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Homologous subfamilies of human alphoid repetitive DNA on different nucleolus organizing chromosomes

(satellite DNA/repeated sequences/DNA sequence/evolution/human)

ARNE LUND JØRGENSEN*, CHRISTOPHER J. BOSTOCK†‡, AND ARNE LETH BAK*

*Institute of Medical Microbiology, The Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark; and †Medical Research Council Mammalian Genome Unit, Kings Buildings, Edinburgh EH9 4JT, United Kingdom

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ABSTRACT **The organization of alphoid repeated sequences on human nucleolus-organizing (NOR) chromosomes 13, 21, and 22 has been investigated. Analysis of hybridization of alphoid DNA probes to Southern transfers of restriction enzyme-digested DNA fragments from hybrid cells containing single human chromosomes shows that chromosomes 13 and 21 share one subfamily of alphoid repeats, whereas a different subfamily may be held in common by chromosomes 13 and 22. The sequences of cloned 680-base-pair *Eco*RI fragments of the alphoid DNA from chromosomes 13 and 21 show that the basic unit of this subfamily is indistinguishable on each chromosome. The sequence of cloned 1020-base-pair *Xba* I fragments from chromosome 22 is related to, but distinguishable from, that of the 680-base-pair *Eco*RI alphoid subfamily of chromosomes 13 and 21. These results suggest that, at some point after they originated and were homogenized, different subfamilies of alphoid sequences must have exchanged between chromosomes 13 and 21 and separately between chromosomes 13 and 22.**

One of the models for genomic change that mediates the evolution of new species or generates major changes within a species involves periodic reorganizations of the genome accompanied by amplification of different families of repetitive DNA. The alphoid family of repetitive DNA is found exclusively in primates and has been studied in human and several monkey and ape species. It is believed that different families of this repeat arose prior to the emergence of several of these species, after which the alphoid families have remained relatively unchanged (1). Although the separation of the branches leading to the great apes and humans took place 6–8 million years ago (2), the most significant human evolution has probably taken place within the last few million years. One might therefore expect to find within the human genome families of the alphoid repeat that have been amplified relatively recently, and evidence for one such family has already been reported (3). Recent studies (4–6) indicate that human alphoid DNA is organized into chromosome-specific subfamilies, formed by the amplification of segments composed of tandemly arranged related copies of the 170-base-pair (bp) (monomeric) or 340-bp (dimeric) repeat units.

Chromosome specificity of subfamilies of alphoid DNA implies that transfer of sequences between nonhomologous human chromosomes occurs very rarely. However, one group of chromosomes, the nucleolus organizing (NOR) chromosomes, appears to undergo recombination between nonhomologues more frequently than do other chromosomes (7, 8), and thus some alphoid families might be expected to be held in common between the NOR chromosomes. We report here studies on the alphoid repetitive DNA in three different human NOR-bearing chromosomes—13, 21, and 22. These chromosomes hold in common a related subfamily of alphoid sequences that has diverged ≈25% from the average sequence of alphoid repeats. The sequences of tetramers of the basic unit of this alphoid family are indistinguishable in chromosomes 13 and 21. Another subfamily is common to chromosomes 13 and 22 but not present in chromosome 21.

MATERIALS AND METHODS

Hybrid Cells, Isolation of Genomic DNA, and Gel Electrophoresis. High molecular weight DNA was extracted as described (6) from normal human embryonic fibroblasts and from the mouse–human hybrid cells Cl21, PgMe25/9, ThyB1-33-6, and PgMe25/8, which contain chromosomes 7, 13, 21, and 22, respectively, as the only detectable human chromosome or subchromosomal fragment (9). The DNAs were digested to completion with various restriction enzymes under conditions recommended by the supplier. Restriction enzymes (*Xba* I, *Taq* I, *Eco*RI, *Bam*HI), T4 ligase, and Klenow DNA polymerase were supplied by Boehringer Mannheim. For Southern blot analysis 15 μg of each of the digested DNAs from the mouse–human cells was loaded on each slot of a 1.5% agarose (Sigma) gel, and 1 μg of *Xba* I- or *Eco*RI-digested total human DNA was loaded and the alphoid fragments were used as length markers. For the isolation of restriction fragments of different lengths, gels were run overnight at 1.5 V/cm in Tris acetate buffer.

