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Long Range Organization of Alphoid Sequence on Human Chromosome 21

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ABSTRACT

One of the major repetitive sequences in the human genome is alphoid DNA, which is organized into a variety of discrete families, defined by both restriction enzyme periodicities and sequence relatedness. The higher-order organization of three different alphoid families found on chromosome 21 was examined here, in order to determine whether members of each family exist as a single cluster or are interspersed with members of other families. DNAs from human-hamster hybrid cell lines containing intact chromosome 21 or fragments of it as the sole human chromosome were digested with restriction enzymes which do not cut within the alphoid families, and subjected to pulsed field gel electrophoresis. Hybridization with probes of different alphoid families was carried out under conditions where they do not cross-hybridize with each other. Members of two alphoid families are found in long clusters at least 1300 kilobases (kb) in length. The pattern of organization of the alphoid clusters on chromosome 21 is a simple subset of that seen in the total genome, suggesting that these alphoid families probably have a chromosome-specific organization. The sizes of the alphoid-sequence containing fragments are distinctly different from one family to another, indicating that members of one family are not significantly intermixed

with those of another. The 340 base pair (bp) EcoRI family appears to be present in a continuous cluster on the the short arm of chromosome 21. The other family (550 bp HindIII) is present on the short arm and in the centromeric region as several separate clusters that are interspersed with unrelated sequences. Alphoid sequence-containing fragments account for at least 25% of the short arm of chromosome 21.

The clustered organizations for the two alphoid families indicate that they may both be involved in the facilitation of Robertsonian translocations which frequently involve the short arm of chromosome 21. On the other hand, their organizations are not consistent with any simple models for the role of alphoid sequences in centromeric function.

LONG RANGE ORGANIZATION OF ALPHOID SEQUENCE ON HUMAN CHROMOSOME 21

by

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VITA

The author, Malini Chawla, born on the 29th of November, 1965, Baroda (India). She did her undergraduate studies at St.Xavier's College, Bombay, graduating in 1987 with a major in Life Sciences and Biochemistry.

Ms. Chawla came to the United States in August of 1988, to pursue her Masters degree at Loyola University of Chicago. She was awarded a graduate assistantship, allowing her to complete the requirements for the degree of Master of Science in 1991.

In March, 1990 she gave a presentation at the regional meeting of the American Society for Cell Biology (ASCB), at Chicago. Soon after that she presented a seminar at the Graduate Research Forum sponsored by the Loyola Chapter of Sigma Xi, and was the recipient of an award. She is also an Associate Member of Sigma Xi. Ms. Chawla also presented her work at the annual meeting of the ASCB in December, 1990.

Presently, Ms. Chawla is in the Ph.D program at Laboratory of Molecular Biology, University of Illinois at Chicago and is receiving a departmental fellowship.

PUBLICATIONS

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INTRODUCTION

The centromeres of eukaryotic chromosomes are specific constricted regions along the chromatin fiber where the spindle fibers attach. They play a fundamental role in chromosome movement during cell division, ensuring proper distribution of replicated chromosomes. Human centromeric regions contain large amounts of repetitive DNA (Manuelidis, 1978). One of the major repetitive DNA sequences present is alphoid DNA (Manuelidis, 1978), basically characterized by tandemly arranged monomeric repeats of approximately 170 bp (Wu and Manuelidis, 1980). Alphoid sequences have also been found on the short arms of acrocentric chromosomes (Manuelidis, 1978). A number of alphoid sequence variants have been identified that apparently arose due to point mutations. These have been classified into families and subfamilies, defined by their sequence relatedness and restriction enzyme periodicities (Willard, 1985; Jorgensen et al., 1986; Willard and Waye, 1987).

Previous studies have emphasized that each human chromosome has its own specific alphoid family (Willard and Waye, 1987b). However this does not preclude the presence of additional alphoid sequences on a given chromosome, and indeed several studies have recently shown the presence of at least two distinctly different alphoid families on the same chromo-

some (Carine et al., 1989; Waye et al., 1987b; Palamidis-Bourtsos, 1989). A recent study done on chromosome 21 (Palamidis-Bourtsos, 1989) has demonstrated that it contains at least three alphoid families, two of which are located at its centromeric region and on the short arm, while a third is present only on its short arm. Precise information on long range organization of multiple alphoid families on a single chromosome was previously difficult to obtain, since DNA fragments of sizes greater than 50 kb remain unresolved in conventional gel electrophoresis. Macro-organization of these sequences can now be studied by using pulsed field gel electrophoresis, a recently developed technique that can resolve fragments as large as 9000 kb (Schwartz and Cantor, 1984; Carle and Olson, 1984; Avdalovic and Furst, 1988).

Alphoid DNAs have been proposed to play a role in centromeric function due to their location at the centromeric regions (Carine et al. 1989) and their sequence similarity with the functional centromeric (CEN) sequences in yeast (Fitzgerald-Hayes et al., 1982). The presence of alphoid DNAs on the short arms of the acrocentric chromosomes has led to proposals that these se-quences may also be facilitating Robertsonian translocations (Jorgensen et al., 1987; Choo et al., 1988). Specific patterns of alphoid DNA organization are predicted by these proposals, but there have not previously been detailed struc-tural studies that could support the suggested involvement of these sequences in the events of

segregation and recombina-tion. Structural study of the paracentromeric region may also ultimately lead to important information concerning possible causes for karyotype abnormalities and genetic disorders.

To study the organization of multiple alphoid families on a single chromosome, chromosome 21 has been selected, since a great deal of its basic structure is already known. Being the smallest chromosome in the human genome it has the largest centromeric DNA / non-centromeric DNA ratio possible, assuming each centromere is the same size (Cooke and McKay, 1978). The region of chromosome 21 involved in this study encompasses the area where Robertsonian translocations frequently occur which also form a significant basis for Down syndrome and other deleterious phenotypes (Stewart et al., 1988).

The present study examines the long range organization of various alphoid families in the total human genome and on human chromosome 21. The study involves determining whether members of these alphoid families are intermixed with each other or exist as separate independent clusters in the genome and particularly on chromosome 21. The location of these alphoid families with respect to each other and to the centromere on chromosome 21 was also examined.

From the results obtained it was concluded that the three alphoid families studied have a simple pattern of organization in the genome and that none of the families are significantly intermixed with each other, but rather exist as separate

independent clusters in the genome. On chromosome 21 the organization of the families is a small subset of that found in the genome, suggesting that these families have a chromosome-specific organization. These alphoid DNA sequences were found to be present in a limited number of clusters on chromosome 21. While one of the families (340 bp EcoRI) is present in a continuous block on the short arm of chromosome 21, another family (550 bp HindIII) is interspersed with some unrelated sequences within its clusters, and is present both on the short arm and in the centromeric regions. The overall clustered organization of these alphoid families is consistent with their proposed role in facilitating Robert-sonian translocations. However their organization is not consistent with any simple models for the role of alphoid sequences in centromeric function.

REVIEW OF LITERATURE

The centromeres of eukaryotic chromosomes are specific constricted regions along the chromatin fiber where the spindle fibers attach. They play a fundamental role in chromosome movement during cell division, ensuring proper distribution of replicated chromosomes. Human centromeric regions contain large amounts of repetitive DNA (Manuelidis, 1978). Repetitive DNA in humans is organized as either interspersed sequences or tandemly repeated sequences. Interspersed repeats exist as single copies present at various loci in the genome. Tandemly repeated sequences have a basic unit of repetition which is present as multiple adjacent copies. One of the major tandemly repeated DNA sequences present in humans is alphoid DNA, which is principally found at the centromeric regions (Manuelidis, 1978). Alphoid sequences are characterized by tandemly arranged monomeric repeats of approximately 170 bp (Wu and Manuelidis, 1980; Willard and Waye, 1987b).

