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Two subsets of human alphoid repetitive DNA show distinct preferential localization in the pericentric regions of chromosomes 13, 18, and 21

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Abstract. We have isolated and characterized two human middle repetitive alphoid DNA fragments, L1.26 and L1.84, which localize to two different sets of chromosomes. In situ hybridization revealed both repeats to have major and minor binding sites on the pericentric regions of several chromosomes. Probe L1.26 maps predominantly to chromosomes 13 and 21. Probe L1.84 locates to chromosome 18. Minor hybridization sites for both probes include chromosomes 2, 8, 9, and 20; in addition, L1.26 revealed minor sites on chromosomes 18 and 22. The binding to these sites strongly depends on hybridization conditions. In Southern blot hybridizations to total human DNA, both L1.26 and L1.84 give the same ladder pattern, with a step size of 170 bp, indicating their presence as tandem repeats, but with different band intensities for each probe. The chromosome-specific nature of particular multimers was confirmed by Southern blot analyses of a human-rodent hybrid cell panel. We conclude that L1.26 and L1.84, with their related sequences, constitute subfamilies of alphoid DNA that are specific for subsets of chromosomes and, in some cases, possibly even for single chromosomes.

The DNA sequences adjacent to the centromeres of human chromosomes have been shown to consist of repeated DNA sequences of varying complexity, organization, and abundancy (Gosden et al., 1975; Mitchell et al., 1979). Some of these have been defined as satellite DNAs on the basis of different isopycnotic centrifugation techniques (Mitchell et al., 1979). Part of the sequences have been shown to be simple sequences consisting of long, uninterrupted arrays of tandemly arranged units of 6–12 bp, whereas others are longer and organized in a more complex manner. Restriction endonuclease digestions of total human DNA also revealed the presence of satellite DNAs. One of these displays a basic repeating unit of 170 bp (Manuelidis, 1976) and is referred to as alphoid DNA because of its homology to the alpha component isolated from the African green monkey (Manuelidis and Wu, 1978). Restriction of genomic DNA with the endonuclease *Eco*RI reveals this family as pronounced 340-bp and 680-bp fragments in ethidium bromide-stained gels (Darling et al., 1982).

It has become apparent that some alphoid DNA sequences can be enriched on specific chromosomes. Using the gel-purified uncloned *Eco*RI 340-bp fragment as a probe, Manuelidis (1978) demonstrated in situ hybridization to the centromeres of all human autosomes, but especially to those of chromosomes 1, 3, 7, 10, and 19. An alphoid 2.0-kb *Bam*HI restriction fragment characterizes the centromere of the X chromosome (Yang et al., 1982; Willard et al. 1983), while Jabs et al. (1984) have identified a 3.0-kb *Bam*HI fragment, prominently enriched on chromosome 6, although the alphoid nature of this fragment still remains to be established.

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Because chromosome-specific repetitive DNA may be useful in the diagnosis of chromosomal rearrangements (Burk et al., 1983), our objective was to identify new repetitive sequences with different chromosome specificities than those already described. In this study, we show that the pericentric regions of chromosomes 13, 18, and 21 can each be characterized by the presence of two middle repetitive alphoid DNA sequences. Furthermore, other chromosomes are shown to harbor related sequences, as they appear as minor binding sites upon in situ hybridization or segregate as specific multimers within a human-rodent hybrid cell panel in Southern blot hybridizations.

Materials and methods

Materials

Restriction endonucleases and Kornberg DNA polymerase were obtained from Boehringer Mannheim. Nylon Gene Screen filters, [α-^{32}P]dCTP (3,000 Ci/mM in Tricine-buffer), and ^{3}H-dTTP (100 Ci/mM) were obtained from New England Nuclear.

The recombinants L1.26 and L1.84 were picked from a library containing 1- to 2-kb fragments of totally *Eco*RI-digested placental DNA. They were cloned in the plasmid pAT153 (Pearson et al., 1982). The human-rodent hybrid cell panel was constructed using standard fusion protocols (Herbschleb-Voogt et al., 1981). From the various hybrid cell lines, high-molecular-weight genomic DNA was prepared as described by Hofker et al. (1985). DNA isolated from human female spleen tissue served as a control.

Reagents used for the chromosomal in situ hybridization included Denhardt's solution (0.02% Ficoll, molecular weight 400,000, from Pharmacia, Sweden; 0.02% polyvinylpyrrolidone, molecular weight 360,000; and 0.02% bovine serum albumin from Serva, Heidelberg), dextran sulfate (Pharmacia, Sweden), formamide (Merck, Darmstadt), and salmon sperm DNA (Serva, Heidelberg).