Isolation of Restriction Fragments. For isolation of restriction fragments containing human alphoid repeat sequences (680-bp *Eco*RI fragments from DNA from chromosomes 13 and 21, and 1020-bp *Xba* I fragments from DNA from chromosome 22; see Fig. 1), up to 50 μg of digested DNA from the relevant mouse–human cells was loaded in each slot of a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and run in Tris acetate buffer containing ethidium bromide (0.5 μg/ml). *Xba* I-digested total human DNA (15 μg) was used as a length marker and was loaded in one slot. Slices 2 mm thick were excised from the gel lanes at positions corresponding to the relevant lengths and placed in preformed wells in another gel (1% agarose). A slit was cut in front of the wells, a piece of NA45 membrane (Schleicher & Schuell) was inserted, and the excised DNA was electrophoresed onto the NA45 membrane. The restriction fragments were eluted from the membrane, ligated into a phage M13mp10 vector (Amersham) and cloned, and recombinant clones were screened for inserts of alphoid repeat sequences. (For details on the isolation, cloning, and screening procedures, see ref. 6.)

DNA Sequencing. The DNA sequencing of recombinant clones containing alphoid repeat sequences was done according to the dideoxy-chain-termination procedure of Sanger *et*

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Abbreviation: NOR chromosome, nucleolus organizing chromosome.

‡Present address: Animal Virus Research Institute, Pirbright, Woking, Surrey GU24 ONF, United Kingdom.

al. (10, 11). The sequence was read on one strand unambiguously in two or more independent preparations of single-stranded DNA of each clone to be sequenced.

^{32}P-Labeled DNA Probes. The probe used in Fig. 1 consisted of a 340-bp alphoid repeat sequence isolated by *Eco*RI digestion from clone αRI-6 (6) and represents the average sequence of the alphoid repeat family (12). The probe used in Fig. 2 consisted of a 680-bp alphoid repeat sequence isolated by *Eco*RI digestion of clone αRI(680),13-5. This originated from chromosome 13 and represents chromosome-specific alphoid repeat sequences. The alphoid repeat sequences of the two probes were labeled with [α-^{32}P]dATP (Amersham; specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq) by chain extension on single-stranded recombinant M13 DNA using sequencing primer (6). The double-stranded labeled inserts were excised by *Eco*RI digestion and isolated by gel electrophoresis as described above. Specific activities of 3–5 × 10^8 cpm per μg of DNA were obtained.

Southern Blot. Southern blotting to nitrocellulose filters (Schleicher & Schuell) was done essentially as described (13). Hybridization, using 10% dextran sulfate (Pharmacia) (14), was done in sealed plastic bags containing 1 × 10^6 cpm per ml of hybridization buffer. In Fig. 1, the stringency of hybridization corresponded to ≈20% mismatch and was done in 6× SSC at 55.5°C (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7), with posthybridization washes in 1× SSC at 54°C. In Fig. 2, the stringency of hybridization was raised and done in 6× SSC at 75.5°C, with washes at 82°C in 1× SSC. The filters were air-dried, placed in plastic bags against x-ray films (Fuji) with an intensifying screen, and exposed at −70°C for a few hours to several days.

RESULTS

Hybridization of the average alphoid probe (Fig. 1) to *Eco*RI digests of DNA isolated from ThyB1-33-6, the hybrid with only human chromosome 21, shows two prominent bands at 680 and 850 bp, with a lower level of hybridization to the 340-bp fragment and those of molecular sizes greater than 850 bp. Bands at these sizes and with the same relative intensities of hybridization are also found in *Eco*RI digests of DNA from PgMe25/9, the human chromosome 13-containing hybrid cell, with some additional fragments of larger size also hybridizing. The 680- and 850-bp fragments on both human chromosomes 13 and 21 also hybridize at high stringency the tetrameric repeat derived from chromosome 13 (Fig. 2), suggesting that these fragments carry closely homologous alphoid sequences.

In contrast, the patterns of hybridization of the average alphoid probe to *Xba* I fragments of DNA derived from chromosomes 13 and 21 (Fig. 1) are clearly distinguishable, and there is only limited hybridization of the chromosome 13-derived alphoid tetramer to *Xba* I fragments of 1020 and 1360 bp in common between chromosomes 13 and 22. It also hybridizes to a *Taq* I fragment of 1870 bp that is held in common between chromosomes 13 and 21 but is not found in chromosome 22, and a *Taq* I fragment of 1360 bp that is common to chromosomes 13 and 22 but is not present on chromosome 21. Taken together, results from hybridizing alphoid probes to Southern blots of these three chromosomes suggest that there are two separate but related subfamilies. One, the chromosome 21 subfamily, is shared by chromosomes 13 and 21, and the other, the chromosome 22 subfamily, is shared by chromosomes 13 and 22.

