10^5 - 10^7 cpm/ml. After hybridization, the filters were washed and autoradiograms were developed as described for screening filters. In most experiments filters were then washed four times at 65° in $0.1 \times$ SSC, 0.1% SDS (34) then several times in $0.1 \times$ SSC at room temperature. The filters were dried and autoradiographed again.

RESULTS

Library Screening. Fig. 1 shows representative results from the screening of the cloned library (23). Radiolabeled AHA-11a DNA (X^+ DNA) hybridized to nearly every plaque (Fig. 1A and 1C) as did 32 P-labeled A9 DNA (X^- DNA, Fig. 1B). However, a few plaques were positive with X^+ DNA on both filters and negative with X^- DNA. Three such plaques are indicated in Fig. 1; twenty eight such plaques were selected from three plates and eight of these were plaque-purified. Fig. 1D-F shows the results of the second plating of one recombinant.

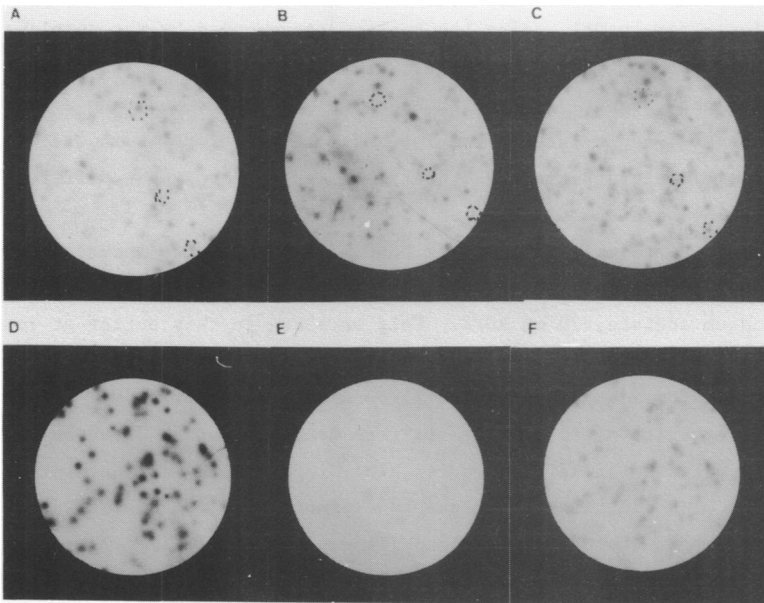


Fig. 1 Screening and Purification of Recombinants. A-C, representative results of screening cloned human library. Replicate filters were hybridized with 32 P-labeled AHA-11a (X^+) DNA (A and C) or with 32 P-labeled A9 DNA (B). Dotted circles indicate three of the plaques picked for purification. D-F, representative results of second plating of one recombinant λ Hb2. Replicate filters were hybridized with 32 P-labeled AHA-11a (X^+) DNA (D and F) or 32 P-labeled A9 DNA (E).

Restriction Patterns of Cloned DNA. Three of the plaque-purified clones were amplified, and the DNA from each was analyzed by agarose gel electrophoresis after digestion with Eco RI. Ethidium bromide staining of the gel revealed three distinct patterns of fragments (Fig. 2). The recombinants have been named λ Hb2, λ Hb4, and λ Hb5; for brevity they will be referred to as b2, b4 and b5. The human insert Eco RI fragments were identified by their lack of co-migration with Charon 4A Eco RI fragments (Fig. 2) and by their lack of homology to 32 P-labeled Charon 4A DNA (data not shown).

The six Eco RI fragments from the human DNA inserted into the recombinant phage b2 add up to 16.0 kb. The ten Eco RI fragments from the human DNA insert of b4 add up to 14.5 kb. The three Eco RI fragments from the human DNA insert of b5 add up to 15.3 kb. Each of these values was the average of at least five measurements.

Also shown in Fig. 2 is the Eco RI restriction pattern of λ H β G1, a well-characterized, previously isolated recombinant from this library (23). This phage contains the δ - and β -globin genes and flanking DNA, genes which have been mapped to human chromosome 11 (9). Thus, in many of the experiments to be described, H β G1 was a convenient control for both single copy and