Southern blotting and hybridization

Restriction enzyme digestion was done under conditions prescribed by the supplier. To ensure complete digestion, a 3-fold excess of enzyme per microgram of DNA was used, and digestion was allowed to proceed for 3 h. Digests were separated in a 0.8% agarose gel in Tris-acetate buffer (22.5mM Tris-acetate [pH 8.3], 10 mM sodium acetate, and 1 mM EDTA), transferred to Gene Screen filters (Southern, 1975), and treated as recommended by the supplier. Hybridization and subsequent washing of the filters were performed at 65 °C as described by Jeffreys and Flavell (1977). The hybridization mixture contained 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 3 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1 mg/ml salmon sperm DNA, 10 × Denhardt's solution, 0.1% SDS, 5% dextran sulfate, and 25–50 ng ^{32}P-nick-translated probe (Rigby et al., 1977) in a final volume of 10–15 ml. Filters were incubated overnight, followed by several washing steps with decreasing concentrations of SSC (Jeffreys and Flavell, 1977). Autoradiography was performed at −70 °C for 1–3 days using Sakura film in conjunction with an intensifying screen.

In situ hybridization

For in situ hybridization we used 72-h phytohemagglutinin (PHA)-stimulated human peripheral blood lymphocyte cultures from healthy male donors. Probes L1.26 and L1.84 were nick-translated using ^{3}H-dTTP as described previously (Rappold et al., 1984a). Hybridization to metaphase chromosomes was performed according to published protocols (Gerhardt et al., 1981; Rappold et al., 1984b), with some modifications. Metaphase spreads were prepared on slides which had been pretreated with 10 × Denhardt's solution. The hybridization mixture contained 0.2–0.5 μg/ml of the radioactive probe together with an excess (500–1,000 ×) of salmon sperm DNA, 2 × SSC, 1 × Denhardt's solution, 50% formamide, and 10% dextran sulfate. The probe and salmon sperm DNA were denatured for 5 min at 100 °C, followed by rapid cooling on ice, before they were added to the mixture. Hybridization was carried out overnight at 40–42 °C in a humidified chamber. Slides were then washed according to either of two procedures:

1. Three changes of 2 × SSC at 65–68 °C, followed by three changes of 0.1 × SSC at room temperature, allowing 30 min for each change (Gerhardt et al., 1981).

2. Three changes of 1 × SSC, 50% formamide (pH 7.0) at 40 °C, followed by three changes of 0.1 × SSC at room temperature, with 20 min for each change (Harper and Saunders, 1981).

Washed slides were dehydrated in ethanol and air dried. Autoradiography was performed as described by Rappold et al. (1984a). The exposure time was 5–6 days at 4 °C.

Chromosome identification

In some experiments, routine G-banding was performed prior to in situ hybridization. Microphotographs of the same metaphase plates were taken before and after hybridization and scored to identify individual chromosomes and localize silver grains. Cells were restained with Giemsa after autoradiography. In other experiments, bromodeoxyuridine (BrdU) and fluorodeoxyuridine (FdU) were added to lymphocyte cultures after 65 h incubation in a final concentration of 10 μg/ml and 0.5 μg/ml, respectively. Six hours later, Colcemid (0.05 μg/ml) was added for 30 min, and chromosome preparations were made (Schempp and Meer, 1983). After autoradiography, the chromosomes were stained using the Hoechst 33258-Giemsa technique introduced by Wolff (1974), with minor modifications (Schempp and Meer, 1983). This procedure results in an R-type chromosome replication pattern, with the BrdU-substituted chromosomal segments colored pale blue and the dT-substituted regions stained dark red.

Results

Alphoid DNA characteristics of L1.26 and L1.84

A dot-blot experiment revealed the repetitive nature of L1.26 and L1.84 (not shown here). They were found to occur about 2,000 times per haploid genome and were remarkable in that they produced a "ladder" of bands when hybridized to *Eco*RI digested genomic DNA in Southern blot hybridizations (Fig. 1, panels A and B, lanes 11). The lengths of these roughly correspond to multiples of 170 bp, which is a strong