Assignments based on hybrid cells need to be approached with some caution because of the possibility for undetected translocations and rearrangements of DNA sequences occurring during the production of the hybrid cell clones. From the point of view of the results reported here, the only hypothetical chromosome rearrangement that could affect the interpretation of the chromosome 13 and 21 comparisons would be that which had specifically translocated a subset of human chromosome 13 alphoid sequences into the human chromosome 21, which remains in the ThyB1-33-6 hybrid cell. This is because all alphoid sequences disappear from the cell upon loss of the human chromosome 21.

Potential artifacts resulting from undetected translocations common to both PgMe25 hybrids are more likely since these hybrid cells were subcloned from the same parent cell line. For example, at first sight the 2.0 and 4.0-kilobase (kb) *Bam*HI bands found in the PgMe25 hybrids could represent material derived from alphoid sequences originally present on the human X chromosome (4, 5) and could have become integrated into one of the mouse chromosomes. We feel that this is probably not the case because the chromosome 13-derived alphoid probe will hybridize to the 2.0- and 4.0-kb

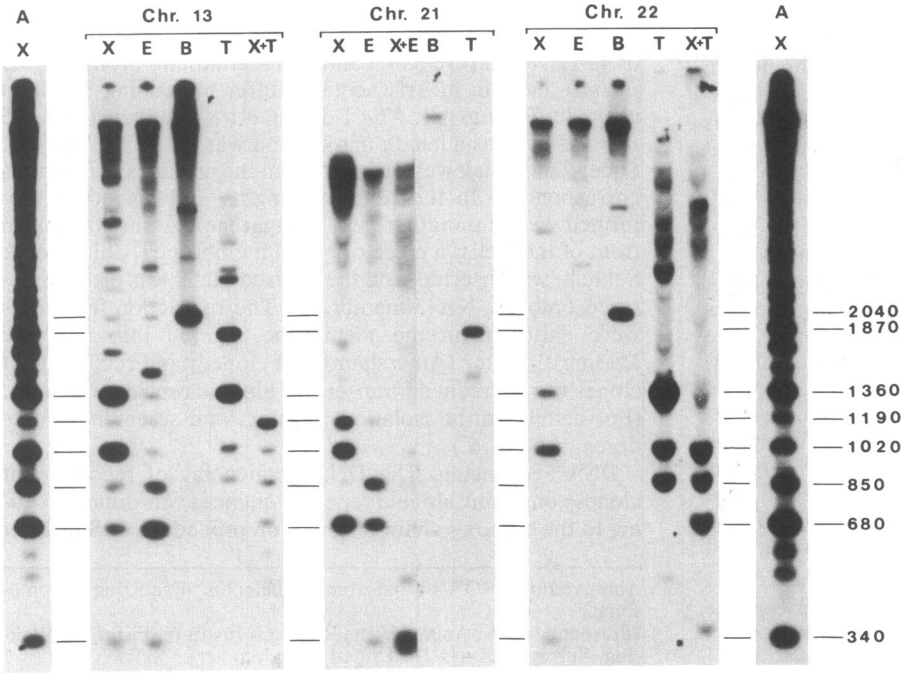

FIG. 1. Hybridization of alphoid repeats in Southern blots of various restriction enzyme digests of DNAs from three different mouse–human hybrid cells: PgMe25/9, ThyB1-33-6, and PgMe25/8, containing human chromosome 13, 21, or 22, respectively, as the only human chromosome. Each lane contained 15 μg of total genomic DNA digested to completion with one or two restriction enzymes, as indicated at the top of the lane. Lanes A contained 1 μg of *Xba* I-digested total genomic DNA from normal human embryonic cells to serve as length markers. Numbers on the right refer to molecular size in bp. Hybridization was at low stringency, allowing for 20% mismatch (55.5°C in 6× SSC and wash at 54°C in 1× SSC). The ^{32}P-labeled probe was prepared from a recombinant M13 clone (αRI-6) with a sequence nearly identical to the average alphoid repeat sequence. The letters at the top of each lane indicate digestion of the DNA with the following restriction enzymes: X, *Xba* I; E, *Eco*RI; B, *Bam*HI; T, *Taq* I. X+T and X+E indicate double digestion with the corresponding restriction enzymes.

