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Genetics

Detection of restriction fragment length polymorphisms at the centromeres of human chromosomes by using chromosome-specific α satellite DNA probes: Implications for development of centromere-based genetic linkage maps

(chromosome X/chromosome 17/repetitive DNA/linkage analysis)

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ABSTRACT **We describe a general strategy for the detection of high-frequency restriction fragment length polymorphisms in the centromeric regions of human chromosomes by molecular analysis of α satellite DNA, a diverse family of tandemly repeated DNA located near the centromeres of all human chromosomes. To illustrate this strategy, cloned α satellite repeats isolated from two human chromosomes, 17 and X, have been used under high-stringency conditions that take advantage of the chromosome-specific organization of this divergent repeated DNA family. Multiple high-frequency restriction fragment length polymorphisms are described for the centromeric region of both chromosome 17 and the X chromosome. Mendelian inheritance of the variants is demonstrated. The X-linked α satellite polymorphisms in particular are highly informative and constitute a virtually unique centromeric DNA marker for each X chromosome examined. Since the strategy we describe is a general one, the α satellite family of DNA should provide a rich source of molecular variation in the human genome and should contribute to the development of centromere-based genetic linkage maps of human chromosomes.**

The value of a genetic linkage map for the human genome has been appreciated for over 30 years because of the possible use of genetic linkage in providing information relevant to the diagnosis of genetic disease (1, 2). However, due to a relative paucity of highly informative genetic markers, linkage analysis has until recently been of little direct medical significance, although regarded as an important genetic concept. Thus, the development of the human gene map over the last 15 years has relied far more on parasexual techniques involving somatic cells than on analysis of recombination in family studies (3, 4). Recently, however, efforts to detect genetic linkage in humans have been enhanced by the recognition of substantial DNA-based variation in the human genome (5) that can be detected by molecular techniques and exploited as genetic markers known as DNA restriction fragment length polymorphisms (RFLPs) (6). To date, more than 800 such RFLPs have been described (7), and multilocus linkage maps based in large part on these DNA markers have been developed for a number of human chromosomes (7–10).

Notwithstanding the demonstrated clinical usefulness of DNA-based genetic linkage analysis for such inherited diseases as Duchenne muscular dystrophy (11, 12), Huntington disease (13), polycystic kidney disease (14), and cystic fibrosis (15–17), most human genetic maps suffer from a lack of precision concerning the relationship between genetic linkage, described in recombination units, and physical landmarks on human chromosomes—namely, bands, telomeres, and centromeres. The location of the centromere in particular is of some interest, since it is the centromere which governs meiotic segregation of chromosomes (18, 19). To be biologically meaningful, genetic maps might ideally be fixed at the centromere, with recombination units increasing outwardly on both chromosome arms.

The development of centromere-based genetic linkage maps of this kind requires the ability to distinguish between homologous centromeres. Previous efforts to define such centromeric polymorphisms have utilized cytogenetic heteromorphisms of constitutive heterochromatin detected by C-banding or Q-banding techniques (20). More recently, variation of centromeric repetitive DNA has been suggested (21), thus raising the possibility that detailed analysis of such polymorphic sequences might provide useful genetic markers in the vicinity of human centromeres.

We describe here a general strategy for detecting high frequency RFLPs in the centromeric region of human chromosomes by molecular analysis of α satellite DNA, a heterogeneous family of tandemly repeated DNA based on a monomer repeat length of ≈170 base pairs (bp) and located in the centromeric region of all human chromosomes (22, 23). Previous reports (24, 25) showed that α satellite DNA in the human genome is organized in a highly chromosome-specific manner, detectable both by restriction enzyme periodicities and by primary DNA sequence. In the present work, we have used cloned α satellite repeats isolated from different human chromosomes to exploit the chromosome-specific nature of human α satellite DNA; specifically, we describe here a number of DNA polymorphisms associated with the centromeric regions of the X chromosome and chromosome 17. Since the strategy we have employed is a general one, the α satellite family of DNA should provide a rich source of molecular variation in the human genome and provide the capability of generating centromere-based genetic linkage maps for human chromosomes.