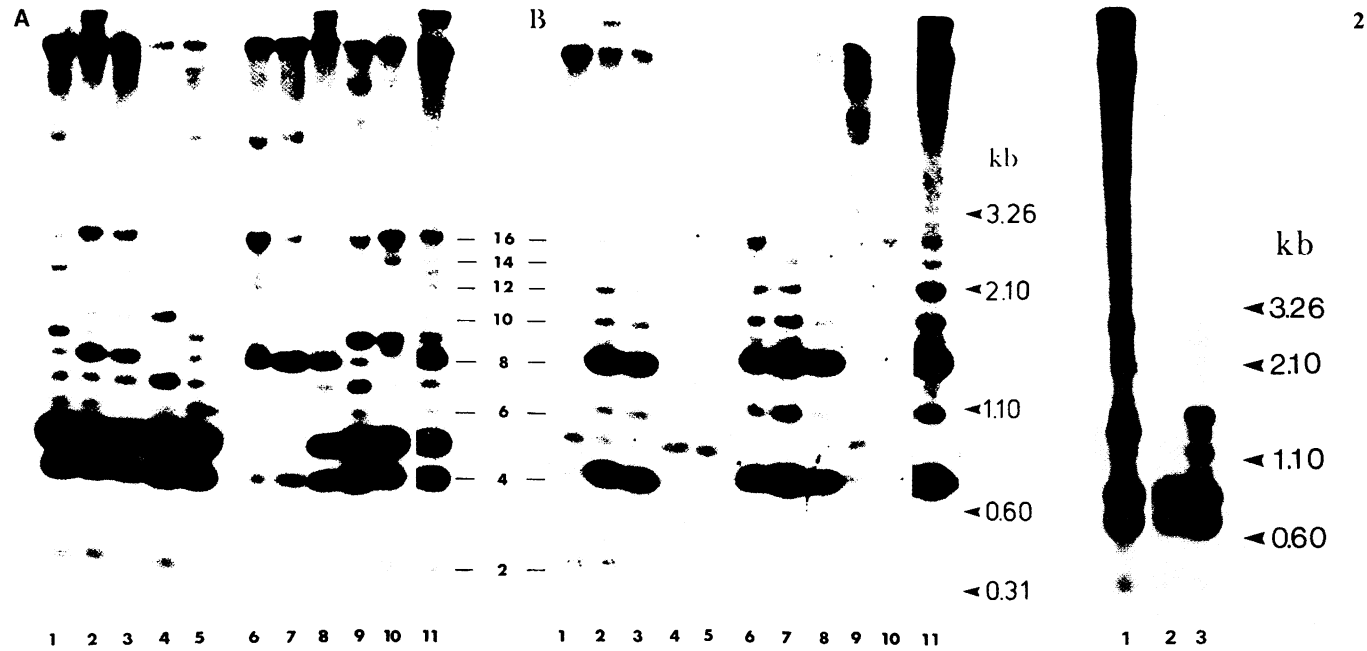

Fig. 1. Hybridization of probe L1.26 (panel A) and L1.84 (panel B) to a series of human-rodent hybrid cell lines. DNA was digested with *Eco*RI. Numbers between the panels indicate multiples of 170 bp. After hybridization with L1.26 (panel A), filters were stripped and rehybridized to L1.84 (panel B). For both panels: lane 1: ADA 1; lane 2: ADA 6; lane 3: ADA 10; lane 4: ADA 13; lane 5: ADA 14; lane 6: A3/G1; lane 7: A3/G12; lane 8: A3/G14; lane 9: 34-2-3; lane 10: PGME4; lane 11: 46,XX. See Table I, top, for chromosome constitution of the hybrids.

Fig. 2. Hybridization of probe L1.26 to two hybrid cell lines with either chromosome 21 (SSC 16–5, lane 2) or chromosome 13 (34-2-3 B3, lane 3) as their only retained human material. Lane 1 is 46,XX total genomic DNA. Digestion was performed with *Eco*RI.

indication that both DNA sequences are members of the alphoid repetitive DNA family (Darling et al., 1982). Both L1.26 and L1.84 appear to hybridize to the same "ladder", but with different intensities per repeat length for each probe. L1.26 itself is 0.85 kb in length, whereas L1.84 is 0.69 kb long. Notably, these particular fragment lengths appear most prominently in hybridizations to *Eco*RI-digested DNA for each probe respectively (Fig. 1, lanes 11, both panels). This implies that each probe is a true representative of its major repetitive component.

Segregation of alphoid multimers within a hybrid cell panel

When hybridized to *Eco*RI-digested DNA from a human-rodent hybrid cell panel (Table I, top), the repeat lengths can be shown to segregate within the panel (Fig. 1, panel A for L1.26, panel B for L1.84, lanes 1–10 for both panels). For L1.84, this segregation is particularly conspicuous for multimers 4 and 8. These multimers are absent from or only weakly present in hybrids ADA1, ADA13, ADA14, 34-2-3, and PG/ME4 (Fig. 1, panel B, lanes 1, 4, 5, 9, and 10, respectively), whereas other cell lines produce strong signals at these positions. For L1.26, a similar phenomenon is seen for multimers 4, 5 to 9, and 16. No hybridization occurs at the 5-, 7-, and 9-mer in hybrids A3/G1 and A3/G12 (Fig. 1, panel A, lanes 6 and 7, respectively), showing that these particular multimers are not abundantly present on the set of human chromosomes present in these hybrids. Conversely, the 8-mer shows up brightly in these hybrids, as well as in three others (ADA6, ADA10, and A3/G14; lanes 2, 3, and 8, respectively).