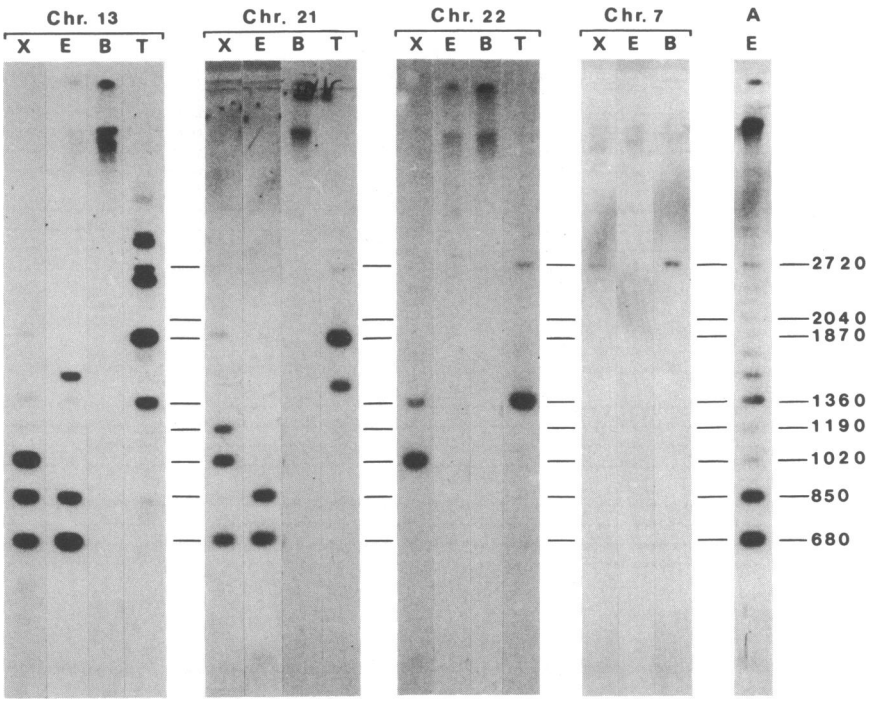

FIG. 2. Hybridization of alphoid repeats in Southern blots of various restriction enzyme digests of DNA from four different mouse–human cells: PgMe25/9, ThyB1-33-6, PgMe25/8, and Cl21 (6), containing human chromosome 13, 21, 22, or 7, respectively, as the only human chromosome. Each lane contained 15 μg of total genomic DNA digested to completion with the restriction enzyme indicated by a letter at the top of the lane. Lane A contained 1 μg of *Eco*RI-digested total genomic DNA from normal human embryonic fibroblasts to serve as length markers. Numbers on the right correspond to molecular size in bp. Hybridization was at high stringency (at 75.5°C in 6× SSC with washes at 82°C in 1× SSC). The ^{32}P-labeled probe was prepared from the recombinant clone αRI(680),13-5 (Fig. 3), which contains alphoid repeat sequences isolated from chromosome 13 and identical to the sequence of the subfamily shared by chromosomes 13 and 21. The letters at the top of the lanes indicate digestion of the DNA with the corresponding restriction enzyme (see legend to Fig. 1).

*Bam*HI fragments present in both PgMe25 hybrids under conditions in which there is no detectable hybridization to fragments of this size in hybrid cells carrying only a single human X chromosome (data not shown). Thus, although the hybrid cells carrying chromosomes 13 and 22 have alphoid repeats in *Bam*HI fragments of the same size as those on the human X chromosome, the sequence of the alphoid repeat would appear to be sufficiently different from that on the X chromosome to be distinguishable by hybridization at high stringency, although it will cross-hybridize under conditions of lower stringency. The presence of *Bam*HI fragments of ≈2.0 and 4.0 kb containing alphoid repeats on chromosome 13, and cross homology of chromosome 13 and 21 alphoid sequences with those of the X chromosome have been noted elsewhere (15). Whatever the origin of the 2.0- and 4.0-kb *Bam*HI fragments, it is the *Eco*RI, *Xba* I, and *Taq* I fragments that indicate homology of alphoid sequences between human chromosomes 13 and 22 and these cannot derive from putative contamination with X chromosome alphoid-repeat DNA since equivalent digests of DNA from a single human X chromosome-containing hybrid cell do not have fragments of the relevant sizes that hybridize the alphoid probes (data not shown).