MATERIALS AND METHODS

DNA Analysis. DNA was prepared from human skin fibroblasts, peripheral lymphocytes, or lymphoblasts or from human–rodent somatic cell hybrids as described (24–27).

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Abbreviations: RFLP, restriction fragment length polymorphism; PIC, polymorphism information content; kb, kilobase(s); bp, base pair(s).

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Methods for restriction endonuclease digestions, electrophoresis, transfer of DNA fragments to nitrocellulose, prehybridization and hybridization of genomic DNA fragments with radioactive probes, washing of filters, and autoradiography have been described (24–26). Completeness of restriction enzyme digestions was monitored by visual inspection of ethidium bromide patterns and by reprobing filters with a second α satellite probe (from a different human chromosome) that hybridizes to a single prominent band in human DNA digested with the particular enzyme used.

α Satellite DNA Probes. For studies of X-linked α satellite, the DNA probe pBamX-E was used (25). This is a 242-bp *Taq* I fragment containing approximately 1.5 monomers of α satellite DNA from the X chromosome (25, 26), subcloned in pUC8. (See legend to Fig. 1 for details of α satellite organization.)

The chromosome 17 α satellite probe used in this study was p17H8, one of several homologous clones isolated from an *Eco*RI plasmid library constructed using DNA from a somatic cell hybrid containing human chromosome 17 (28). Plasmid p17H8 contains a 2.7-kilobase (kb)-long *Eco*RI insert that has been mapped to chromosome 17 with an extensive somatic cell hybrid panel (27, 28). Complete nucleotide sequence analysis of p17H8 has shown that the insert consists of 16 tandemly repeated α satellite monomers, each 65–91% identical to monomers from the human X chromosome (25).

Data Analysis. DNA samples were obtained from a random group of unrelated, predominantly Caucasian individuals and from several large Utah families being studied for various inherited disorders. The polymorphism information content (PIC) for chromosome 17 RFLPs was determined as described (6). For X-linked RFLPs, since males are hemizygous, the PIC was modified to be formally equivalent to heterozygosity. To obtain the maximum-likelihood estimate of the recombination fraction between two polymorphisms, the computer program LIPED (29) was used.

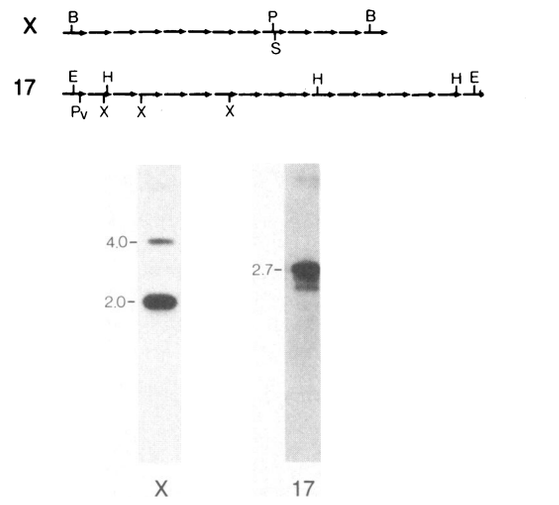

FIG. 1. Organization of α satellite DNA from human chromosomes X and 17. (*Upper*) Restriction maps and monomer organization. The X chromosome *DXZ1* higher-order repeat unit is 2.0 kb long, comprised of 12 tandemly repeated 171-bp monomers, represented by the arrows. Individual monomers are 65–85% identical in sequence to each other. Independent higher-order repeat units are >99% identical (25). Restriction sites: B, *Bam*HI; P, *Pst* I; S, *Sst* I. The chromosome 17 *D17Z1* higher-order repeat unit is 2.7 kb long, comprised of 16 tandem monomers. Individual monomers are 60–85% identical to each other. Independent higher-order repeat units are >98% identical (unpublished data). Restriction sites: E, *Eco*RI; H, *Hin*dIII; Pv, *Pvu* II; X, *Xba* I. (*Lower Left*) Southern blot analysis of *DXZ1* family. Human genomic DNA was digested with *Bam*HI and hybridized to labeled pBamX-E. Pattern of bands reflects the higher-order repeat unit and a small proportion of its 4.0-kb multimer (26, 30). (*Lower Right*) Southern blot analysis of *D17Z1* family. Human genomic DNA was cleaved with *Eco*RI and hybridized to labeled p17H8. Pattern of bands reflects the major higher-order repeat unit and minor related domains from chromosome 17. Sizes of major bands are indicated in kb.