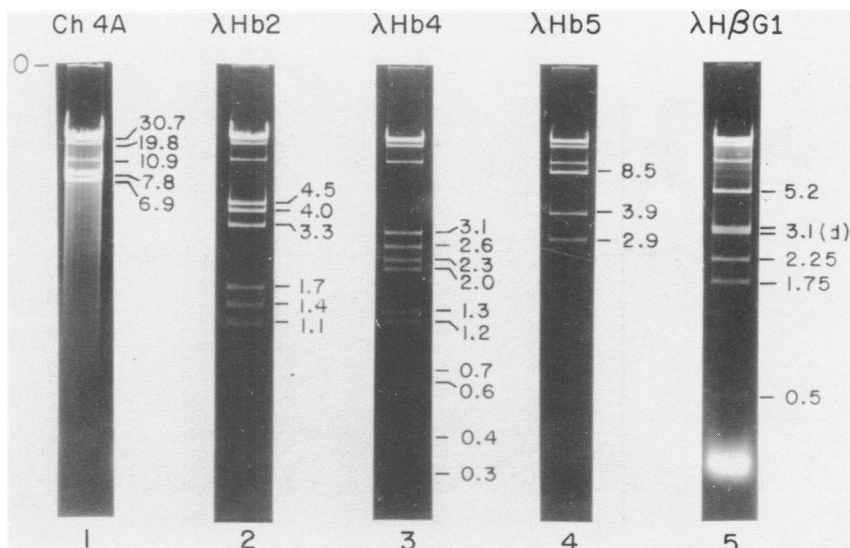


Fig. 2 Ethidium Bromide Patterns of Eco RI Digested Recombinant DNAs Separated on 1% Agarose Gels. The numbers refer to the fragment lengths in kb; 0 indicates the origin. Lane 1, 0.5 μ g Charon 4A DNA; lane 2, 0.25 μ g λ Hb2 DNA; lane 3, 0.25 μ g λ Hb4 DNA; lane 4, 0.25 μ g λ Hb5 DNA; lane 5, 0.25 μ g λ H β G1 DNA (23).

certain reiterated autosomal sequences.

Homology to Human X Chromosome. To show directly that the recombinants b2, b4 and b5 were homologous to the human X chromosome, we probed Eco RI digests of AHA-11a (X^+) DNA and of back-selected AHA-11aAz (X^-) DNA with each ^{32}P -labeled phage DNA in a blot experiment (Fig. 3); *i.e.*, fragments from Eco RI restriction digests of DNA were fractionated by electrophoresis, transferred to filters (32) and hybridized with ^{32}P -labeled DNAs. Radiolabeled b2 DNA hybridized to one major fragment in AHA-11a DNA at 3.3 kb as well as to a large number of other fragments giving the effect of a smear. Mixing experiments confirmed that the major Eco RI fragment in AHA-11a DNA was the same size as the b2 3.3 kb Eco RI fragment (data not shown). Labeled b4 DNA and labeled b5 DNA both hybridized as smears to AHA-11a DNA without any evidence of major fragments. Each recombinant cross-reacted very faintly (less than 1% of the binding to X^+ DNA, as estimated from autoradiograms) with back-selected AHA-11aAz (X^-) DNA or mouse A9 DNA, but this binding was completely eliminated by washing the filter in 0.1 x SSC, at 65°. Washing the filters with 0.1 x SSC at 65° also released some of the DNA hybridized to X^+

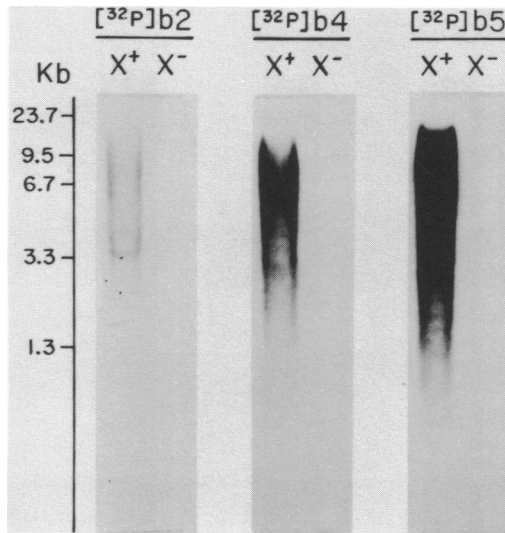


Fig. 3 Homology to Human X Chromosome. Hybridization of ^{32}P -labeled recombinant DNAs (3×10^6 cpm/ml hybridization mix) to 10 μ g of Eco RI digested AHA-11a (X^+) DNA or AHA-11aAz (X^-) DNA. The exposure for each autoradiogram was 5.5 hr. Numbers indicate migration of some of the molecular weight standards and the size (3.3 kb) of the prominent fragment visible in the first lane. Filters were not washed in 0.1 x SSC.

DNA, but did not alter the hybridization pattern. Clearly, each recombinant was homologous to many Eco RI restriction fragments of human X chromosome DNA.

Test for X Chromosome Specificity. If the sequences of b2, b4 and b5 which were homologous to reiterated X chromosome DNA were confined to the X chromosome, then the intensity of the hybridization should be directly proportional to the number of X chromosomes in the target DNA. The X-specificity of these recombinants was tested with a blot experiment in which three human DNAs, normal male (46,XY), normal female (46,XX) and a 47,XXX cell line, were digested with Eco RI, and probed with phage DNA. All three ³²P-labeled recombinant DNAs hybridized to all three human DNAs equally well (data not shown). Thus, the reiterated sequences in these recombinants were not X-chromosome specific and are likely representatives of the most reiterated X chromosome sequences described earlier (4).