The fact that the three NOR-bearing chromosomes have some fragments in common that hybridize the chromosome 13-derived tetramer at high stringency indicates the presence of related repeats in all three chromosomes. To explore this further, we have isolated and sequenced four tetramer (680 bp) units cloned at random from *Eco*RI digests of each of chromosomes 13 and 21 and four dimers from each of two hexamer fragments (1020 bp) picked at random from *Xba* I digests of chromosome 22. The sequences in Fig. 3 show that the *Eco*RI tetrameric fragments in chromosomes 13 and 21 are indistinguishable. Preliminary sequence data on cloned 680-bp alphoid repeats from another and independent isolate of chromosome 13 indicate the presence of the same tetrameric sequence. All the tetramers that were sequenced consist of two closely related, but distinct, dimer units containing both an *Xba* I (dimer I, 2′ monomer) and an *Eco*RI (dimer II, 2′ monomer) restriction site. As shown in Fig. 1, a double digestion of chromosome 21 DNA with these two enzymes liberates alphoid homologous sequences in bands of ≈510 and ≈340 bp expected on the basis of the tetrameric sequences. This indicates that any fragment longer than 680 bp in either the *Eco*RI or *Xba* I digest may also contain sequences related to these tetramers.

Fig. 3 also shows that the sequence of the 22-73 cloned insert of the 1020-bp *Xba* I fragment of chromosome 22 differs slightly from the 22-82 cloned insert. This shows that the two original *Xba* I fragments may have been located in separate domains, each containing a slightly different amplification unit. Nevertheless, the sequences of these chromosome 22-derived hexamer fragments are clearly related to the sequence of the tetramers derived from chromosomes 13 and 21 (≈60% homology in subfamily-specific positions, see Fig. 3).

The sequence of the eight *Eco*RI tetramers from chromosomes 13 and 21 deviates from the average alphoid sequence by ≈25%. Setting aside examples where at least two repeats have the same changes in the same position (subgroups of the subfamily), and counting the deletion of 4 bases (positions 84–87; clone 21-208,I-1) as one event, the extent of deviation in non-subfamily characteristic positions is 0.3%. This compares with 0.6% deviation among the 18 dimer members of the chromosome 7 subfamily (6).

The majority of the alphoid repeats on chromosome 7 are present in *Eco*RI fragments of 340, 680, and 1020 bp, but, as shown in Fig. 2, these do not contain sequences that are homologous to those of the *Eco*RI tetramers of chromosomes 13 and 21. Hybridization at moderate stringency (75.5°C in 6× SSC and wash at 74°C in 1× SSC) showed the same results (data not shown). When analyzing the significance of different levels of hybridization between complex repeated sequences, it is sometimes difficult to distinguish between the relative contributions from differing quantities and differing degrees of homology. Nevertheless, considering the stringency of hybridization used in Fig. 2, the faint band at ≈2.7 kb could indicate that small amounts of sequences similar to that of the subfamily studied may be present in chromosome 7, and the faint ladder of bands in the *Eco*RI-digested total genomic DNA also suggests that small amounts of a closely related subfamily may be present in other parts of the genome. If this is true, the concept of chromosome-specific subfamilies would have to be modified accordingly.

DISCUSSION

From our data, it seems clear that the alphoid repeats studied here conform to the general pattern of organization being