It appears, therefore, that various multimers of both L1.26 and L1.84 may be assigned to different sets of chromosomes, which, in certain cases, may be reduced to individual chromosomes. Compare, for example, signals obtained with both probes in hybrids ADA1, ADA3, ADA14, and PG/ME4 (lanes 1, 4, 5, and 10, respectively). Because of the repetitive nature of both probes, however, conventional concordance

Table I. Listing of chromosomes in the hybrid cell panel and intensity of hybridization signals obtained with L1.26 and L1.84

Chr. No.	Total obs.	Hybrid											
		ADA1	ADA6	ADA10	ADA13	ADA14	A3G1	A3G12	A3G14	34-2-3	PGME4	34-2-3B3	SCC16-5
1	6	+	+	+	+	+					?[a]		
2	0												
3	5	+			?[a]	+	+		+				
4	6	+				+	+	+	+		+		
5	6	+	+	+		+			+		+		
6	4		+	+					+		+		
7	3		+						+		+		
8	9	+	+	+	+	+	+	+	+		+		
9	4	+		+					+		+		
10	3					+	?[a]	+					
11	6				+	+		+	+		+		
12	7	+	+		+	+			+	+	+		
13	8	+	+	+	+	+				+	+	+	
14	6	+	+	+		+		+	+				
15	5		+	+	+			+	+				
16	1	+											
17	6		+	+			+	+	+		+		
18	5		+	+			±[b]	+	+				
19	6	+	+	+	+		+		+				
20	4	+				?[a]	+		+				
21	8	+	+	+	+	+			+		+		+
22	7	+	+	+		+	+	+	+				
X	4		+		+		+		+				
Y	4	+				+	+	+					
Total		15	15	13	10	14	11	10	18	2	12	1	1
Multimer (L1.26)		Intensity of signal[c]											
4		3	3	2	3	3	1	1	2	4	3	3	3
5		4	4	3	4	4	0	0	2	4	3	3	3
6		4	4	2	3	4	2	2	1	4	1	3	0
7		3	3	3	4	3	0	0	1	4	1	3	1
8		1	3	2	0	1	3	3	3	1	0	1	0
9		3	1	1	1	2	0	0	0	4	4	4	0
10		3	3	3	5	3	2	3	0	0	0	0	0
12		0	3	0	0	2	3	0	0	0	0	0	0
13		4	3	3	1	3	3	0	0	1	1	1	0
14		0	2	2	0	0	2	1	0	1	4	1	0
16		1	3	2	0	1	4	2	1	3	4	0	0
Multimer (L1.84)		Intensity of signal[c]											
4		0	2	2	0	0	2	4	2	0	0	0	0
5		4	3	2	4	4	0	0	0	4	3	2	2
6		0	1	1	0	0	2	3	1	0	0	0	0
8		0	2	2	0	0	2	3	2	0	0	0	0
9		0	1	0	0	0	1	2	1	0	0	0	0
10		0	1	1	0	0	2	2	1	0	0	0	0
12		0	1	1	0	0	2	2	1	0	0	0	0
14		0	1	1	0	0	1	2	1	1	1	0	0
16		1	2	1	0	1	3	1	1	1	2	0	0

[a] Chromosome not identified unambiguously.

[b] Chromosome scored negative, but enzyme marker present.

[c] Intensity of signals given as a percentage of relative strength in total human DNA: 0 = absent or very weak; 1 = 25%; 2 = 50%; 3 = 100%; 4 = 200%; 5 = 400%.