Functional centromeric sequences (CEN) have been identified in yeast (S.cerevisiae), that are responsible for segregation of sister chromatids during cell division (Bloom and Carbon, 1982; Fitzgerald-Hayes et al., 1982; Carbon, 1984; Stinchcomb et al., 1982; Saunders et al., 1988). Some

sequence similarity has been found between the yeast centromeric region and human alphoid sequences (Fitzgerald-Hayes et al., 1982). A high degree of similarity has also been found both in repeat length and nucleotide sequence between alphoid repeats of lower primates and humans (Musich et al., 1980). This suggests an evolutionary relationship between satellite sequences in higher eukaryotes and functional centromeric sequences in yeast, and thus perhaps the involvement of alphoid sequences in centromeric function.

Alphoid sequences have also been found to be present on the short arms of acrocentric chromosomes, which are known to be very frequently involved in Robertsonian translocations. These translocations are recombinations between whole acrocentric chromosome arms (Therman, 1986). Because of its location, alphoid DNA has been proposed to play a role in facilitating Robertsonian translocations (Jorgensen et al., 1987; Choo et al., 1988).

Alphoid Repetitive DNA

Alpha DNA was first found in African green monkey where it comprises 15-20% of the total DNA (Singer, 1982). A basic repeating unit of 172 bp is observed when this DNA is digested with the restriction enzyme Hind III (Rosenberg et al., 1978). This sequence is organized into extremely long tandem arrays (Madhani et al., 1986).

Sequences very similar to alpha DNA have been found in all primates studied including humans (Wu and Manuelidis,

1980). The basic characteristics shared by all alphoid sequences are that they occur as tandemly arranged monomeric repeats of approximately 170 bp (Wu and Manuelidis, 1980) and that all families thus far isolated are located principally in centromeric regions (Manuelidis, 1978). There are at least 500,000 copies of this sequence in the total human genome (Schmookler-Reis and Goldstein, 1980).

In humans, alphoid DNA was originally identified as tandemly repeated 340 or 680 bp EcoRI fragments in which the 340 bp dimer was composed of two 170 bp monomer units (Wu and Manuelidis, 1980). A number of other sequence variants have been identified that apparently arose due to point mutations. These variants have been classified into families and subfamilies according to their degree of sequence relatedness (Willard and Waye, 1987b; Jorgensen et al., 1987). Two alphoid sequences are defined as belonging to separate families if they have a >25% sequence divergence with respect to each other, while members of the same subfamily will differ in sequence by no more than 4% (Willard, 1985; Jorgensen et al., 1986; Willard and Waye, 1987b). These alphoid sequences are distributed among many chromosomes but the copies on individual chromosomes tend to be more similar to each other than they are to copies on other chromosomes (Waye et al., 1987; Waye et al., 1987b).

To date, at least eleven different alphoid families have been identified, each consisting of multiple subfamilies

(Willard, 1985; Jorgensen et al., 1986; Willard et al., 1987; Willard and Waye, 1987b). Some of these families have been shown to be chromosome-specific (Waye and Willard, 1985; Waye and Willard, 1989) while a number are found on multiple chromosomes (Doering et al., 1988; Manuelidis, 1978; Jabs et al., 1984). Previous studies have emphasized that each human chromosome has its own specific alphoid family (Willard and Waye, 1987b). However this does not preclude the presence of additional alphoid sequences on a given chromo-some. For example, Carine et al. (1989) showed that four different tandemly repeated DNA sequences are present on chromosome 1. Three of these were different alphoid families. A study done by Waye et al. (1987) demonstrated the presence of two distinctly different alphoid sequences on chromosome 7. A recent study of chromosome 21 has demonstrated the presence of two families of alphoid DNA at its centromeric region and on the short arm, and a third family only on its short arm (Palamidis-Bourtsos, 1989). The detailed long range organi-zation of these multiple alphoid families on chromosome 21, or on any other chromosome, is still unknown.

To study the organization of multiple alphoid families on a single chromosome, human chromosome 21 has been chosen. Chromosome 21 is advantageous for such studies since a great deal of its basic structure is already known (VanKeuren et al., 1986; Choo et al., 1988b; Gardiner et al., 1988; Meijer et al., 1989). Also, since it is the smallest chromosome in

the human genome it has the largest centromeric DNA / non-centromeric DNA ratio possible, assuming each centromere is the same size (Cooke and McKay, 1978), and thus allows for easier analysis of alphoid sequences in the centromeric region.

Molecular Biology of Chromosome 21

Chromosome 21 is one of the five acrocentric chromosomes in the human genome, the other four being chromosomes 13, 14, 15 and 22. It has between 1000 and 2000 genes (Cooke and McKay, 1978) spread over 50,000 to 60,000 kb of DNA (Harris et al., 1986; Cooper and Hall, 1988). The short arm comprises 20,000-25,000 kb while the long arm is 30,000-35,000 kb in length (Gardiner et al., 1988). The phenomenon of non-disjunction that sometimes occurs at the centromere of chromosome 21 during meiosis results in a genetic disorder known as trisomy 21 or Down syndrome (Stewart et al., 1988; Cooper and Hall, 1988). Robertsonian translocations involving the short arm of chromosome 21 frequently occur and are also a significant basis for trisomy 21 and other deleterious phenotypes (Stewart et al., 1988). A number of studies indicate that only a discrete portion of the long arm of chromosome 21 (21q22.1-21q22.2) must be present in three copies in order to cause the Down syndrome phenotype (Stewart et al., 1988).

Five alphoid families have been found to be present on chromosome 21. Each of these families is also present on at least several other chromosomes in the genome. A detailed

description of each of these families is presented below.

1. 340 bp or 680 bp EcoRI family: This was the first alphoid sequence to be studied in humans. Digestion of the human genome with the restriction enzyme EcoRI reveals two prominent fragments of length 680 bp and 340 bp (Manuelidis, 1976; Manuelidis, 1978b). Sequence studies have shown two tandem subunits of 171 and 169 bp comprise the 340 bp dimer. The 680 bp repeat is composed of two 340 bp dimers in which the EcoRI site is missing at the junction (Wu and Manuelidis, 1980). The copy number of the 340-680 bp family is approximately 220,000 copies per haploid genome, with about 175,000 copies of the 340 bp repeat and 45,000 copies of the 680 bp tetramer (Schmookler-Reis and Goldstein, 1980; Palamidis-Bourtsos, 1989). This alphoid sequence constitutes about 2-3% of the total human genome (Manuelidis, 1978b; Schmookler-Reis and Goldstein, 1980) and is present on multiple chromosomes. It is particularly abundant at the centromeres of chromosomes 1, 3, 7, 10 and 19 and is also found at the centromeres of all the acrocentric chromosomes. In situ studies have suggested that telomeres of some chromosomes may also bear these sequences (Manuelidis, 1978b). The 340-680 bp EcoRI family consists of a number of variants which have been classified into subfamilies, defined by their sequence relatedness (Willard, 1985; Jorgensen et al., 1986; Jorgensen et al., 1987; Willard and Waye, 1987). At least five subfamilies of the 340-680 bp EcoRI family have been found on chromosome 21.

Three of these are "340" bp subfamilies while two of them belong to the "680" bp subfamilies. Each of these subfamilies has its own distinct pattern of organization both in the total genome and on chromosome 21. Despite their differences in organization all of these subfamilies share some characteristics, including a lack of variants containing internal HindIII sites, and presence of numerous higher multimers containing BamHI sites (Palamidis-Bourtsos, 1989). The total copy number of the 340-680 bp EcoRI family on chromosome 21 has been estimated to be approximately 8000 copies (Palamidis-Bourtsos, 1989).