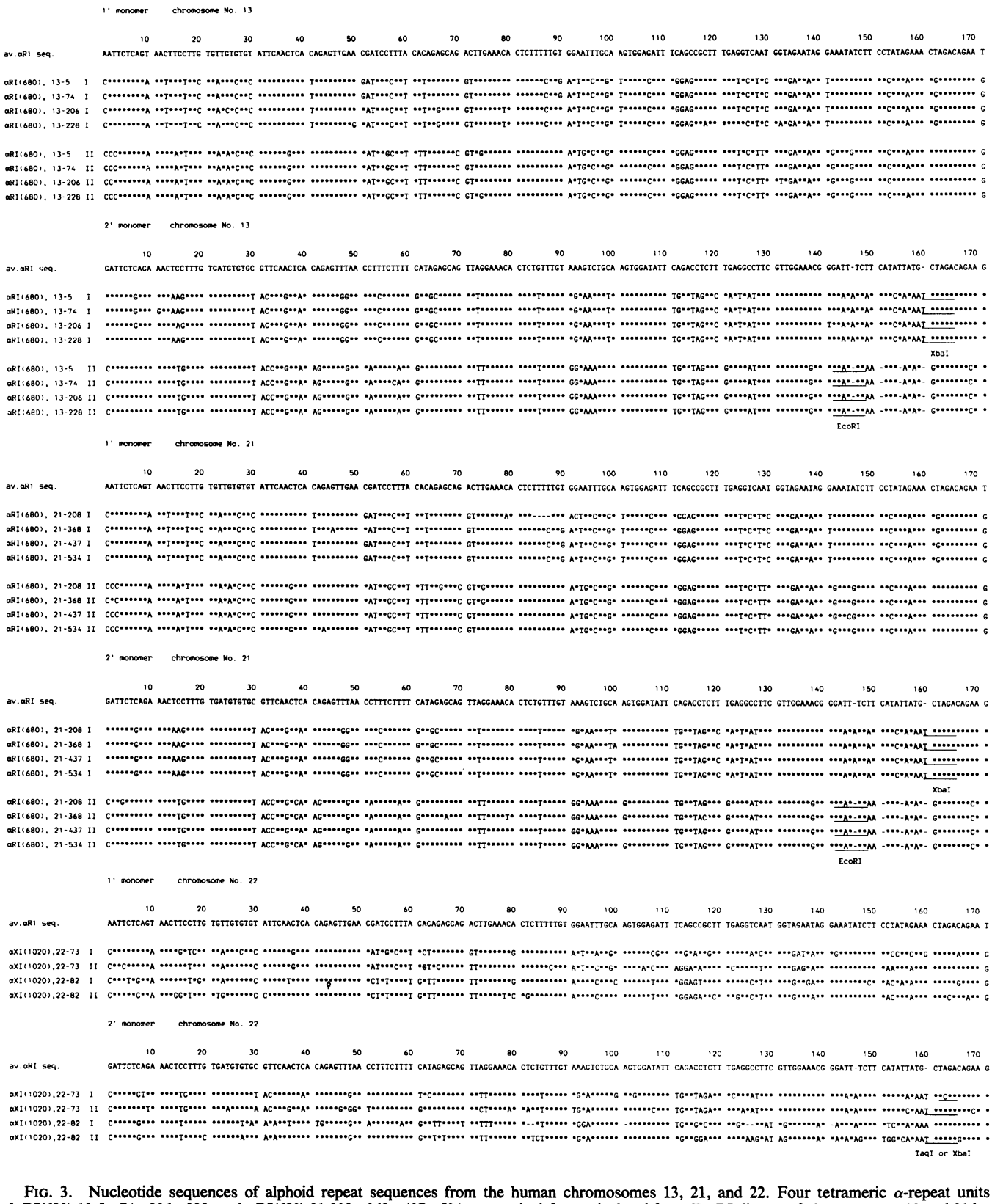

FIG. 3. Nucleotide sequences of alphoid repeat sequences from the human chromosomes 13, 21, and 22. Four tetrameric α-repeat units [αRI(680),13-5, -74, -206, -228, and αRI(680),21-208, -368, -437, -534, respectively] were isolated from *Eco*RI digests of chromosomes 13 and 21 by excision of the 680-bp *Eco*RI bands (see Fig. 1) and cloned in phage M13. Each tetramer consisted of two dimers (I and II) and, by using the dideoxy-chain-termination method of sequencing, the complete sequence was obtained by sequencing one dimer from each end of the tetramer. Asterisks indicate identical nucleotides, and a dash (−) indicates a deletion of a nucleotide relative to an average alphoid repeat sequence (12). The characteristic *Eco*RI site in the average sequence, which is normally used as endpoints of the alphoid repeat dimer (6), is absent because of a point mutation at position 1, changing A to C. A new *Eco*RI site in the tetrameric sequence has arisen in the second dimer (II) at positions 141–148 by a point mutation at position 144, changing T to A. To facilitate comparison of these sequences with that of the average alphoid sequence, the sequences derived here have therefore been rearranged and aligned with the average *Eco*RI dimer. Similarly, two hexameric alphoid repeat units [αXI(1020), 22-73 and -82] were isolated from the 1020-bp *Xba* I band of chromosome 22 (see Fig. 1) and cloned. Under the cloning procedure in phage M13, the 1020-bp insert in clone αXI(1020), 22-82 suffered a deletion of 90 bp. The clones were sequenced from both ends to obtain the sequences of the four dimers shown in the figure.