Cross Reaction between Recombinants. The sequence homology among the three cloned recombinants was examined by blot experiments probing each Eco RI digested DNA with each ³²P-labeled recombinant (Figs. 4A, 4B, 5A). The homology between each Eco RI digested recombinant and ³²P-labeled H β G1 was also determined (data not shown). The results can be summarized as follows. One insert fragment of b2 (4.0 kb) was homologous to three insert fragments of b4 (3.1 kb, 2.3 kb, 2.0 kb) and to all b5 insert fragments. H β G1 and b2 did not share any detectable homology. The recombinants b4 and b5 shared extensive homology with each other. One or both of the 3.1 kb Eco RI fragments of H β G1 shared homology with three insert fragments of b4 (3.1 kb, 2.6 kb, 2.3 kb) and with all b5 insert fragments; these homologies might be due to Alu family sequences known to be present in H β G1 (35,36), although this remains to be shown directly.

When the filters (Figs. 4 and 5) were washed in 0.1 x SSC at 65 $^{\circ}$, little, if any, of the ³²P-labeled b5 DNA hybridized to b5 DNA was removed, as judged by comparing autoradiograms exposed for equivalent times before and after the low salt wash (data not shown). Similarly, the low salt, high temperature wash did not change the intensity of any of the fragments of b2 DNA hybridized to ³²P-labeled b2 DNA (data not shown). However, the low salt, high temperature wash differentially melted off ³²P-labeled b4 DNA hybridized to the b4 human insert fragments (Fig. 5B). Five of the b4 human insert fragments lost so much hybridized radiolabeled DNA that an 8-fold longer exposure (corrected for decay of isotope) was required to obtain autoradiographic bands of about the same intensity as before the low salt wash. The 3.1 kb and 2.3 kb fragments lost less of their radioactivity and

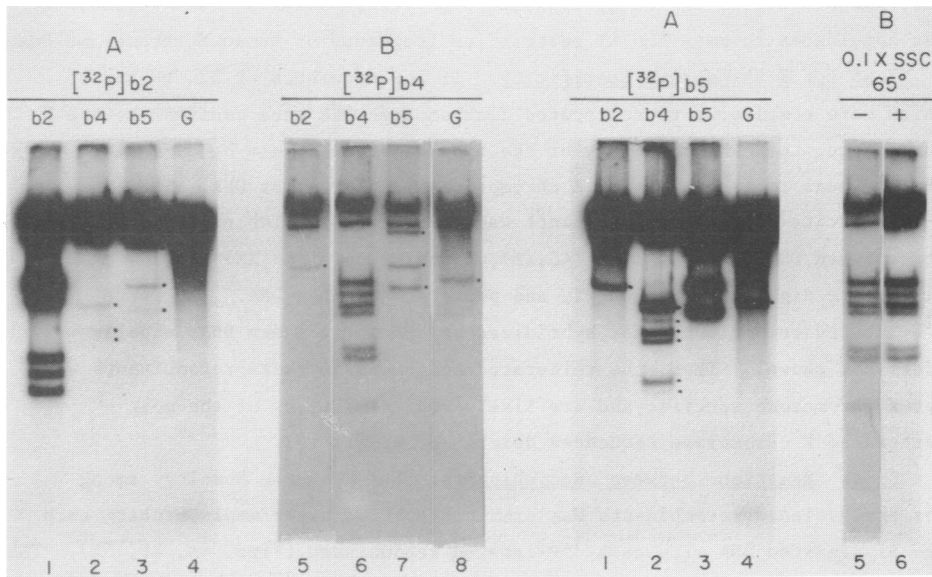


Fig. 4 (Left) Cross Reaction between Recombinants. Eco RI digested recombinant DNAs (0.25 μ g) hybridized with 32 P-labeled DNAs (10^5 cpm/ml hybridization mix for 42 hr). Hybridization of 32 P-labeled b2 DNA (A) or 32 P-labeled b4 DNA (B) to λ Hb2 (lanes 1 and 5), λ Hb4 (lanes 2 and 6), λ Hb5 (lanes 3 and 7) and λ HBG1 (G, lanes 4 and 8). Cross-reacting human insert fragments are indicated with dots. Autoradiograms exposed for 65 hr (A), or 7 hr (B). Filters were not washed in 0.1 x SSC. See Fig. 2 for ethidium bromide patterns of these digests.

Fig. 5 (Right) Cross Reaction between Recombinants. Eco RI digested recombinant DNAs (0.25 μ g) hybridized with 32 P-labeled DNAs (10^5 cpm/ml hybridization mix for 42 hr). A, hybridization of 32 P-labeled b5 DNA to λ Hb2 (lane 1), λ Hb4 (lane 2), λ Hb5 (lane 3) and λ HBG1 (lane 4). Cross-reacting human insert fragments are indicated by dots; autoradiogram exposed for 65 hr. B, self-hybridization of 32 P-labeled b4 DNA to b4 DNA before (lane 5) and after (lane 6) wash of filter in 0.1 x SSC at 65 $^\circ$. Autoradiogram exposed for 7 hr (lane 5) or 56 hr (lane 6, corrected for isotopic decay). See Fig. 2 for ethidium bromide patterns of these digests.

appeared somewhat darker on the second film than on the first. Note the great increase in intensity of the vector fragment bands in the exposure after the wash, showing that well-paired hybrids were not dissociated in this experiment. These results suggest that b4 contains at least one internally repeated sequence element which is not identical in all copies.