Studies by Jorgensen et al. (1987) have shown that chromosomes 13, 21 and 22 have a common subfamily of the 340 bp EcoRI family that has diverged approximately 25% from the average sequence for this family. The sequences of the basic tetrameric unit of this alphoid family are indistinguishable on chromosome 13 and 21. Another subfamily is common to chromosomes 13 and 22 but absent from chromosome 21. These results suggest that homogenization may have occurred between each of two different blocks of alphoid DNA on the same chromosome (13) independent-ly with blocks of alphoid DNA on different chromosomes (21 and 22). This conforms to the general pattern of organization that has been proposed for alphoid DNA, i.e., large amplifica-tion units composed of a fixed order of several different but related smaller repeat units (Jorgensen et al., 1987).

2. BamHI 2.0 kb family: This family with BamHI sites regularly spaced 2.0 kb apart is largely specific to the human X-chromosome, and maps to the pericentromeric or proximal long arm region of the X-chromosome (Yang et al., 1982; Willard et al., 1983). Members of this family have also been shown to be present in other regions of the genome, including chromosome 1 (Carine et al., 1989) and chromosome 21 (Palamidis-Bourtsos, 1989). The 2.0 kb fragment contains twelve monomer units of 171 bp in length, each one having 68-73% homology to the 340 bp EcoRI consensus sequence (Yang et al., 1982; Waye and Willard, 1985). A number of sequence variants of the 2.0 kb fragment are found in the total genome and a subset of these is present on chromosome 21. While the 2.0 kb organization may be specific for the X-chromosome, on chromosome 21 this family is predominantly organized as 1.36 kb AluI and EcoRI repeats (Palamidis-Bourtsos, 1989). The 2.0 kb BamHI family has a different organization in the total genome and on chromosome 21 than does the 340-680 bp EcoRI family (e.g. variants containing internal HindIII sites can be seen in this family both in the genome and on chromosome 21). The copy number has been estimated to be 32,000 copies per haploid genome, with only 50 copies present on chromosome 21 (Palamidis-Bourtsos, 1989).

3. pHH550 family: The 550 bp HindIII family is a recently-isolated alphoid family (Doering et al., 1988). Members of this family are unusually heterogeneous in sequence

with variant sequence classes located in separate genomic domains. Restriction sites in pHH550 do not appear with a 170 bp spacing, but rather as non-integral multiples of the monomer repeat, further emphasizing the heterogeneity in the family. It is found on chromosome 21 as well as in other regions of the genome, and exhibits chromosome-specific organization. On chromosome 21 it is seen to be predominantly organized as 1.1 kb AluI and EcoRI fragments, but on other chromosomes many other variants have been observed (Palamidis-Bourtsos, 1989). The copy number for pHH550 has been estimated to be 44,000 copies per haploid genome, with 2500 copies present on chromosome 21 (Palamidis-Bourtsos, 1989). Hybridization analysis has shown that, in contrast to other alphoid families, pHH550 has a high degree of similarity to sequences found in all primates tested, including monkeys. A great degree of similarity has also been found between pHH550 and the human alphoid consensus sequence. These findings suggest that pHH550 is an evolutionarily old sequence closely related to the one from which other human alphoid sequences diverged (Carnahan et al., 1989).

4. p308: The p308 family is an alphoid sequence that is present on multiple chromosomes in the human genome. In situ hybridization studies have shown that the p308 family is present on centromeres of all human autosomes and the X chromosome. It is significantly enriched on chromosomes 1, 3, 12, 19, and is most prominent on chromosome 6. This repeti-

tive sequence is organized primarily as tandem 3 kb BamHI repeats containing one TaqI site, or else it is organized into BamHI and TaqI repeats of variable size that have some chromosome specificity (Jabs et al., 1984). Although variable in organization from one chromosome to another, p308 seems to be present in centromeric heterochromatin of essentially all human chromosomes (Jabs et al., 1984) indicating that a significant component of pericentromeric DNA is similar for all human chromosomes. This alphoid sequence family is evolutionarily conserved since sequences similar to p308 have also been found in the genomes of mouse leukocytes and fibroblasts, and some p308 repeats in the murine genome have been found to be present in a 3.0 kb BamHI and TaqI organization (Jabs et al., 1984). Studies with hamster-human hybrid cell lines containing only human chromosome 21 show p308 localized at the centromere. Cells containing fragments of chromosome 21 with its centromere hybridized with the probe p308, while cells containing fragments of chromosome 21 without a human centromere showed no hybridization to the probe (VanKeuren et al., 1986). Approximately 10^5 copies of this sequence have been estimated in the diploid genome (Jabs et al., 1984). Since p308 hybridizes weakly to 2.0 kb BamHI tandem repeats on the X chromosome, it has also been suggested that p308 is related to the X-enriched 2.0 kb BamHI repeat described by Yang et al. (1982) and Willard et al. (1983).

5. pTRA2 alphoid clone: Another recently isolated

alphoid family is represented by the pTRA2 clone. This clone of 3.9 kb was first obtained from a chromosome 21 library. It is found on all human chromosomes and is particularly abundant on the acrocentric chromosomes. The presence of this family preferentially on the acrocentrics suggest an evolutionary process consistent with recombination exchange of sequences between these nonhomologues. It has been suggested that during the nose-to-nose association or 'fusion' of acrocentric chromosomes during meiosis I, recombinational exchange between these nonhomologous chromosomes takes place (Choo et al., 1988). The study further suggested that such exchanges are more frequent among chromosomes 13, 14 and 21 than between chromosomes 15 and 22, since there are many more copies of the pTRA2 sequence on the former than the latter chromosomes. On chromosome 21, the higher order repeat unit of this family is the 3.9 kb HindIII cluster. This higher order repeat is also produced by restriction digests with RsaI and MspI. The copy number for this family on chromosome 21 has been estimated to be >100 (Choo et al., 1988).

Alphoid Centromeric Map of Chromosome 21

Recently, an alphoid centromeric map of chromosome 21 has been generated using somatic cell lines containing fragments of chromosome 21. Three families and five subfamilies of alphoid DNA have been located on the short arm and centromeric region of chromosome 21. Each of these families has a distinct pattern of organization on the chromosome. This

mapping showed that all members of the 340-680 bp EcoRI family on chromosome 21 are located on the short arm outside the centromere. While both the pHH550 and 2.0 kb BamHI families have some members located in the centromeric region, most are on the short arm outside the centromere (Palamidis-Bourtsos, 1989). As indicated above, the p308 family is located in the centromeric region of chromosome 21 (VanKeuren et al., 1986). All the families are completely absent from the long arm of chromosome 21 (Palamidis-Bourtsos, 1989). Since both the 2 kb BamHI and 550 bp HindIII families are present on the centromere and the short arm they cannot each be in one continuous block. This indicates that at least one of these families is present in more than one cluster (Palamidis-Bourtsos, 1989). The present study involves characterizing in greater detail the long range organization of these alphoid families on chromosome 21.