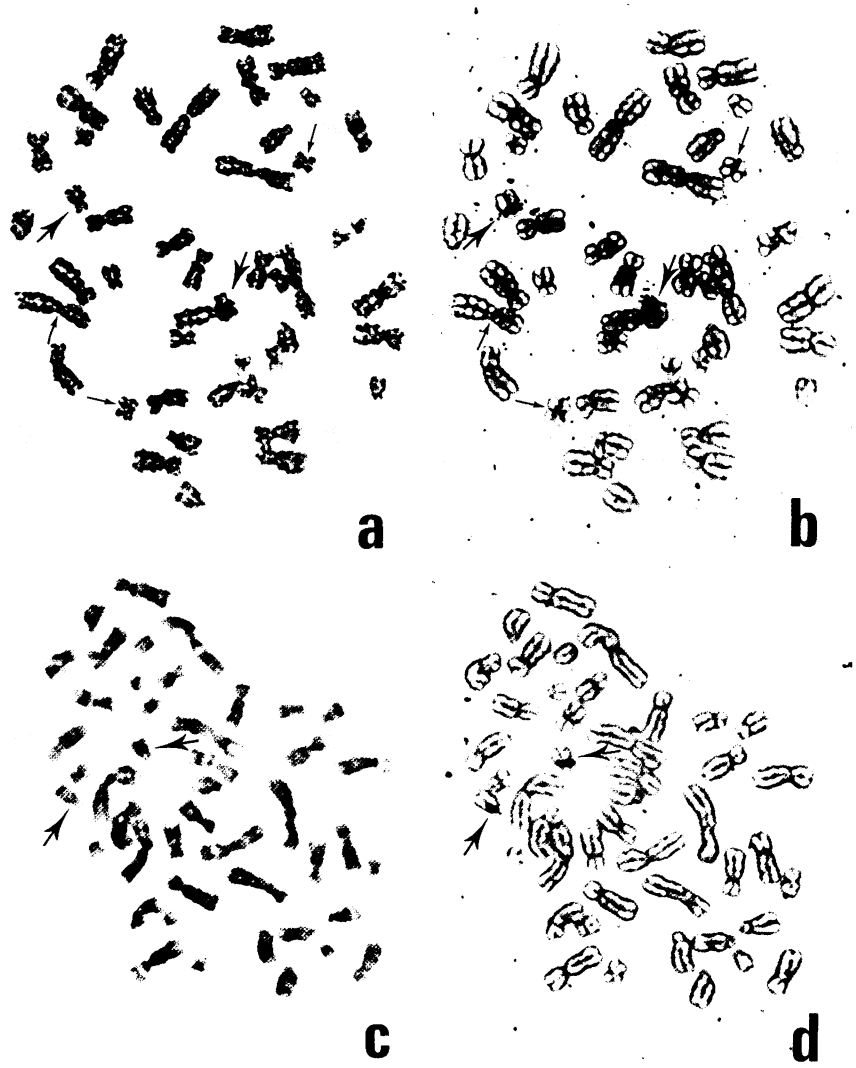

Fig. 3. (a, c) Trypsin-Giemsa banded metaphase spreads from a male human lymphocyte culture. **(b, d)** The same metaphase spreads after in situ hybridization and autoradiography with probe L1.84 following different washing procedures (see text). Large arrows point to silver grains over the pericentric region of chromosome 18, indicating a consistent major hybridization site of L1.84. Small arrows **(b)** indicate minor hybridization sites of L1.84 at the pericentric regions of chromosomes 2 and 20, which are not apparent under more stringent hybridization conditions **(d)**.

analysis, as used for mapping single copy sequences, is not easily applicable. We have therefore resorted to a modified procedure. In each hybrid, the obtained signal per multimer was represented by a number corresponding to its intensity compared to the relative intensity of that multimer in total human DNA (see legend of Table I). This is shown in the middle portion of Table I for L1.26 and in the bottom portion of Table I for L1.84. When the data for a particular multimer are compared with the chromosome constitution of the hybrids (Table I, top), one can determine which chromosome gives the best fit for these signals. A chromosome was specifically excluded when a cell line contained it but failed to produce a signal. The absence of both signal and chromosome was regarded as indecisive, as was the absence of chromosome and presence of signal. The latter category is created by the propensity of somatic cell hybrids to undergo chromosome breakage with retention of a cytogenetically unidentifiable fragment. Thus, the highest values for multimer 6 of L1.26 can be seen to cosegregate with chromosome 13 in the panel. Multimers 7 and 9, although less clear, also principally cosegregate with chromosome 13. Data for multimers 4 and 5 fit equally well to both chromosomes 13 and 21. The mapping of the latter two, which make up at least 50% of the L1.26-related sequences, to chromosomes 13 and 21 was confirmed by Southern blot hybridization experiments with two hybrid cell lines, each containing one of these chromosomes as their only retained human material. The results (Fig. 2) show that both multimers hybridize equally well to both hybrids (lane 2, chromosome 21; lane 3, chromosome 13), as well as to total DNA (lane