Identification of Recombinant Restriction Fragments Bearing Sequence Elements Homologous to Reiterated Genomic DNA. Since the reiterated sequences represented in the recombinants have homology to human autosomes as well as to the human X chromosome, we wanted to know 1) which fragments from the recombinants actually carried the reiterated sequences and 2) whether the

fragments homologous to autosomes were the same as the fragments homologous to the X chromosome. To answer these questions we digested b2, b4 and b5 DNAs with restriction enzymes, ran replicate agarose gels and transferred the fragments to nitrocellulose filters. Another gel of H β G1 DNA digested with Eco RI was run and transferred at the same time. The filters were hybridized for 140 hr with one of four 32 P-labeled genomic DNAs: 1) AHA-11aAz DNA (X $^{-}$), 2) AHA-11a DNA (X $^{+}$), 3) AHA-16e DNA (X $^{+}$ + 12 autosomes) or 4) AHA-16eAz DNA (7 autosomes).

The results with Eco RI digests are shown in Fig. 6 and summarized in the Eco RI columns of Table I. The results with other enzymes (Kpn I, Hae III, Alu I, Xba I, and Bam HI) are also summarized in Table I. As expected, there was no detectable hybridization to the human insert fragments of b2, b4 or b5 with 32 P-labeled AHA-11aAz DNA (Fig. 6, lanes 1-3). When Eco RI digests of H β G1 were probed with radiolabeled AHA-11a DNA, only the 3.1 kb doublet fragment of H β G1 DNA was detected (Fig. 6, lane 4). When H β G1 DNA was probed with 32 P-labeled AHA-16e DNA (X $^{+}$ + 12 autosomes including chromosome 11), the 5.2 kb and the 3.1 kb doublet Eco RI fragments were detected (Fig. 6, lane 5),

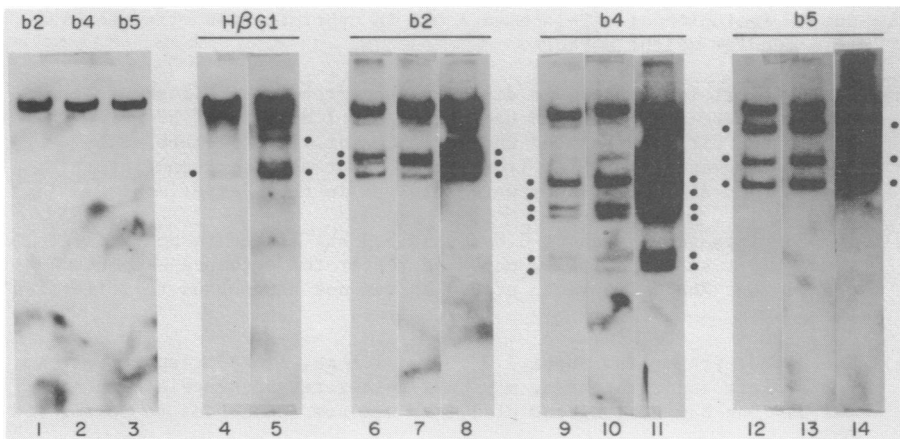


Fig. 6 Recombinant Fragments Homologous to Reiterated Genomic DNA. Eco RI digested recombinant DNAs (0.25 μ g) hybridized with 32 P-labeled genomic DNAs (10^7 cpm/ml hybridization mix for 140 hr). Recombinant DNAs were λ Hb2 (lanes 1, 6-8), λ Hb4 (lanes 2, 9-11), λ Hb5 (lanes 3, 12-14) and λ H β G1 (lanes 4-5). Lanes 1-3 hybridized with 32 P-labeled AHA-11aAz (X $^{-}$) DNA; lanes 4, 6, 9, 12 hybridized with 32 P-labeled AHA-11a (X $^{+}$) DNA; lanes 7, 10, 13 hybridized with 32 P-labeled AHA-16eAz DNA (7 autosomes); lanes 5, 8, 11, 14 hybridized with 32 P-labeled AHA-16e DNA (X $^{+}$ + 12 autosomes). Hybridization to human insert fragments indicated by dots. See Fig. 2 for ethidium bromide patterns of these DNAs. The Charon 4A vector arms hybridized in the experiment because the hybridization mixtures included 32 P-labeled λ DNA to bind to molecular weight standards. Filters were not washed in 0.1 x SSC. Autoradiograms were exposed for 90 hr (lanes 1-3) or 52 hr (lanes 4-14).