RESULTS

Description of Chromosome-Specific α Satellite Repeats. Organization of the long centromeric tandem arrays of α satellite DNA monomers specific for individual human chromosomes can be characterized by the length of the amplified repeat unit, by the particular restriction enzyme(s) used to visualize the repeat in digests of genomic DNA, and by its primary DNA sequence. As described previously (25, 26, 30), α satellite on the human X chromosome is comprised of some 5000 tandem copies of a 2.0-kb repeat (designated *DXZ1*), each one of which consists of 12 directly repeated α satellite monomers (Fig. 1). To reflect the hierarchical nature of tandem arrays of repeated DNAs such as α satellite, we refer to larger multiple-monomer repeated units such as this as the "higher-order" repeat unit, in order to distinguish it from its constituent monomer repeats. This 12-monomer higher-order repeat unit can be visualized with a number of enzymes that cleave X chromosome DNA only once within the 2.0-kb repeat, including *Bam*HI (Fig. 1), *Pst* I, and *Sst* I (25, 26, 30).

α satellite DNA on chromosome 17 is characterized by a tandem, 16-monomer higher-order repeat unit, approximately 2.7 kb long, that can be visualized by use of the enzymes *Eco*RI or *Pvu* II (Fig. 1) (28). Each chromosome 17 contains an estimated 1000 copies of the 16-monomer repeat (designated *D17Z1*), as well as lesser amounts of other repeat lengths corresponding usually to 15- and 14-monomer higher-order repeats (Fig. 1).

X-Linked α Satellite RFLPs. To detect RFLPs for α satellite DNA from the human X chromosome, we screened DNA from a series of unrelated male individuals with restriction enzymes known from previous work to cut only rarely within the higher-order repeat unit on the X chromosome (25, 26). In such digests, because of the tandem nature of the repeats, most of the X chromosome α satellite is found in extremely large restriction fragments that migrate in the unresolved region of standard agarose gels. With certain enzymes, however, a number of smaller fragment lengths are resolved. For example, in *Hin*dIII-digested human DNA, a series of bands are observed that appear as a "ladder" of fragments ranging in size from 2.0 kb to >20 kb (Fig. 2*A*). Variation between different individuals appears as extra or missing "rungs" in the ladder. The particular pattern of bands is virtually unique for each genomic sample analyzed, suggesting a high frequency of polymorphic restriction sites among the tandem copies of α satellite on the X chromosome. Similar results were obtained with a number of different enzymes, including *Xba* I, *Eco*RI, *Bgl* II, and *Msp* I, in addition to *Hin*dIII. An example of the variation observed in *Xba* I-cleaved genomic DNA is shown in Fig. 2*B*. The observed patterns are highly reproducible for a given individual, with respect both to the pattern of bands and to the relative intensity of each band.

Because of the highly X-specific nature of the probe under the conditions used (24, 25), we presumed that the observed patterns reflect variation in α satellite on the X chromosome. To demonstrate this directly, we performed both family and somatic cell hybrid mapping studies. Somatic cell hybrids derived from independent fusion experiments between rodent cells and different human 46,XX female cell strains were analyzed for the *DXZ1 Hin*dIII polymorphism. Such analyses were performed for four sets of hybrids, utilizing four different human female parental strains. Representative data from one hybrid set are shown in Fig. 3*A*. For each set of