Pulsed Field Gel Electrophoresis

One way to study the detailed long range organization of alphoid sequences on a single chromosome and in the total genome requires the use of pulsed field gel electrophoresis (PFGE). It is imperative to have large fragments of DNA for analysis when studying long range organization, but conventional gel electrophoresis cannot resolve fragment sizes exceeding 50 kb. These high molecular weight fragments remain at the top of the gel as an unresolved fraction (Fangman, 1978). This size limitation is overcome by pulsed field

electrophoresis techniques which cause the larger molecules to periodically reorient from one electric field direction to another (Schwartz and Cantor, 1984; Carle and Olson, 1984). Here the electrophoretic mobility of DNA depends strongly on the pulse time (duration of applied field) and the nature of the applied field (uniform or non-uniform) (Schwartz and Cantor, 1984; Carle and Olson, 1984). The single most important determinant of mobility in pulsed field gel electrophoresis is the interval at which the direction of the electric field is switched. Schwartz and Cantor (1984) suggested that the time required for DNA to reorient when the field is switched is size dependent. Thus for any given switch interval, molecules smaller than a specific size will have time to both reorient and migrate under the influence of the new electric field. Since larger molecules take longer to reorient than smaller molecules, the fragment mobility becomes a function of size. Once reoriented, these molecules continue to move through the gel in an elongated conformation. This is not the case in regular gel electrophoresis where fragments longer than 50 kb migrate with size-independent mobilities, presumably because they present a cross-section to the gel matrix that is independent of their lengths. Furthermore, the mobility and the molecular size in PFGE are almost linearly related as compared to the logarithmic relationship between the two in conventional gel electrophoresis (Smith et al., 1987). The present study uses a specific kind of PFGE, the

Beckman Transverse Alternating Field Electrophoresis (TAFE) system, that allows study of fragments as large as 9000 kb (Avdalovic and Furst, 1988). In this system the alternating fields are orthogonal to one another but transverse to the plane of the gel so that each lane is subjected to the same electric field (Avdalovic and Furst, 1988).

PFGE has been used to resolve large domains of alphoid DNA generated by digestion with enzymes which cleave rarely within the genomic DNA and even more infrequently in alphoid DNA. Thus, macro-organization of human centromeric regions has been examined using this method (Jabs et al., 1989). The most striking characteristics observed for the human centromeric regions were their chromosome-specific macro-organization and high frequency of macro restriction fragment length polymorphisms (RFLPs) (Jabs et al., 1989).

Long-Range Organization of Alphoid DNA

Several studies have been done on the long-range organization of particular alphoid families. In some cases members of a given alphoid family are present in large clusters, uninterrupted by other sequences, including members of other alphoid families (Tyler-Smith and Brown, 1987; Jorgensen et al., 1987; Waye and Willard, 1989). Other investigations indicate that alphoid sequences family members are not found in a single continuous block but exist in multiple clusters (Choo et al., 1987; Palamidis-Bourtsos, 1989).

In one investigation the 2.9 kb alphoid repeat specific to chromosome 3 has been found to contain 17 tandem monomers of approximately 171 bp each. The study demonstrated that the chromosome 3 higher order repeat units are localized in large domains, at least 1000 kb in length. Each of these arrays comprises 500 or more copies of an approximately 2.9 kb tandem repeat or a 2.55 kb higher order repeat unit. In this study some RFLPs were detected by PFGE, and these may be due to variability in the length of the domains containing these repeats (Waye and Willard, 1989).

Another study has been carried out involving the alphoid family found on the Y chromosome. In this study organization of the alphoid DNA was determined on each of the Y chromosomes present in two somatic cell hybrids. In each a simple major block of alphoid DNA is present, approximately 475 kb long on one chromosome and about 575 kb on the other. The structure of the two blocks was found to be very similar, although restriction enzyme sites for AvaII are present on one block but not on the other. The alphoid DNA within each block is organized into tandemly repeated units, most of which are about 5.7 kb long and some variants about 6.0 kb in length. These 5.7 kb and 6.0 kb units are made up of tandemly repeated 170 bp units (Tyler-Smith and Brown, 1987). Thus this study suggests that long range polymorphisms of tandemly repeated sequence families may be frequent.

Long-range organization of alphoid families on chromosome

1 has been recently studied, and in this case alphoid sequences are not found in a single continuous block but exist in multiple clusters. A study done by Carine et al. (1989) examined the amounts of four different human tandemly repeated DNA sequences in a series of human-hamster hybrid cells containing fragments of human chromosome 1. These minichromosomes had suffered deletions extending into the pericentromeric and centromeric regions that contain the tandemly repeated DNA sequences. In most cases the deletions resulted in loss of portions of several different tandem families rather than the complete loss of any one family, thus indicating interspersion of blocks of tandem repeats. These results were further confirmed by PFGE. Several large restriction fragments from one of the hybrid cell lines were found to hybridize with both satellite III DNA and alphoid EcoRI dimer, indicating that the two families of tandem repeats were interspersed (Carine et al., 1989).

Another study by Choo et al. (1987) indicated that the alphoid sequence predominantly found on chromosome 17 is arranged in multiple clusters rather than a continuous block. The higher order organization of this sequence was studied using a series of cosmid clones. Different arrangements of alphoid and non-alphoid DNA were observed in different cosmids. One group of them contained only alphoid DNA. Another contained alphoid DNA at one end and non-alphoid DNA at the other. A third group contained alphoid DNA at both

ends with non-alphoid DNA in the middle. This collection of cosmid clones containing a total of 374 kbs of insert was found to contain a total amount of 20-23% of non-alphoid DNA. This suggested that the human alphoid DNA arrays are relatively frequently interrupted by other non-alphoid DNA. Since none of the clones contained alphoid DNA flanked on both sides by non-alphoid DNA, it was inferred that relatively short alphoid arrays are not common. The sizes of the interspersed non-alphoid DNA regions were found to be heterogenous in size ranging from 6-24 kbs. Thus, this alphoid DNA consists of multiple clusters with an irregular and complex pattern of organization (Choo et al., 1987).

Evolution of Alphoid DNA

The repetitive nature of the alphoid sequences suggests that amplification of short ancestral repeats was the mode of origin of alphoid DNA. Saltatory replication and unequal recombination are the two processes that have been proposed to account for this amplification. The general principle of the saltatory model is that at various times a group of repeating units may be suddenly amplified laterally to generate a large number of identical tandem copies. Then the identity of the copies is lost as mutations accumulate in them. The two steps proposed for generating the 340 bp EcoRI dimer are: 1) the generation of a dimer whose subunits diverged and 2) amplification of this dimer template (Wu and Manuelidis, 1980; Dover, 1982; Waye and Willard, 1985; Lewin, 1987). A dimer composed

of two variant monomers may occur in a number of possible ways (Wu and Manuelidis, 1980). An ancestral 170 bp sequence is redundantly replicated to give a dimer and the units of this dimer with time acquire sequence divergence. A second possibility is that two repeated interspersed variant copies of a sequence are recombined by unequal crossing over. A third possibility is that tandem variants within the genome recombine to give the dimer with base variation in each subunit. Amplification takes place as the final event in all cases, producing long tandem arrays of alphoid DNA. Such continued rounds of saltation could result in a chromosome-specific alphoid family.

An alternative to saltation for amplification of the alphoid sequence is unequal crossing over (Smith, 1976). In this recombination event the two recombining sites are present at non-identical but analogous locations in the two parental repetitive DNA arrays. The assumption is that unequal crossing over occurs frequently between homologues. Crossovers can occur much more frequently than usual in long tandem arrays since they have extensive regions of homology in each of many different alignments. This mechanism will result in an expansion of established arrays and a spreading of variant sequences along tandem arrays at a rate which is much faster than, and independent of, the mutation rate. Starting with a sequence of DNA in the genome that is not maintained by natural selection, this process will act to impose uniformity

or homogenization on the tandem array involved, resulting in repeats with a single sequence. Once a region has become repetitious, mutations occurring will be either eliminated or expand to occupy the entire sequence (crossover fixation). (Smith, 1976).

A second prediction of the unequal recombination model is that sequence uniformity or homogeneity can spread only through a single linkage group. This means that alphoid sequences of non-homologous chromosomes must evolve independently by crossover fixation, as they cannot recombine with one another, resulting in the formation of chromosome-specific alphoid families. Also a recent mutation within an alphoid sequence spreads initially through copies close to the origin of the mutation, by the process of crossover fixation. Thus chromosome-specific domains, as well as domains within a particular chromosome, are possibly a result of a mutation that has spread from its original site of occurrence to adjacent areas but has not filled the entire block. Therefore, if unequal recombination is a significant process in the maintenance of homogeneity, then repeats near the ends of the block would exhibit more divergence (Devilee et al., 1986; Jorgensen et al., 1987).