TABLE I Restriction Fragments of Recombinants Bearing Sequence Elements Homologous to Reiterated Human DNA^a (sizes in kb).

b2 digested with				b4 digested with		b5 digested with	
Eco RI	Kpn I ^b	Hae III ^c	Alu I ^c	Eco RI	Xba I ^d	Eco RI	Bam HI ^e
4.5	1.8	1.3	2.3	3.1	6.6	8.5	3.1
4.0	1.6	1.1	1.7	2.6	1.1	3.9	1.0
3.3	1.2	0.9	1.3	2.3		2.9	0.8
		0.6	1.1	2.0			
		0.5	0.9	1.3			
		0.3	0.8	1.2			
			0.6				
			0.5				
			0.4				
			0.3				

- a) These fragments were all homologous to the following three ³²P-labeled genomic DNAs: AHA-11a (X⁺), AHA-16e (X + 12 autosomes) and AHA-16eAz (7 autosomes only). These fragments had no homology to back-selected AHA-11aAz DNA (e.g., see Fig. 6).
- b) Two larger Kpn I fragments, 5.0 kb and 6.4 kb, linked respectively to vector left and right arms, may have reiterated sequence elements but this experiment could not detect such sequences linked to vector DNA due to inclusion of ³²P-labeled λ DNA in hybridization mixes to bind to molecular weight markers.
- c) Human insert fragments were distinguished from vector fragments in a control experiment in which Hae III or Alu I digests of b2 were hybridized with ³²P-labeled Charon 4A DNA or with ³²P-labeled b2 insert fragments (purified from a gel). Not all the human insert Hae III and Alu I fragments were resolved in these experiments.
- d) Two Xba I fragments, about 0.8 kb and 5.3 kb, linked respectively to vector left and right arms, may have reiterated sequence elements; see b. One Xba I fragment of 0.7 kb was not homologous to reiterated DNA.
- e) Two Bam HI fragments, about 7.7 kb and 1.6 kb, linked respectively to vector left and right arms, may have reiterated sequence elements; see b. One Bam HI fragment of 1.1 kb was not homologous to reiterated DNA.

but the smaller Eco RI fragments (0.5 kb, 1.75 kb and 2.25 kb) were not observed even after a 3-fold longer exposure. This most likely means that single copy sequences were not detected in these experiments, because the 1.75 kb Eco RI fragment contains the 3' coding sequences for δ-globin as well as flanking sequences contained within a 3.1 kb Pst I fragment thought to be single-copy DNA (37).

The results summarized in Table I show that for the four enzymes used to digest b2, there were no qualitative differences in the b2 fragments detected by DNA from a hybrid containing only the human X chromosome, by DNA from a hybrid containing 7 human autosomes and no human X chromosome, or by DNA from a hybrid containing 12 human autosomes plus the human X chromosome. This strongly suggests that the sequence elements in b2 homologous to genomic reiterated sequences are localized in the same b2 fragments regardless of the chromosomal source of labeled human reiterated sequences. Furthermore, washing the filters with $0.1 \times \text{SSC}$ at 65° did not result in differential loss of hybridization to any of the fragments, but rather a uniform loss of 50-75% of the hybrids, estimated by comparing autoradiographic exposures. Thus, by this rough measure of thermal stability, the reiterated autosomal sequences related to b2 and the reiterated X chromosome sequences related to b2 are indistinguishable. Similar conclusions pertain to the localization of sequence elements in b4 and b5 homologous to genomic reiterated DNA and to the relatedness of the autosomal and X chromosome members of these families of reiterated DNA.

Chromosomal Organization and Distribution of Sequence Elements in b2 Homologous to Reiterated DNA. Since both the autosomal hybrid DNA (AHA-16eAz) and the X^+ hybrid DNA (AHA-11a) hybridized to the same fragments of b2, b4 and b5, we wanted to know if these reiterated sequences from the clones had similar organization on autosomes and the X chromosome. To answer this question we did several blot experiments in which restriction enzyme digests of 46,XY, 46,XX, 47,XXX, AHA-11a or AHA-16eAz DNA were transferred to filters and then probed with ^{32}P -labeled b2, ^{32}P -labeled b4 or ^{32}P -labeled b5 DNA. No distinct pattern of restriction fragments was observed when ^{32}P -labeled b4 DNA or ^{32}P -labeled b5 DNA was used to probe genomic DNAs digested with either Eco RI or Bam HI (data not shown), nor were any distinct fragments observed when ^{32}P -labeled b2 DNA was used to probe genomic DNA digested with Bam HI (data not shown). Thus these digests did not reveal any organizational patterns of the reiterated sequences. These experiments did indicate, however, that there was little, if any, homology between any of our recombinants and a prominent 2 kb Bam HI human DNA fragment that is apparently X chromosome-specific (our unpublished results and refs. 15 and 38). Likewise, none of the recombinants had significant, if any, homology to the prominent fragments of 0.68 kb and 0.34 kb resulting from Eco RI digestion of human DNAs and thought to be centromeric (14) (see Fig. 7 for results with ^{32}P -labeled b2 DNA; results with ^{32}P -labeled b4 and b5 DNAs not shown).