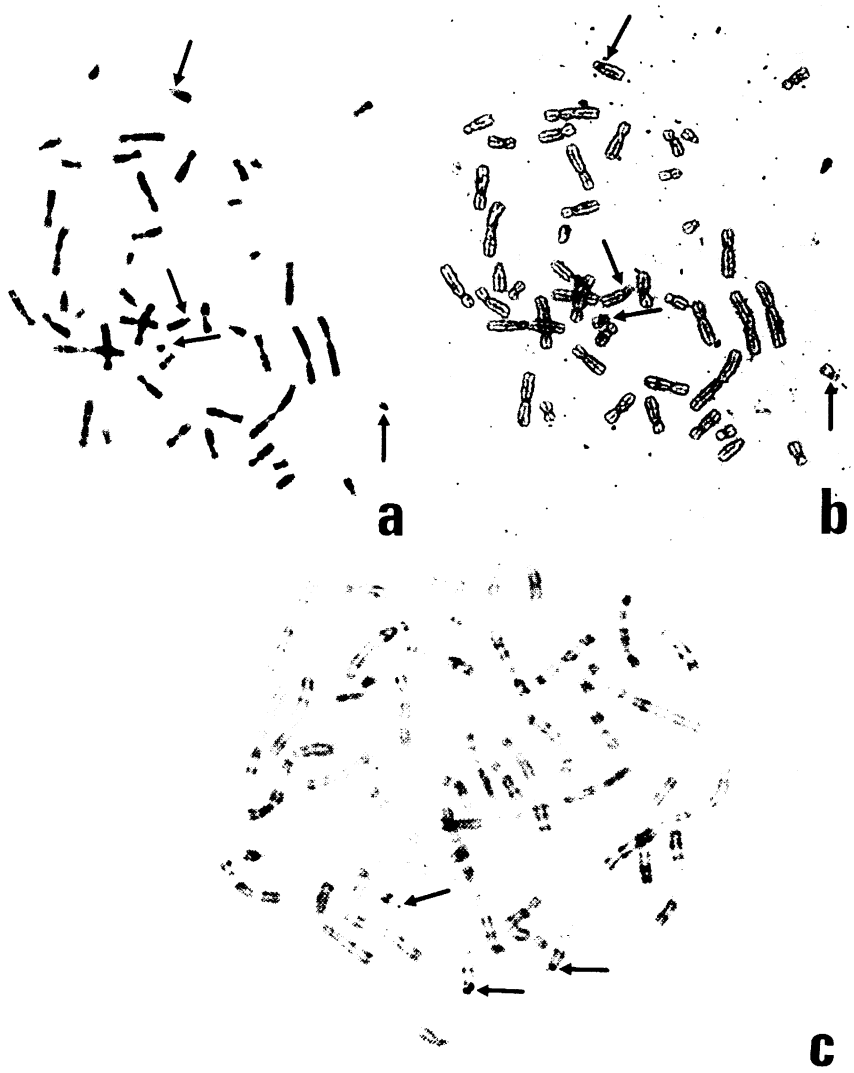

Fig. 4. (a) Trypsin-Giemsa banded metaphase spread from a male human lymphocyte culture. **(b)** The same metaphase spread after in situ hybridization and autoradiography with probe L1.26. Arrows point to silver grains over the pericentric regions of chromosomes 13 and 21. **(c)** Metaphase spread with R-type banding pattern obtained from male human lymphocyte culture after in situ hybridization with L1.26. Arrows indicate the pericentric regions of both chromosomes 13 and one chromosome 21.

1), indicating that these particular multimers are located on these chromosomes in about the same abundancy. For L1.84, the data for multimers 4, 6, 8, 10, and 12 fit best to chromosome 18 (Fig. 1; Table I). Other multimers of both L1.26 and L1.84 do not show clear cosegregation patterns and, therefore, probably map to a more complex set of chromosomes.

In situ hybridization

Figure 3 shows two representative G-banded metaphase plates before (a, c) and after (b, d) in situ hybridization with probe L1.84. Metaphase plates shown in Fig. 3a and b were obtained using procedure 1 for relaxed washing of the slides after hybridization (see the materials and methods section), whereas procedure 2 (stringent washing) was applied to the metaphase plates shown in Fig. 3c and d. In both experiments, the pericentric region of chromosome 18 was heavily labeled (large arrows in Fig. 3). Results obtained with L1.26 are illustrated in Fig. 4. It shows a representative metaphase plate with an R-type banding pattern after in situ hybridization using washing procedure 1. Silver grains are present over the centromeric regions of chromosomes 13 and 21 (arrows).