Satellite associations(SA) and nucleolus organizing region (NOR) associations are other phenomena that may play significant roles in the genomic evolution of alphoid sequences. Satellite associations are obvious during mitotic

and meiotic metaphases. The centric regions of the acrocentric chromosomes are brought together, giving a chance for chromosome material to exchange (Hansson, 1979). NOR associations have been seen in the metaphase stage of cell division presumably as a result of the fact that all nucleolus organizer regions get together to participate in the formation of the nucleolus (Jackson-Cook et al., 1985). Here again the possibility for genetic exchange exists. Thus there appears to be a good deal more "cross talk" among the acrocentric, NOR bearing chromosomes than is possible for other chromosomes (Jackson-Cook et al., 1985). These phenomena could play a role in amplification of alphoid sequences by moving them between acrocentric chromosomes. Hence it would not be surprising to find similar alphoid sequences on the different acrocentric chromosomes, and indeed one example of this has been observed (Jorgensen et al., 1987).

Possible Function of Alphoid DNA

The function of alphoid DNA is at present unknown. However there are some theories regarding its possible role in centromere function and its organization-dependent facilitation of Robertsonian translocations (Carine et al., 1989; Stewart et al., 1988). Due to the location of alphoid DNA at the centromere (Manuelidis, 1978) and its sequence similarity with the functional CEN sequence of the yeast S.cerevisiae (Fitzgerald-Hayes et al., 1982), alphoid sequences have been proposed to play a role in centromere function. If this is

so, then information about their organization may suggest the manner in which they function (Figure 1). If the different alphoid families are clustered into independent blocks at the centromere then each block may have a separate role to play in centromere function as a whole, or some of these blocks may not even play any significant role at all. On the other hand if these families are interspersed with each other in multiple blocks, then the centromere may contain redundant functional units composed of several alphoid families (Carine et al., 1989).

The short arm region of chromosome 21 that is being examined in the present study is known to be frequently involved in Robertsonian translocations. These translocations are recombinations of whole chromosome arms between acrocentric or telocentric chromosomes. Robertsonian translocations, as a rule, are the result of an exchange in meiosis or mitosis, and not of breakage and rejoining at fragile sites (Therman, 1986). Various hypotheses have been put forward to explain the relatively high incidence of Robertsonian translocations involving the different acrocentrics. One view is that the centric heterochromatin and satellite stalks are prone to breakage (Therman, 1986). Another proposal is that chromosome exchange takes place through the participation of acrocentric chromosomes in satellite and NOR associations, which play a significant role in bringing together the centric regions of the acrocentric chromosomes (Therman, 1986). Hence

if the alphoid families exist in long separate clusters in the centromeric and short arm regions of acrocentric chromosomes, then it is highly probable that they play a role in any out-of-register recombination (Jorgensen et al., 1987; Choo et al., 1988), that may occur during such associations. Referring to Figure 2A it is apparent that even if the two chromosomes are not aligned and are out-of-register, there is still a possibility for cross-over between homologous regions. Alternatively if these families exist interspersed with each other then they are less likely to facilitate out-of-register recombination or Robertsonian translocation (Figure 2B), since the homologous regions no longer overlap when the two chromosomes lie out-of-register.

The present study will help to further elucidate overall alphoid organization and so clarify its possible involvement during the events of chromosome segregation and recombination. This structural study of the centromeric region may thus ultimately lead to important information concerning possible causes for karyotype abnormalities and genetic disorders.

MATERIALS AND METHODS

Sources of DNA

Total genomic DNA was obtained from the normal diploid human fibroblast line 3348B (received from N.I.G.M.S. Human Genetic Mutant Cell Repository). Cells from passage thirteen through passage twenty were used in the study. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO) and antibiotics (GIBCO penicillin-streptomycin mixture at a concentration of 50 units/ml of penicillin G sodium and 50 mcg/ml of streptomycin sulfate). Cells were maintained at 37^0C in approximately 5% CO_2.

Chromosome 21 DNA was studied using Chinese hamster-human hybrid cell lines where chromosome 21 material is the sole human component (received from Dr. M. Cummings at University of Illinois, Chicago). The cell line 153-E9A was derived from the fusion of the auxotrophic hamster cell line ade$^-$C with human lymphocytes (Moore et al., 1977). The parental hamster cell line lacks GARS (glycineamide ribonucleotide synthetase) and thus requires purines for normal growth (Patterson et al., 1981). The 153-E9A line was chosen due to its ability to grow on hypoxanthine-free medium, and in order to retain the human

chromosome 21, the cell line is grown under selection. It is routinely grown in purine- and/or hypoxanthine-free medium such as alpha MEM (Minimal Essential Medium) or DMEM containing 5-10% dialyzed fetal bovine serum, and antibiotics (Sigma antibiotic/antimycotic mixture, at concentrations recommended by the vendor). This cell line has been cytogenetically characterized and found to contain the full chromosome 21 (Moore et al., 1977).

The cell line 153-E7BX was also derived from the fusion of the auxotrophic hamster cell line ade$^-$C with human lymphocytes. This cell line is a sister cell line of 153-E9A, and contains the long arm and the centromere of chromosome 21 translocated to a CHO (Chinese hamster ovary) chromosome. To retain this portion of human chromosome 21, the cell line must be grown under selection, and is routinely cultured in the same medium as cell line 153-E9A.

The cell line 2Fu'1 was derived from the fusion of the auxotrophic hamster cell line Urd$^-$C and human lymphocytes (Patterson et al., 1983). This cell line contains the long arm of chromosome 21 translocated to a CHO chromosome (Patterson et al., 1983; Jabs et al., 1984). The short arm of chromosome 21 and most or all of the centromeric material are absent in this cell line (Patterson et al., 1983; VanKeuren et al., 1986). Although there is a uridine requirement for growth, there is no selection used to maintain the human chromosome fragment in the cell line, and the line is karyo-

typically stable. It is routinely maintained in Ham's F12 medium supplemented with 5-10% calf serum, uridine ($3x10^{-5}$M/ml),and antibiotics (Sigma antibiotic/antimycotic mixture, using a concentration recommended by the vendor).

All three hamster/human hybrid cell lines used in this study are sensitive to overcrowding and die soon after reaching confluence. Thus, they are passaged or harvested prior to becoming confluent. All of these cell lines have been recently characterized and found to have the expected karyotypes.

All tissue culture work was done under aseptic conditions to maintain sterility.

Preparation of DNA samples

Sterile conditions were maintained throughout the preparation and treatment of DNA. All solutions were autoclaved and glassware or plasticware was sterile.

Medium was removed from cells growing in flasks and the monolayers were washed with PBS (8 g/liter NaCl, 0.2 g/liter KCl, 1.15 g/liter Na_2HPO_4, 0.2 g/liter KH_2PO_4, pH 7.0-7.2). Then cells were harvested by incubation for 10-20 minutes at 37^0C in a 0.06% trypsin solution (2.5% stock, Gibco Laboratories), diluted in PBS. The cells were pelleted, counted and resuspended in PBS to a concentration of 8-10 x 10^6cells/ml. The cell suspension, maintained at 45^0-50^0C, was mixed with an equal volume of 2% LMP agarose (Low Melting Point, Beckman) in

PBS, that was also at 45^0-50^{0}C. This mixture was pipetted into a gel mould and left on ice for 1-2 hours to let the agarose gel. The plugs formed were 0.2cm X 0.2cm X 2.5cm, and each contained five gel lanes worth of DNA. The final amount of genomic DNA was 0.7 ug/lane and hybrid cell DNA was 0.8 ug/lane. The greater amount of hybrid cell DNA was required to detect the lower amounts of alphoid DNA in these cell lines relative to genomic DNA.