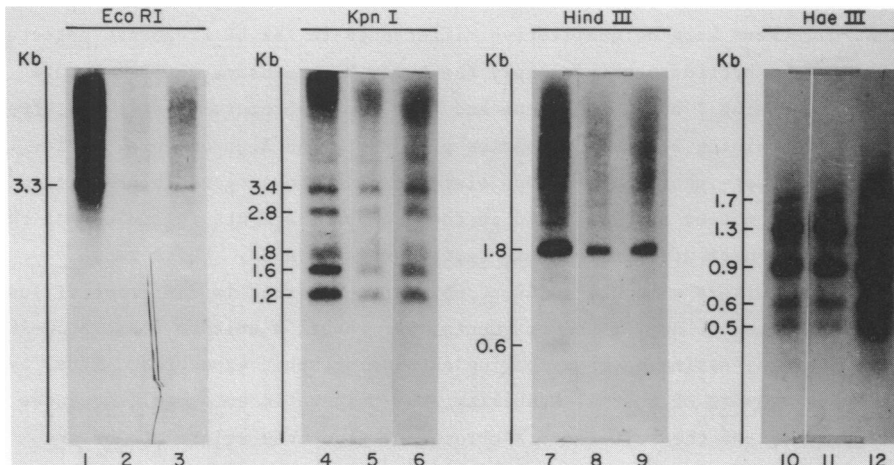


Fig. 7 Chromosomal Organization of Fragments Homologous to b2. Digests of 47,XXX DNA (1 μ g; lanes 1, 4, 7, 10), AHA-11a (X^r) DNA (10 μ g; lanes 2, 5, 8, 11) or AHA-16eAz (7 autosomes) DNA (10 μ g; lanes 3, 6, 9, 12) were probed with ³²P-labeled b2 DNA (2 x 10⁶ cpm/ml hybridization mix, lane 1-3, 10-12 or 4 x 10⁵ cpm/ml hybridization mix, lanes 4-9). Lanes 1-3, Eco RI digests; lanes 4-6, Kpn I digests; lanes 7-9, Hind III digests; lanes 10-12, Hae III digests. Digests were run on 1% (lanes 1-9) or 1.5% (lanes 10-12) agarose gels. The numbers refer to the lengths of the genomic fragments indicated. Autoradiograms exposed for 6 hr (lanes 1-3, 10), 21 hr (lanes 11 and 12), 72 hr (lanes 4, 6-9) or 200 hr (lanes 5). Filters were not washed in 0.1 x SSC.

However, digestion of genomic DNA with either Eco RI or Kpn I followed by hybridization to ³²P-labeled b2 DNA revealed an ordered arrangement of genomic b2 homologs. Furthermore, the organization of b2 homologs on the X chromosome was indistinguishable from the organization of these homologs on autosomes. Specifically, we observed that a 3.3 kb fragment was present in each Eco RI digest (Figs. 3 and 7, lanes 1-3). There was also a considerable amount of hybridization to fragments of many other sizes, producing a smear. The ethidium bromide-stained digest had a faint band at 3.3 kb when only one microgram of DNA was loaded onto the gel; if more DNA was loaded, this fragment did not stand out from the background smear of DNA. Kpn I digests of 47,XXX, AHA-11a or AHA-16eAz DNA revealed five major fragments of 3.4 kb, 2.8 kb, 1.8 kb (a doublet), 1.6 kb and 1.2 kb homologous to ³²P-labeled b2 DNA in addition to several minor bands (Fig. 7, lanes 4-6). These same fragments were observed with 46,XY and 46,XX DNA. There is again evidence of a smear of hybridization indicating sequence elements homologous to b2 on Kpn I fragments of many different lengths. The major Kpn I fragments appear to be the same size as ethidium bromide staining fragments in the human genomic digests.

We next surveyed three more enzymes to see how conserved the chromosomal organization of b2 homologs might be. When 47,XXX, AHA-11a, or AHA-16eAz DNA was digested with Hind III, one major fragment (1.8 kb) hybridized to ³²P-labeled b2 DNA (Fig. 7, lanes 7-9). The ethidium bromide-stained Hind III digests also revealed a major fragment of 1.8 kb and several minor bands, one of which (0.6 kb) was the same size as a fragment homologous to b2 DNA. The autoradiogram also revealed other minor fragments homologous to b2 DNA as well as a smear of hybridization to fragments greater than 2.4 kb. Hae III digests of each genomic DNA revealed six major fragments of 1.7 kb, 1.3 kb, 0.9 kb, 0.6 kb, 0.5 kb and 0.3 kb in each DNA homologous to ³²P-labeled b2 DNA (Fig. 7, lanes 10-12). In addition there was a suggestion of another fragment of 1.4 kb. Three fragments (1.3 kb, 0.9 kb, 0.3 kb) in ethidium bromide stained Hae III digests of human DNA were the same size as fragments homologous to ³²P-labeled b2 DNA. Alu I digests of each genomic DNA probed with ³²P-labeled b2 DNA revealed two major fragments (0.7 kb and 0.5 kb) as well as five other fragments (1.1 kb, 0.9 kb, 0.6 kb, 0.4 kb, 0.25 kb) superimposed on a high background smear (data not shown).

The most striking feature of all these digests is that there were no observable differences in the pattern of ³²P-labeled b2 hybridization to human DNA (46,XY; 46,XX; or 47,XXX) or to seven human autosomes in a mouse background (AHA-16eAz) or to the human X chromosome in a mouse background (AHA-11a). Furthermore, there was no qualitative change in the hybridization pattern after the filters were washed at 65° in 0.1 x SSC, though there was an apparently equal loss (estimated to be about 75%) of radioactivity from each fragment. That the pattern of fragments homologous to b2 on the X chromosome is the same as that on autosomes has been confirmed (data not shown) with two other independently derived mouse-human hybrids: cell lines XVI-18A and VII-2 HAT, containing human autosomes, but no human X. One of these hybrids, VII-2 HAT, contains only a single human chromosome, an 11p/17q translocation.