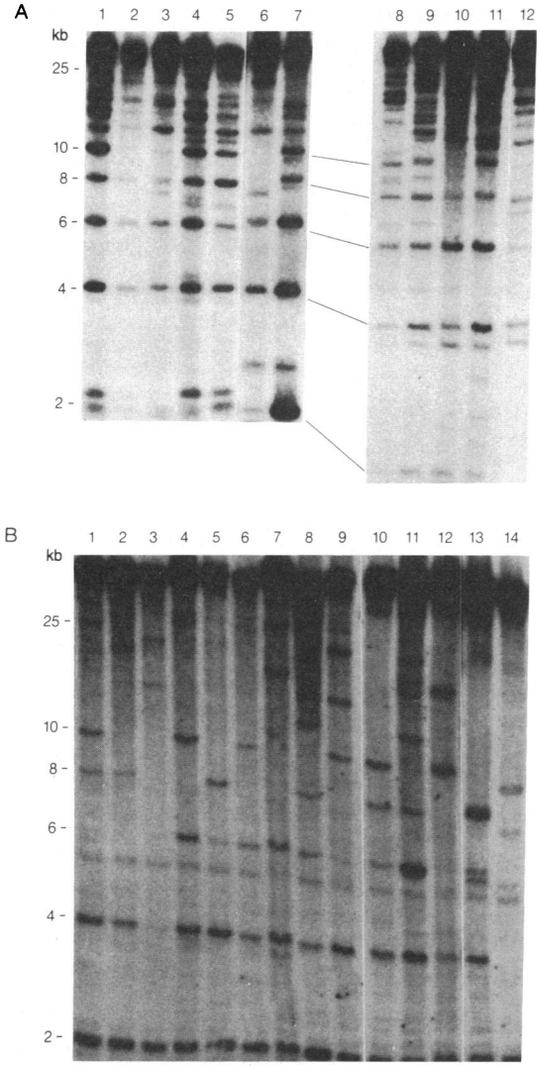

FIG. 2. Polymorphic variation of X-linked α satellite. Genomic DNA from unrelated males was digested with *Hin*dIII (*A*) or *Xba* I (*B*) and hybridized to labeled pBamX-E. Sizes of representative bands are indicated at left. Completeness of enzyme digestions was monitored as described in *Materials and Methods*. All lanes showed a single prominent band after rehybridization of filters to a second α satellite probe from a different chromosome.

hybrids, two types of patterns were observed; the patterns segregated independently of the human autosomal content of the hybrids and correlated only with the presence of one or the other of the two X chromosomes from the human parental cells, as determined by RFLP analysis using other X-linked DNA markers (data not shown).

In addition, Mendelian inheritance of the *Hin*dIII polymorphism was demonstrated in three large multigeneration kindreds. As expected for an X-linked marker, two types of patterns could be demonstrated in DNA from sons of heterozygous women (Fig. 3 *B* and *C*). Furthermore, unique fragment lengths observed in fathers were absent from all sons but present in daughters, consistent with X-linkage (data not shown).

Conventional definition of alleles clearly is not possible for a genetic marker consisting of thousands of copies of a repetitive DNA element, spanning several million base pairs of DNA (26). Nonetheless, the frequency of any given fragment length can be calculated using male DNAs with a single X chromosome. *DXZ1* fragment lengths were initially scored only as present or absent, without regard to intensity