The cells in the plugs were then lysed to release DNA into the agarose. The plugs were incubated in five volumes of ES (0.5M EDTA, pH 9,with 1% sarkosyl) at 50^{0}C for 1-3 hours, and then for four days with gentle shaking at 50^{0}C in five volumes of fresh ES containing 2 mg/ml proteinase K (Bethesda Research Laboratories, BRL). This latter solution was changed once every 24 hours. Then plugs were rinsed twice for 15 minutes each with five volumes of ES at room temperature. Plugs were next given four washes of 1-2 hours each at room temperature in 20-25 volumes of $T_{10}E_1$ (10mM Tris, 1mM EDTA; pH 7.4) containing 1mM PMSF (phenyl methyl sulfonyl fluoride), by agitating gently on a rotator. The plugs were then rinsed with several changes of distilled water at room temperature and then with 20-25 volumes of $T_{10}E_1$ (pH 7.4), at least three times over 2-3 hours at 4^{0}C. One final overnight wash was done in $T_5E_{0.5}$ (5mM Tris, 0.5 mM EDTA; pH 7.4) at 4^{0}C. The plugs were stored in $T_5E_{0.5}$ at 4^{0}C until used.

Restriction Enzyme Digests

Restriction enzymes were purchased from Bethesda Research Laboratories (BRL), and the buffers recommended by the manufacturer were used for the digestion reactions. All the enzymes used are known not to cut within the alphoid sequences, thus generating fragment lengths of at least 30 kb. The digestion conditions involved incubation of plugs in 5 volumes of 1x restriction buffer, containing 50 ug/ml BSA (bovine serum albumin) and 7mM B-mercaptoethanol, with 30-40 units of restriction enzyme per lane of DNA. The digestions were incubated for 15 hours at 37^{0}C. The enzyme reactions were stopped by first rinsing the plugs at room temperature twice for 15 minutes each with at least 1 ml of $T_{10}E_1$ (10mM Tris, 1mM EDTA; pH 8.4) containing 0.5% N-lauroyl sarkosyl and then three times for 20-25 minutes each in $T_{10}E_1$ (pH 8.4). Double digests involved digesting with two different enzymes separately on two sequential days under the same conditions described above. After the first digestion, the plugs were rinsed with $T_{10}E_1$, pH 8.4, alone to remove the first enzyme. The second enzyme was then added with its own reaction buffer and the plugs incubated for another 15 hours, after which the reaction was stopped as described above.

Standard Gel Conditions and DNA Transfer

One percent LE agarose (Beckman Gene Line) gels were made in TAFE buffer (10 mM Tris, 0.5 mM EDTA, 43.5 mM acetic acid),

and then cooled to 4^0C to mould. The running buffer (same as gel) was cooled to 15^0C. DNA containing plugs were cut into 0.5 cm long pieces, and gently pushed into the gel lanes with a pipette tip. The wells were sealed with 1% LE agarose. The gel was placed in the electrophoresis box containing the precooled buffer. A ramped protocol was used for running the gel. The run comprised of two stages: the first was 30 minutes long with a current of 170 mA and 4-second pulse time, to drive the DNA into the gel; the second stage extended over 20 hours with a current of 150 mA and pulse time of 60-seconds (Avdalovic and Furst, 1988b). The pulsed field gel system used here is the Gene Line System from Beckman.

Yeast (Saccharomyces cerevisiae) chromosome markers (Beckman) were run in every gel to calibrate the fragment sizes in the sample lanes. The yeast chromosomes range in size from 260-1500 kb (Figure 3).

Gels were stained with ethidium bromide, photographed (Figure 3) and then the DNA from the gels was transferred to nylon membranes (Gene Screen Plus hybridization transfer membranes; NEN Research Products) by the alkali Southern blot method (Reed and Mann, 1985).

DNA Probes and Hybridization

Three different human alphoid repetitive DNA sequences, cloned in pBR322, were used for these studies: 1. pHE340-9 is a standard 340 bp EcoRI alphoid repeating sequence (Doering et

al., 1986). This family is found on multiple chromosomes in the human genome (Manuelidis, 1978b). 2. pHH550-31 is a 550 bp long repeat and represents the 550 bp HindIII family. The family has been found to be present on chromosome 21 as well as other chromosomes (Doering et al., 1988). 3. pXBRI is a BamHI 2.0 kb alphoid repeat primarily found on the X chromosome (Yang et al., 1982). The human LINES-1 (long interspersed repetitive sequences) family was represented by the probe pHK 1.8-44, a 1.8 kb KpnI fragment, cloned in pBR322. This sequence has previously been shown to be found on many chromosomes but only on the short arm of chromosome 21 (Doering et al 1988b). All probes were labelled with ^{32}P by nick-translation (Rigby et al., 1977) to an average specific activity of 2.14×10^8 cpm/ug.

The blots were prehybridized at 37^{0}C for 5 hours in hybridization solution (50% Formamide; 1.0 M NaCl; 50 mM Tris, pH 7.5; 1% sodium dodecyl sulfate - SDS; 10 ug/ml heat denatured E.coli DNA) and then heat denatured probe was added to a concentration of 6 ng/ml and hybridization proceeded at 37^{0}C overnight.

The membranes undergoing standard washes were washed twice at room temperature for 10 minutes with 2X SSC (1X SSC is 150 mM NaCl, 15 mM sodium citrate, 0.1 mM EDTA), twice at 60^{0}C for 30 minutes with 2 X SSC plus 1% SDS and twice at room temperature for 30 minutes with 0.5X SSC. This allowed a mismatch of 16% for the pHH550-31 probe, and 17.2% for the

pHE340-9 clone. The hybridization conditions used for the pXBRI probe were more stringent, allowing only a 4.5% bp mismatch. In this case hybridization and the first and third sets of washes were done under standard conditions, but the second set of washes was done with 0.1 X SSC plus 1% SDS at 65^{0}C. Membranes were air dried slightly and autoradiographed using Kodak XAR film and Cronex intensifying screens. Exposure times depended on the probe being used. Blots probed with pHE340-9 and pHH550-31 required an exposure time ranging from 1-3 days with a single intensifying screen while with probe pXBRI the time required was about 2-2.5 weeks with two intensifying screens.

In some instances, after an exposure was obtained, the blot would be stripped of the probe and rehybridized with another (Figure 4). These blots were stripped by either of two methods: 1. Incubated in 0.4 M NaOH at 42^{0}C for 30 minutes, followed by a wash in 0.1X SSC, 0.1%SDS, 0.2 M Tris pH 7.5 at 42^{0}C for an additional 30 minutes. 2. Boiling method: The blots were boiled for 30 minutes in a bag containing 10mM Tris (pH 7.5), 1mM EDTA and 1% SDS. In both cases, the blots were then air dried slightly and autoradiographed using Kodak XAR film and intensifying screens to make sure that no residual probe remained on the blot before rehybridizing with another probe.

RESULTS

Selection of Appropriate Enzymes

To study and compare the macromolecular organization of alphoid DNA in the whole genome and on chromosome 21 it is imperative to have large pieces of DNA for analysis. Determining whether one or more alphoid families are in the same region on a single chromosome and characterizing their location with respect to each other and to the centromere all requires the chromosome to be cut into a small number of fragments that are large in size. Thus the initial part of the study was focused on selecting appropriate restriction enzymes that would create a limited number of alphoid sequence-containing fragments of size greater than or equal to 50 kb.