Table II summarizes the sizes of the genomic restriction fragments homologous to b2 and compares these sizes to those of the corresponding b2 digests. These results are based on at least two independent experiments with each DNA preparation. Examination of Table II reveals that most (19/33) of the b2 fragments correspond to fragments in the genomic digests, but clearly not all of the b2 fragments were observed in genomic digests. This latter observation can be explained by the results in Fig. 6 which indicate that some fraction of the b2 insert DNA (*i.e.*, at least the 1.7 kb, 1.4 kb, and 1.1 kb Eco RI fragments) is homologous to genomic single copy DNA since a fragment of

TABLE II Comparison of b2 Restriction Fragments and Genomic DNA Restriction Fragments Homologous to b2 (sizes in kb).

Eco RI		Hind III		Kpn I		Hae III		Alu I	
b2	genomic	b2	genomic	b2	genomic	b2 ^e	genomic	b2 ^e	genomic
4.5	-	6.4 ^b	-	6.4 ^c	-	-	1.7	2.7	-
4.0	-	4.9	-	-	6.2	-	1.4	2.3	-
3.3 ^a	3.3	-	3.7	-	5.5	1.3	1.3	1.7	-
1.7	-	2.3 ^b	2.3	5.0 ^c	-	1.1	-	1.3	-
1.4	-	1.8 ^a	1.8	-	4.6	0.9	0.9	1.1	1.1
1.1	-	-	1.4	-	3.7	0.6	0.6	0.9	0.9
		-	1.1	-	3.4	0.5	0.5	0.8	0.8
		0.6 ^a	0.6	-	2.8	0.3	0.3	0.6	0.6
				1.8 ^a	1.8 ^d			0.5	0.5
				1.6 ^a	1.6			0.4	0.4
				1.2 ^a	1.2			0.3	0.3

- a) These fragments of b2 have been shown to co-migrate with their corresponding genomic fragments by direct mixing experiments (data not shown).
- b) The 6.4 kb human Hind III fragment is linked to the left vector arm; the 2.3 kb human Hind III fragment is linked to the right vector arm.
- c) The 5.0 kb human Kpn I fragment is linked to the left vector arm; the 6.4 kb human Kpn I fragment is linked to the right vector arm.
- d) This band is a doublet; the faster-moving band of the doublet co-migrates with the 1.8 kb b2 Kpn I fragment.
- e) These fragments are only some of the Hae III and Alu I fragments of human insert DNA, i.e., those fragments resolved on the gels; See Table I, footnote c.

H β G1 DNA homologous to a genomic single copy sequence was not detected in that experiment (Fig. 6). Preliminary experiments using solution hybridization techniques also suggest that some of the b2 insert DNA is homologous to genomic single copy DNA. From control blot experiments (data not shown), comparing the intensity of hybridization of ³²P-labeled b2 DNA to known amounts of b2 DNA or genomic DNA digested and immobilized on filters, we estimate that our hybridization conditions can detect fragments present as few as 20 times in genomic DNA, although most of the fragments detected in genomic digests appear to be present several hundred to a thousand times in the human genome. Thus any hybridization to single copy genomic sequences in the experiment shown in Fig. 7 would almost certainly be obscured by the intense hybridization of b2 to genomic reiterated sequences.

Table II also shows that some genomic fragments homologous to b2 sequences

did not have matching fragments in b2. This suggests that there is at least one sequence element in b2, homologous to reiterated genomic fragments, which has a different restriction site in b2 from that in the bulk of such genomic homologs. Such microheterogeneity in b2 might be due to a point mutation and consequent loss of a restriction site in b2 or to changes after cloning the DNA (e.g., see refs. 36,39).

Comparison of Tables I and II reveals that most (16/22) of the b2 fragments carrying sequence elements homologous to genomic reiterated DNA matched the sizes of reiterated fragments in genomic DNAs homologous to b2. However, since nearly one-third of these b2 fragments (Table I) did not match a genomic fragment (Table II), it is likely that b2 contains members of at least two families of reiterated genomic DNA sequences, one of which is responsible for the hybridization pattern observed in Fig. 7.