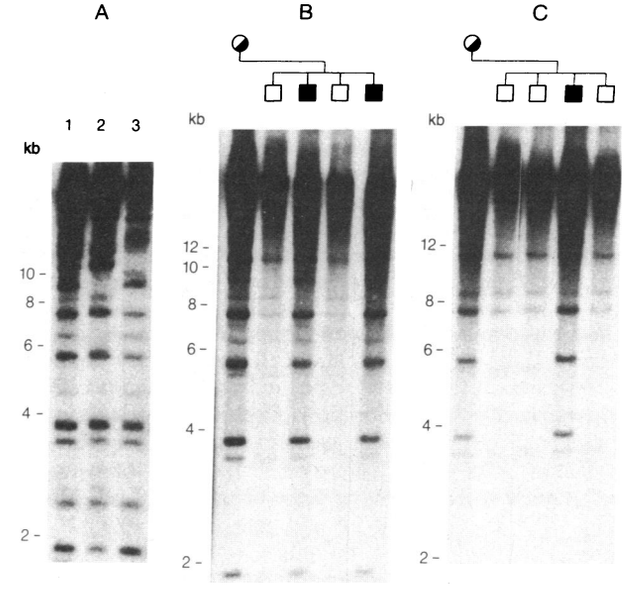

FIG. 3. X linkage of α satellite RFLPs. DNAs were digested with *Hin*dIII and hybridized to labeled pBamX-E. (*A*) Segregation in hybrids. DNAs are from 46,XX cells (lane 1) and from two hybrids (lanes 2 and 3) containing different X chromosomes from the human parental cells. (*B* and *C*) Mendelian inheritance of α satellite RFLPs. Pedigrees are indicated above lanes. Symbols in the pedigrees indicate presumed genotypes of X-linked RFLPs. Sizes of representative bands are indicated.

of hybridization. Data for representative fragment lengths are presented in Table 1. Haplotypes can be generated to express the X α satellite genotype of any particular X chromosome. Considering only the 3.7-, 5.0-, 9.0-, and 10.0-kb *Hin*dIII bands for illustrative purposes (see Fig. 2*A*, for example), 11 of the 16 possible haplotypes were observed among 42 randomly selected X chromosomes, with the most frequent haplotype having a frequency of 0.24. The polymorphism information content (PIC, ref. 6) for this single analysis, which does not consider band intensity or any variable band lengths other than the four being scored, is 0.87. A similar analysis for three of the polymorphic *Xba* I bands (6.0, 8.0, and 10.0 kb; Fig. 2*B*) revealed eight haplotypes with a PIC of 0.82. The combined PIC for the seven fragment lengths was 0.93. Clearly, if one considered all of the polymorphic

Table 1. Frequencies of α satellite RFLPs in a Caucasian population

		Polymorphism			
DNA	Enzyme	Fragment length, kb	n*	Present	Absent
DXZ1†	*Hin*dIII	3.7	42	0.74	0.26
		5.0		0.36	0.64
		9.0		0.31	0.69
		10.0		0.79	0.21
	Xba I	6.0	17	0.77	0.23
		8.0		0.47	0.53
		10.0		0.53	0.47
D17Z1‡	*Eco*RI	0.5	112	0.38	0.62
		1.5		0.35	0.65
		2.0		0.37	0.63
	Pvu II	2.2	112	0.36	0.64

*Number of chromosomes.
†Frequencies were determined empirically by scoring male DNAs for the presence or absence of the indicated fragment.
‡Presence of the indicated fragment was scored as a dominant trait. The frequencies shown were calculated indirectly from the proportion of individuals in whom the fragment was absent.

fragment lengths observed with multiple enzymes and distinguished markedly different hybridization intensities as well, the PIC for these *DXZ1* polymorphisms would approach unity.

α Satellite RFLPs of Chromosome 17. A different strategy was employed to search for polymorphisms involving chromosome 17 α satellite. Restriction enzymes were used that were known to cleave within the higher-order repeat unit (28). Variant bands were observed in digests of human genomic DNA with a number of enzymes, including *Eco*RI (e.g., Fig. 4) and *Pvu* II. Extensive analysis of these patterns has defined four major *D17Z1 Eco*RI haplotypes, as determined by examining the genotypes of individual chromosomes 17 isolated in somatic cell hybrids, by following transmission of these haplotypes in families, and by cloning and sequencing of various polymorphic repeat lengths. Although each of the major patterns can be subdivided by consideration of minor fragments or fragment intensity, the four major *Eco*RI haplotype patterns are (*i*) 2.7 kb only; (*ii*) 2.0/1.5/0.5 kb; (*iii*) 2.0/0.5 kb; and (*iv*) 1.5/0.5 kb. The patterns shown in Fig. 4, therefore, reflect different diploid combinations (homozygous or heterozygous) of these four *D17Z1* haplotypes.