A quantitative evaluation of several experiments performed with L1.84 and L1.26 using the two different washing procedures is presented in Fig. 5. This analysis confirms the major localizations of L1.84 to chromosome 18 and of L1.26 to chromosomes 13 and 21. However, consistent minor binding sites become apparent at the pericentric DNA of several other autosomes in experiments using washing procedure 1 (e.g., the small arrows in Fig. 3). Notably, minor hybridization sites at the pericentric DNA of chromosomes 2,

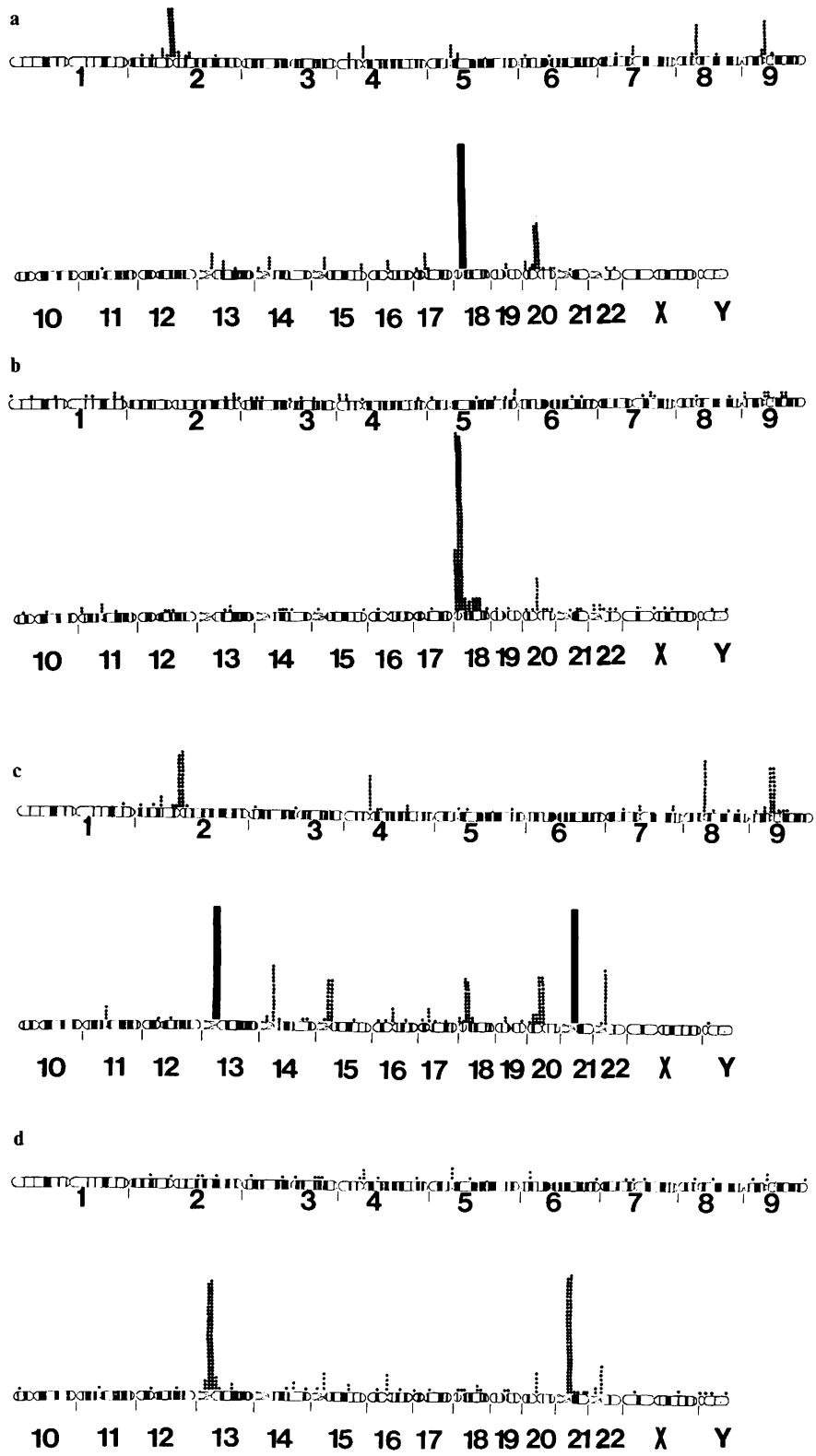

Fig. 5. Schematic representation of trypsin-Giemsa banded mid-metaphase chromosomes, illustrating positions of silver grains in metaphase spreads from male human lymphocyte cultures after in situ hybridization with probes L1.84 and L1.26 and autoradiographic exposure for 5–6 days. Each dot represents the chromosomal localization of one silver grain. **(a)** Distribution of silver grains in 8 metaphase spreads after in situ hybridization with L1.84 (washing procedure 1). The pericentric region of chromosome 18 was so heavily labeled that individual silver grains could not be distinguished. **(b)** The same as **a**, using washing procedure 2; 21 metaphases were scored. **(c)** Distribution of silver grains in 6 metaphase spreads after in situ hybridization with L1.26 (washing procedure 1). Because of heavy labeling, no individual grains could be distinguished on the pericentric regions of chromosomes 13 and 21. **(d)** The same as **c**, using washing procedure 2; 15 metaphases were scored.