Since the organization of b2 homologs on the X chromosome is indistinguishable from their organization on autosomes, we were able to estimate, by a blot experiment, the fraction of certain b2 sequences found on the X chromosome. To do this we made use of a Chinese hamster-human hybrid cell line, cl 2D (21), whose only human chromosome is the X, making up 3.2% of the hybrid genome as determined by chromosome measurements. In preliminary experiments we determined, as expected, that the pattern of restriction fragments, generated by several enzymes and homologous to b2, was the same on the human X chromosome in cl 2D as on the human X in AHA-11a (see Fig. 8 for Hind III digest; other data not shown). Varying amounts of Hind III digested cl 2D DNA or Hind III digested 45,XO DNA (of which 2.5% is X chromosome DNA, ref. 40) were hybridized with ^{32}P -labeled b2 DNA. As shown in Fig. 8, the intensities of hybridization to the major 1.8 kb Hind III fragment were approximately equal in the lanes with 0.5 μg of 45,XO DNA and 5 μg of cl 2D DNA. There was no cross-reaction between ^{32}P -labeled b2 DNA and Chinese hamster DNA. Thus, by comparing the two lanes of about equal hybridization, the fraction of genomic b2-related Hind III 1.8 kb fragments found on the X chromosome in 45,XO DNA was calculated as $(\mu\text{g } 45,\text{XO DNA})(\text{percent of } 45,\text{XO DNA that is X DNA})/(\mu\text{g cl } 2\text{D DNA})(\text{fraction of cl } 2\text{D DNA that is human X DNA}) = (0.5)(2.5)/(5)(0.032) = \sim 8\%$. Since only 2.5% of 45,XO DNA is X DNA, this result suggests an approximate three-fold enrichment of these sequences on the X relative to expectation if dispersal of these sequences on all human chromosomes were equal according to mass of DNA. This result suggests an interesting, asymmetric, X chromosomal distribution of at least some of the b2 sequences, although clearly this interpretation requires additional

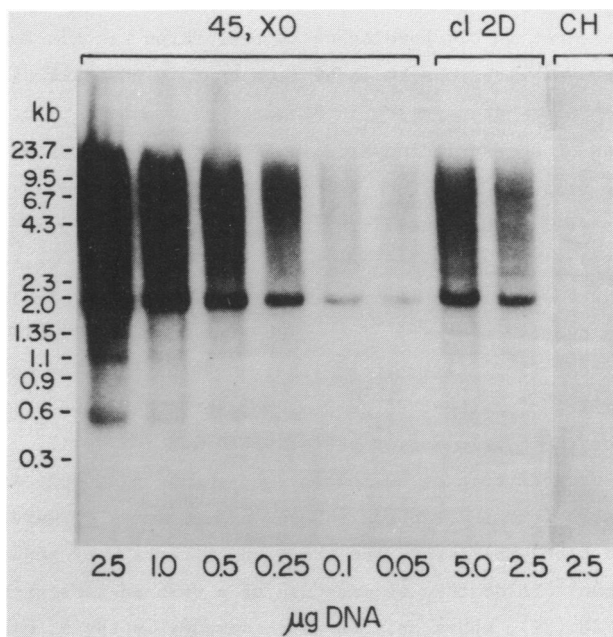


Fig. 8 Estimate of Fraction of Genomic b2-related 1.8 kb Hind III Fragments Homologous to the X Chromosome. Hind III digests of 45, XO DNA, cl 2D DNA, or Chinese hamster (CH) DNA were probed with ^{32}P -labeled b $_2$ DNA (7.5×10^5 cpm/ml hybridization mix for 89 hr). The amount of target DNA in each lane is indicated in the figure. Numbers indicate fragment lengths of molecular weight markers. Filter was not washed in $0.1 \times \text{SSC}$. Autoradiogram was exposed for 14 hr.

experimental verification.

DISCUSSION

The screening method described here is designed to detect recombinants with homology to the human X chromosome. The three recombinants analyzed in detail so far all carry some sequences homologous to reiterated X chromosome DNA (Figs. 3, 6). The restriction fragments with homology to the X chromosome are the same fragments homologous to reiterated autosomal DNA (Fig. 6). Thus these recombinants probably are representatives of a class of human-specific X chromosome reiterated sequences described earlier (4) which are represented on both the X and autosomes and account for about 6% of the X chromosome DNA. This class of X chromosome sequences is represented several thousand times in the human genome and several hundred times on the X chromosome (4).

Our data (Fig. 7 and Table II) indicate considerable intragenomic

conservation of restriction sites around genomic sequences homologous to b2 on the X chromosome as well as on autosomes. Indeed, the results of probing human DNA, AHA-11a DNA and AHA16eAz DNA suggest that the homologs of b2 have the same organization on autosomes as on the X chromosome, although we do not yet know how many or which autosomes have homology to the reiterated elements in b2. Despite the conservation of restriction sites around these reiterated b2 homologs, the sequence homology is not perfect, since hybrids of b2 and its genomic homologs are partially dissociated in 0.1 x SSC at 65°. It remains to be seen whether, upon detailed analysis, the sequence elements of b2 homologous to reiterated genomic DNA have the same degree of homology with the autosomal homologs as with their X chromosomal homologs, although our data so far indicate that this is the case.

The conserved organization of b2 homologs is in contrast to the organization of one well-characterized, homogeneous reiterated DNA, mouse satellite DNA. The mouse X chromosome carries satellite DNA sequences lacking abundant restriction enzyme sites for three enzymes whereas these same enzymes digest the satellite sequences found in genomic mouse DNA (12). Our results also contrast with those obtained with the more heterogeneous human satellite fractions (15,39) and with the autosomal sequences homologous to the human Y chromosome-specific 3.4 kb Hae III fragment (41,42). We note here that this conserved organization has certain features in common with the transposable elements found in Drosophila, yeast and prokaryotes (43-45).

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