built up for alphoid DNA; namely, large amplification units composed of a fixed order of several different but related smaller repeat units. Such an organization requires that the sequences in families of repeats are maintained closely similar by a process termed homogenization, suggested to be mediated by unequal crossing-over or mechanisms akin to gene conversion (16, 17). Counteracting this movement to homogeneity are the effects of sequence divergence brought about by normal mechanisms of random mutation. There must also be rare events that bring about occasional dispersion of subfamilies of repeats between two or more nonhomologous chromosomes. Each of these processes operates in dynamic organisms within fluid populations and thus might be expected to have very different effects, depending on the precise biological and temporal contexts in which they occur.

It is possible to imagine several possible ways in which identical tetrameric alphoid sequences came to be present on chromosomes 13 and 21, but we will consider only three. Firstly, by chance, independent homogenization processes could have resulted in identical sequences evolving in these blocks of alphoid DNA. Secondly, homogenization processes could be operating between different homologues to prevent divergence of blocks of alphoid DNA. Thirdly, following homogenization of one chromosome, a portion of a subfamily could have been transferred between nonhomologous chromosomes by recombination. If this hypothetical recombination event had happened recently in evolutionary time, any independent homogenization processes operating on the two blocks of alphoid DNA, now separated on different chromosomes, may not have had sufficient time to cause the isolated sequences to diverge. Although our data do not allow an unequivocal distinction between these possibilities, we feel the last option best fits the available data.

The Southern blots in Fig. 2 show that, although the sequences of the cloned tetramers from chromosomes 13 and 21 are identical, the overall structures of the amplification units in these two chromosomes cannot be absolutely identical. This is because the patterns of hybridization to *Eco*RI fragments of > 680 bp from the two chromosomes differ. Thus, if the explanation of the near identity lies in the translocation of a portion of an alphoid subfamily from either chromosome 13 to chromosome 21, or vice versa, the translocated portion could not have been identical to the portion left behind, or the separated portions may have begun to diverge.

In contrast to the close homology of alphoid DNA between chromosomes 13 and 21, chromosomes 22 and 21 appear quite distinct. None of the four different dimer sequences found in chromosome 22 can be present in chromosome 21, as none of the chromosome 22-derived dimers contained restriction sites with positions characteristic of those found on all the fragments from chromosome 21. In addition, stringent hybridization of the chromosome 22 1020-bp *Xba* I fragment to blots of chromosome 21 DNA detects no alphoid DNA (data not shown). However, chromosome 22 is related to chromosome 13 by the observation that about one-half of the alphoid DNA on chromosome 22 is closely homologous to about one-half the alphoid DNA on chromosome 13 and would appear to form a chromosome 13/22 subfamily, which is related to, but distinct from, the chromosome 13/21 subfamily. If chromosome translocations are the basis for between-chromosome similarities, the chromosome 13/22 homology must represent a separate, and earlier, recombination.

The only way to explain these results on the basis of *in situ* homogenization processes would be to postulate that homogenization can occur between each of two different blocks of alphoid DNA on the same chromosome (2) independently" with blocks of alphoid DNA on different chromosomes (21 and 22). We feel that "cross-talk" between the different NOR-bearing chromosomes may occur at a higher frequency than for other chromosomes, but not selectively so. For example, Robertsonian translocations are not especially frequent between chromosomes 13 and 21, being of the same frequency as those between chromosomes 13 and 22 and between chromosomes 21 and 22 (18, 19). Our data suggest that homogenization occurs predominantly on different homologues, establishing independently chromosome-specific subfamilies with different characteristic lengths of sequence register (6, 20). The chromosome 13/21 subfamily of alphoid repeats has 0.3% deviation among the different tetrameric sequences. Assuming a drift of ≈0.13% per million years for neutral mutations in hominids (2, 21), this would mean that the subfamily was amplified some 2.3 million years ago, presumably on either chromosome 13 or chromosome 21. Occasional exchanges between nonhomologues must occur, at least in the NOR-bearing chromosomes studied here, to ensure some homogenization between nonhomologues. The fact that the alphoid repeats on chromosome 13 are indistinguishable from those on chromosome 21 would suggest that the transfer of alphoid sequences between these two chromosomes has occurred very recently in the evolution of the hominid line.

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