8, 9, and 20 are seen with both probes. Other weak hybridization sites include similar regions on chromosomes 4, 14, 15, 16, and 17. Furthermore, probe L1.26 shows a small, but significant, degree of binding to the pericentric DNA of chromosome 18, where the most prominent signal is observed with L1.84.

Discussion

The results show that the pericentric regions of some human chromosomes can be distinguished by specific fractions of middle repetitive alphoid DNA sequences, with L1.26 for chromosomes 13 and 21 and L1.84 for chromosome 18.

In Southern blotting hybridization experiments, both sequences produce a ladder of bands as a result of the specific spacing of endonuclease restriction sites within the tandem organization. The lengths of these bands correspond to multiples of 170 bp for both L1.26 and L1.84 (Fig. 1), indicating that both probes are members of the repetitive alphoid satellite DNA family (Manuelidis, 1976; Darling et al., 1982). We have subsequently sequenced both L1.26 and L1.84 and found them to contain respectively five and four tandemly organized copies of this 170-bp alphoid monomer unit (Devilee et al., 1986). The units are $74 \pm 7\%$ homologous to the reported 170-bp consensus sequence (Wu and Manuelidis, 1980) and also to each other. Furthermore, the mean cross-homology between L1.26 and L1.84 is 75%. It appears, therefore, that they represent two divergent subfamilies of alphoid DNA. Hence, while the entire alphoid DNA family is believed to be present in the centromeres of all human chromosomes, our data, and those of others (Manuelidis, 1978; Willard et al., 1983; Wolfe et al., 1985), suggest that specific variants of the 170-bp unit may be highly enriched on certain chromosomes. If so, it may be possible to clone a specific alphoid satellite member for each of the 24 human chromosomes. As such, L1.84 represents a chromosome 18–specific member, whereas L1.26 demonstrates a strong cross-homology with alphoid DNA sequences principally located on chromosomes 13 and 21.

The weak cross-homologies observed in hybrid cells are compatible with in situ hybridization of both L1.26 and L1.84 to chromosomes 2, 4, 8, 9, 14 to 17, and 20. The size of the in situ hybridization binding site and degree of homology may explain the variable appearance of these sites when treated with various washing procedures. Other parameters, such as the quality of the chromosome preparations or small variations in the denaturing of chromosomal DNA or in the hybridization conditions, may also be important. Since these parameters are difficult to control precisely, we cannot exclude that some chromosomes which did not reveal in situ hybridization with the two probes so far might do so under alternative hybridization conditions. Of interest is that L1.26 hybridizes weakly to chromosome 18 in situ, whereas L1.84 does not hybridize to chromosomes 13 and 21, indicating that either the cross-homologies between L1.26 and L1.84 are insufficient to produce consistent signals under the conditions used or, and more likely, other subsets of alphoid DNA are present on chromosome 18 which are more closely related to L1.26 than are L1.84 sequences, illustrating the heterogeneity of alphoid DNA sequences on one chromosome.

Some special considerations should be taken into account when using a hybrid cell panel to map a repetitive probe. First, the complex distribution of the probe hampers conventional concordance analysis. However, in the case of L1.26 and L1.84, which appear to be enriched on one or two chromosomes, a "best fit" analysis, as presented here, can prove useful. The mapping (Fig. 1, Table I) of the prominent repeat lengths of both L1.26 and L1.84, which represent either the most homologous or the most abundant sequences, are in agreement with the results obtained from in situ hybridization. Clearly, subclones of hybrids differing only in the presence of a single chromosome are extremely informative, especially if the repetitive sequence is more dispersed. Second, within a hybrid cell line, only a fraction of the cells may contain a particular chromosome. As a consequence, signals for a repeat length putatively located on this chromosome will be relatively weaker, thereby interfering with its correct mapping. Furthermore, undetected translocations can misguide the unwary cytogeneticist, leading to faulty conclusions about the chromosome constitution of the cell line. In situ hybridization identifies the chromosomes directly and, simultaneously localizes the DNA sequences to specific regions on these chromosomes. Southern blotting of a hybrid cell panel, on the other hand, reveals interesting aspects about the organization of repetitive probes on the various chromosomes which would not be apparent from in situ hybridization, such as which

multimer is located on which chromosome. Our results clearly show that both techniques complement each other.

The availability of chromosome-specific probes will be useful in the study of hypotheses on alleged functions of alphoid DNA (Manuelidis, 1982). Furthermore, when one considers their location to chromosomes 13, 18, and 21, L1.26 and L1.84 will become valuable tools for the detection in undividing cells of the three most common trisomies in man (Cremer et al., in preparation).

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