Total genomic DNA from fibroblast cells was digested with a number of different restriction enzymes. These enzymes have been previously shown to cut rarely within the alphoid families, and thus yield alphoid-sequence containing fragments greater than 30 kb in size (Palamidis-Bourtsos, 1989). Restriction enzymes tried were BamHI, HindIII, PstI, PvuI, ClaI, KpnI, NotI, MluI, SfiI, and SstII. Digestions with the enzymes SstII, NotI, MluI, and SfiI resulted in alphoid sequence-containing fragments greater than 1500 kb when either

the pHE340-9 or pHH550-31 probes were used. Therefore most of the alphoid DNA remained in the unresolved fraction at the top of the gel (Figure 5). Hence, these enzymes were not used in future experiments. Digestion of genomic DNA with KpnI resulted in degradation of the DNA. A control sample with only KpnI reaction buffer and no enzyme also led to degradation, indicating that the buffer itself was activating some nucleases still present in the DNA plugs. Thus, KpnI was also rejected from the list of enzymes for genomic digestion. Digestion of DNA from hamster-human hybrid cells with KpnI did not show any degradation, apparently because of fewer nucleases present in those DNA samples (see below). When genomic DNA was digested with the enzymes BamHI, HindIII, PstI, PvuII or ClaI, 5-20 bands were detected by each of the three alphoid probes. These bands ranged in size from 50 to 1400 kb (Figure 5; Table I). Some of the bands were very intense while others were relatively faint. The degree of intensity of a band normally indicates the copy number of the alphoid family on the fragment. A small amount of each alphoid family is seen to be present in the unresolved fraction in many of the enzyme digests (Figure 5; Table I).

Several examples of genomic DNA digested with various restriction enzymes and hybridized with alphoid probes are shown in Figure 5. ClaI and PvuII digests of genomic DNA yield a very small number of fragments containing alphoid families. Hybridization with the pHE340-9 probe reveals 5-6

prominent bands which range in size from approximately 250 to 1000 kb. Some other minor bands are also detected (Figure 5; Table I). BamHI and PstI digests on the other hand create a larger number of fragments containing alphoid sequences. As seen in Figure 5, hybridization with the probe pHH550-31 reveals 9-10 bands in BamHI and PstI digests. Most of these bands are equally intense, ranging in size from approximately 200 to 1400 kb (Figure 5; Table I). Digestion of genomic DNA with HindIII creates at least seven fragments that hybridize to the probe pXBRI. Most of these fragments are relatively low in molecular weight, ranging in size from about 100 to 1000 kb (Figure 5; Table I).

Organization of Various Alphoid Families in the Total Genome

Members of these three alphoid families could be intermixed with each other in the genome or exist in separate independent clusters. In the former case, some of the same restriction fragments would be observed to hybridize with more than one alphoid probe. On the other hand, if the alphoid families are present as separate clusters, then the different alphoid probes would detect fragments of entirely different sizes in any particular enzyme digest (Figure 4). Therefore, to determine the organization of the alphoid families the restricted DNA on the filters was hybridized with the probe for one family, that probe removed and then the filter rehybridized with another probe. The banding patterns obtain-

ed by hybridization with each of the three different family probes were then compared.

A BamHI digest hybridized to the probe 340-9 reveals a number of bands of equal intensity, ranging in size from 110 to 1220 kb (Figure 6; Table I). The same digest on hybridization with the probe pHH550-31 contains relatively fewer bands, ranging in size from approximately 300 to 1290 kb (Figure 6; Table I). The two alphoid probes do not detect any high molecular weight bands which are the same size, indicating that members of the two families are not significantly intermixed (Figure 6; Table I). The organization of these two families was then compared to that of the 2kb BamHI family. In this case the family is seen to be contained in a few fragments, very different in size from those observed with the pHE340-9 or pHH550-31 probes. These range in size from about 250 to 1250 kb (Table I). Thus, all three alphoid families seem not to be significantly intermixed with each other but rather exist in separate clusters.

A PvuII digest when hybridized with the probe pHE340-9 reveals 6-7 prominent bands and a few minor ones. Fragments range in size from 265 to 1065 kb (Figure 7; Table I). The same digest probed with pHH550-31 has 6-7 predominant fragments of sizes ranging from 260 to 1440 kb (Figure 7; Table I). The banding patterns with these two alphoid family probes are distinctly different (Figure 7; Table I). Thus the PvuII digest confirms that these two alphoid families are not

significantly intermixed with each other (Figure 7; Table I).

Total genomic DNA digested with HindIII and hybridized to the pHE340-9 probe shows a number of bands of equal intensity. The fragments range in size from 170 to 1300 kb (Figure 8; Table I). The same digest when probed with pHH550-31 showed an entirely different banding pattern. The number of bands was about the same as seen with the pHE340-9 probe, but their size ranged from 135 to 1300 kb (Figure 8; Table I). Hybridization with the pXBRI probe reveals at least seven fragments in this digest, most of which are of relatively low molecular weight. The bands obtained with this probe were different in size from those obtained with either the pHE340-9 or pHH550-31 probes (Figure 8; Table I). Thus, with HindIII digests too, members of the three alphoid families do not appear to be significantly intermixed with each other in the total genome.

The banding pattern obtained with all three alphoid probes was observed to be the same in genomic DNA from cells of culture passages 13 to 18. This indicated that there is no major loss or reorganization of these repetitive sequences over at least five passages. An example is a HindIII digest of genomic DNA obtained from either passage 13 or passage 18 (Figure 9); both of these samples had identical banding patterns when hybridized to the probe pHH550-31. Another example is a BamHI digest of DNA obtained from either passage 14 or 18; both samples revealed identical banding patterns on hybridization with the probe pHE340-9 (Figure 9). This result

is contradictory to that of Schmookler-Reis and Goldstein (1980), where a decrease in copy number of the 340-680 bp EcoRI family was reported during serial passage of human diploid fibroblasts. They reported a total decrease of approximately 23% in the number of repeats in this family over the course of 21 population doublings (Schmookler-Reis and Goldstein, 1980). If there was any loss or reorganization of alphoid DNA during the cell passages used in the present study, a loss of fragments or a change in their size would have been observed. Even though the total number of population doublings used in the present study was about one half of those used by Schmookler-Reis and Goldstein, some detectable loss of these sequences might have been expected, if the rate of loss is constant with passage number.

Organization of Various Alphoid Families on Chromosome 21

Comparison of organization of three alphoid families in the genome and on chromosome 21

Digestion of total genomic DNA with various restriction enzymes revealed a simple banding pattern with all three alphoid probes. From this it can be concluded that all three families have a simple organization in the total genome. This could mean that these alphoid families are present on a small number of chromosomes, that each have their own distinct clusters, or on many chromosomes whose multiple alphoid clusters are all of approximately the same size. These possibilities can be distinguished by examining the organi-

zation of alphoid families on a single chromosome (chromosome 21 in the present study). If the alphoid families are present as a few distinct clusters on each chromosome then on chromosome 21 an alphoid probe would detect only a few of the bands observed in the total genome. On the other hand, if the same number of clusters of approximately the same size are present on all the chromosomes, then when DNA digests of chromosome 21 are hybridized with each probe a banding pattern will result that is not significantly different from that seen in the total genome. Hence the banding pattern for a single chromosome would not be a subset of the banding pattern of the total genome.