Mendelian inheritance of the polymorphic *D17Z1* repeat forms has been demonstrated in large three-generation kindreds. Examples of two-generation inheritance are shown in Fig. 5 for polymorphisms detected by *Eco*RI and *Pvu* II. As indicated in the figure, it is possible within families to use the intensity of fragment lengths as an indication of homozygosity or heterozygosity for the polymorphism. The frequency of the major polymorphic fragment lengths in a Caucasian population is given in Table 1 for the enzymes *Eco*RI and *Pvu* II.

Since the *D17Z1* family comprises an estimated 10^6 bp of DNA, we investigated genetic linkage between the polymorphisms detected by *Eco*RI and *Pvu* II. No obligate recombination events were detected in 54 informative meioses, including 11 phase-known meioses. These data indicate that the two polymorphisms are linked at an estimated recombination distance of 0 centimorgans [confidence limits (31) of 0–3 centimorgans] with a peak lod score (logarithm of the odds in favor of linkage) of 18.3. Combined haplotypes for the *Eco*RI and *Pvu* II *D17Z1* polymorphisms were scored for 43 unrelated Caucasian individuals. Strong disequilibrium was noted between the presence of the 2.2-kb *Pvu* II band and the presence of the 1.5-kb and 0.5-kb *Eco*RI bands. The calculated PIC for this analysis was 0.43. In practice, this polymorphism can be made more informative than the PIC might indicate by considering either the intensity of bands or the variation of minor fragments (e.g., Fig. 5).

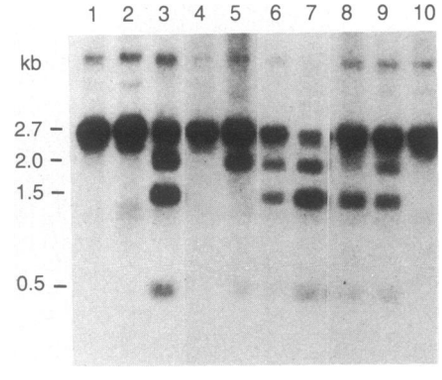

FIG. 4. Polymorphic variation of chromosome 17 α satellite. Genomic DNA from 10 unrelated individuals was digested with *Eco*RI and hybridized to labeled p17H8. Sizes of detected fragments are indicated.

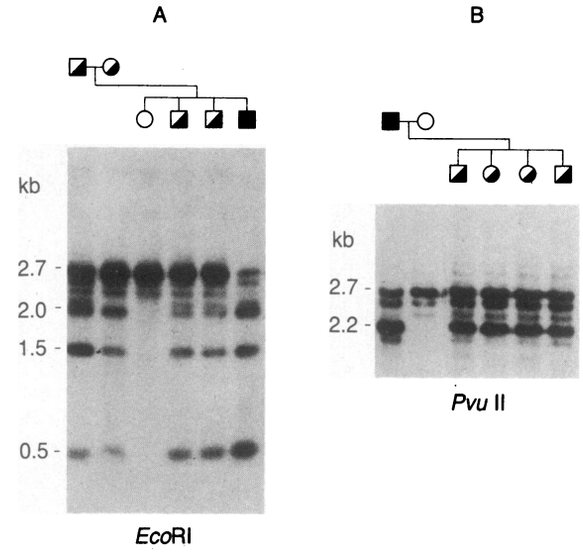

FIG. 5. Mendelian inheritance of chromosome 17 α satellite RFLPs. Genomic DNAs were digested with *Eco*RI (*A*) or *Pvu* II (*B*) and hybridized to labeled p17H8. Sizes of detected fragments are indicated. Pedigrees are indicated above lanes; symbols indicate presumed genotype of individuals for the RFLPs, based on grandparental genotypes (not shown) and consideration of the presence and intensity of polymorphic fragments.

DISCUSSION

In this report, we describe detection of RFLPs in the vicinity of the centromeres of two human chromosomes, utilizing α satellite repeated-DNA probes. Our description of α satellite DNA as "centromeric" is based on localization of this repeated-DNA family by *in situ* hybridization using a variety of different α satellite DNA probes (21, 23, 24, 30, 32–35). Formal proof of the centromeric location of these RFLPs will require linkage analysis with other genetic markers previously localized to positions on chromosome arms near the centromere.

The molecular basis of the complex polymorphisms described here remains uncertain. For the X-linked RFLPs, in which variant bands represent only a minor proportion (an estimated 25–100 copies each) of the total *DXZ1* repeat population on a given X chromosome, it is likely that the polymorphic fragment lengths reflect the hierarchical organization of tandem repeat families, characterized in this case by duplication and amplification of *DXZ1* domains marked by the presence of a single new restriction site for an enzyme such as *Hin*dIII or *Xba* I (Fig. 3). For RFLPs revealed by enzymes that cut within the higher-order repeat unit, as illustrated here by the chromosome 17 α satellite polymorphisms, a slightly different mechanism must be considered. These polymorphisms must have either arisen quite early during evolution of the α satellite family or achieved a high copy number by processes such as sequence conversion or unequal crossing-over, which are capable of mediating the spread of a variant copy throughout a tandem array of repeats (36, 37).

Since α satellite DNA is organized in a chromosome-specific manner at the centromeric region of each human chromosome, the strategy we have presented is likely to be a general one applicable to the development of a centromere-based genetic linkage map for any human chromosome. The critical components of this strategy are (*i*) isolation of representative α satellite DNA probes from the chromosome

of interest; (*ii*) demonstration of chromosome-specific hybridization using that probe; and (*iii*) demonstration of RFLPs.

With regard to isolation of α satellite probes, it should be straightforward to isolate representative α satellite clones from the entire genome. α satellite probes have been isolated and mapped to nearly one-half of the human chromosomes already (refs. 21, 24, 25, 28, and 32–34, and unpublished data), and certain α satellite probes hybridize generally to all human chromosomes at lowered stringencies, thus facilitating identification and cloning of additional repeats (24, 35).

The second aspect of this strategy, chromosome-specific hybridization, depends heavily on the probe and the experimental conditions chosen. For some chromosomes, such as chromosome 17, one can utilize the full higher-order repeat unit length and achieve essentially specific hybridization patterns under highly stringent conditions (Fig. 1). For other chromosomes, such as the X chromosome, extensive analysis of subfragments may be required before a suitable probe fragment is found (25). Since the human α satellite family is characterized by substantial DNA sequence divergence (22, 25, 32–35) and since even individual chromosomes can exhibit a wide range of different monomer sequences (e.g., ref. 25), a number of combinations of probe and stringency may be required to establish optimal chromosome-specificity.

As illustrated by the results presented here, the third part of the strategy, demonstration of useful RFLPs with α satellite DNA probes, can be achieved in either of two general ways once chromosome-specificity is established. First, one can use enzymes that cleave within the higher-order repeat unit for a given α satellite array. This approach is illustrated here with *Eco*RI and *Pvu* II for chromosome 17 α satellite (Figs. 4 and 5). In this case, the RFLPs detected were marked by substantial disequilibrium, which, if found to be a general feature of such RFLPs, may limit their information content. Alternatively, one can use enzymes that only rarely cleave within the higher-order α satellite repeat unit to search for RFLPs. This approach has been applied to X-linked α satellite in this study (Fig. 2). Based on our experience, these RFLPs are largely independent of each other and in linkage equilibrium, thus increasing their usefulness. Indeed, the X-linked RFLPs described here provide a virtually unique DNA marker for the centromeric region of each X chromosome, which should be extremely valuable not only for contributing to a linkage map of the X chromosome but also for analysis of X chromosome nondisjunction.

Such polymorphisms are not limited to the two chromosomes studied here; we have also detected useful RFLPs for α satellite from human chromosomes 1, 7, and 11 (unpublished data), and RFLPs have been detected by using an apparently related repeated DNA probe from chromosome 6 (21). Thus, the available data strongly suggest that the α satellite DNA family will be a rich source of polymorphic DNA markers for the development and use of centromere-based genetic linkage maps of the human genome.

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