A BamHI digest of genomic DNA hybridized to the probe pHE 340-9 revealed about fifteen bands, while only two of these were detected on chromosome 21 (153-E9A DNA). These two fragments on chromosome 21 have relatively low molecular weights of sizes 100 and 200 kb. A significant fraction of this family on chromosome 21 is seen to remain unresolved at the top of the gel (Figure 6; Table I), and also a number of smaller fragments, less than 100 kb in size, that run off the gel in PFGE are known to exist (Palamidis-Bourtsos, 1988). PstI and HindIII digests of chromosome 21 DNA show the presence of this alphoid family on only 6-8 fragments as opposed to the numerous fragments seen in the total genome. The most predominant fragments in the PstI digest are 365 and 420 kb in size (Figure 10; Table I). Two other prominent

fragments are of sizes 630 and 610 kb. These fragments are not seen in the genomic set of bands. This can be explained by the presence of polymorphisms since 3348B and 153-E9A are not sister cell lines. A single site mutation might have created an RFLP. In the HindIII digest, 5 of the 8 fragments seen on chromosome 21 are relatively more prominent. These range in size from 270 to 575 kb. In this digest also one polymorphic fragment is observed of size 1390 kb which falls between the 1285 and 1460 kb fragments in the genomic DNA. Even clearer demonstrations that the 340 bp EcoRI family organization on chromosome 21 is a small subset of that in the genome are seen with ClaI, KpnI and PvuII digests. In these enzyme digests the family is seen to be present on only one or two high molecular weight fragments of chromosome 21, ranging in size from approximately 250 to 1200 kb. (Figure 11; Table I). On the other hand, in the genome, ClaI digests show 5-6 prominent bands and PvuII shows 6-7 bands with the pHE340-9 probe. A small amount of the family is seen to be present in the unresolved fraction of all enzyme digests. Since polymorphisms were not seen with most enzyme digests it can be concluded that the polymorphisms observed have arisen due to point mutations and not large deletions. In the latter case variable cluster lengths would have been observed in all enzyme digests. From the results obtained particularly with the enzymes that cut rarely (ClaI, KpnI and PvuII) it is apparent that the 340 bp EcoRI family is present in a limited

number of clusters on chromosome 21 that are a subset of that found in the total genome.

The organization of the 550 bp HindIII family on chromosome 21 is also a small subset of that found in the total genome. This is well illustrated by the example of a BamHI digest of genomic and chromosome 21 DNA hybridized to the pHH550-31 probe. The family is seen to be present on only three fragments of chromosome 21, as opposed to the numerous fragments in the genome. The sizes of these fragments are 350, 700, and 1200 kb (Figure 6; Table I). In ClaI and KpnI digests of chromosome 21 the family is found on single fragments of sizes 1240 and 1355 kb respectively (Figure 12; Table I). Chromosome 21 DNA digested with enzymes like PstI, HindIII and PvuII show relatively more fragments (2-5) bearing the family but still less than that seen in the total genome. The most predominant fragments in the PstI digest are 880 and 920 kb in size. These fragments are not seen in the genomic set of bands and therefore, are probably also polymorphisms. HindIII and PvuII digests reveal that the family is present on 4-5 fragments which range in size from 250 to 1000 kb. On the other hand in a HindIII genomic digest 12-15 fragments are seen and in a PvuII genomic digest 6-7 fragments are observed (Figures 8 and 11, Table I). A small amount of the family is seen to remain unresolved in all enzyme digests. Again, since most of the enzymes do not show polymorphic fragments there is no length variability in this family either, and polymorphisms

seen in the PstI digest must be due to point mutations in either of the non-sister cell lines, i.e., 3348B and 153-E9A. The small number of fragments seen, particularly with the rare cutters (ClaI, KpnI, PvuII), suggests that the 550 bp HindIII family is organized in a limited number of clusters on chromosome 21.

The organization of the 2 kb BamHI family on chromosome 21 could not be detected by our methods. The amount of DNA used in each sample lane was not sufficient to detect the low copy number of this family on chromosome 21 under the high stringency conditions used (Materials and Methods).

Presence of two alphoid families as separate domains/clusters on chromosome 21

Since the different alphoid families being studied were observed not to be significantly intermixed with each other in the total genome, the same was expected to be true for chromosome 21. This was confirmed by hybridizing digests of the hamster-human hybrid cell line 153-E9A DNA with the probe for one family, removing the probe and rehybridizing with another probe. Examples of these results are described below.

A BamHI digest of the 153-E9A DNA containing human chromosome 21 was probed with pHE340-9. Two bands were detected each of low molecular weights (110 and 200 kb). The same digest probed with pHH550-31 revealed three high molecular weight fragments of sizes 350, 700, and 1200 kb. Thus the banding patterns obtained with the two probes are

entirely different (Figure 6; Table I). In ClaI digests the probe pHE340-9 detects two fragments of sizes 850 and 1000 kb, while only one fragment of size 1240 kb is seen with the probe pHH550-31 (Figure 12; Table I). Similarly, two fragments of sizes 880 and 1160 kb are detected by the probe pHE340-9 in a KpnI digest while only one fragment of size 1355 kb is seen with the probe pHH550-31. Several other enzymes confirmed these results indicating that the two families are present on separate fragments (Figures 6, 7, 8, 10; Table I). All enzyme digests have varying amounts of alphoid DNA in the unresolved region of the gels (Figures 6, 7, 8, 10).

From the above observations it was concluded that on chromosome 21 the two different alphoid families, 340 bp EcoRI and 550 bp HindIII, are not significantly intermixed but rather exist in separate independent clusters.

Organization of two alphoid families on chromosome 21 with respect to the centromere

In order to determine the location of each alphoid family on chromosome 21 with regard to the centromere, and to determine whether these families were present in single or multiple clusters on the chromosome, three different hybrid cell lines were used. The different cell lines carry copies of chromosome 21 that have deletions in the short arm and the centromeric regions (see Materials and Methods for details). If an alphoid family is present on just the short arm region of chromosome 21 then DNA from cell line 153-E9A (containing

the full chromosome 21) would hybridize with that probe, while DNAs from cell lines 153-E7BX (lacking the short arm region of chromosome 21) and 2Fu^{r}1 (lacking the short arm and the centromeric regions of chromosome 21) would not hybridize to the probe. If the family is present on the short arm region and the centromeric region of chromosome 21 then the probe for that family will hybridize to DNA from cell lines 153-E9A and 153-E7BX but not 2Fu^{r}1. And finally, if the family is present on the long arm as well as the short arm then DNA from all three cell lines would hybridize to that probe.

If the family is present in a continuous block extending from the short arm into the centromeric region, then digests of DNA from cell line 153-E7BX will be missing fragments seen in 153-E9A and have at least one fragment whose size is different from that found in 153-E9A. On the other hand, if the family is present as separate clusters in two different locations, or present as a continuous block that is periodically interrupted by unrelated sequences, then the cell line 153-E7BX will be missing fragments seen in 153-E9A, but will have no fragments differing in size from ones in 153-E9A. This assumes that the 153-E7BX breakpoint is in the interrupted region.

BamHI digests of DNAs from the cell lines 153-E9A, 153-E7BX and 2Fu^{r}1 were compared upon hybridization with the two different alphoid probes. It was observed that the 340 bp EcoRI family was present only in the cell line 153-E9A, which

contains the complete chromosome 21 (Figure 13; Table I). The probe did not hybridize to DNA from the other two cell lines indicating that the family is present only on the short arm region and is absent from the centromeric region. On the other hand, the same digest when probed with pHH550-31 shows the presence of the family in cell lines 153-E9A and 153-E7BX but not in 2Fu'1 (Figure 13; Table I). These findings indicate that the 550 bp HindIII family is spread over both the short arm and the centromeric regions. The same results for these two alphoid families were obtained with various other enzymes (Figure 14; Table I) and confirm the findings of Palamidis-Bourtsos (1989).

In the lane containing the cell line 153-E7BX only the one fragment of size 250 kb and the unresolved fraction can be detected as opposed to three fragments in 153-E9A (Figure 13; Table I). This fragment in cell line 153-E7BX is the same size as one seen in the DNA from 153-E9A. The other fragments observed in 153-E9A are missing in 153-E7BX. These results suggest that either a portion of this family is located in a totally different location distal from the centromere or the family is present as a continuous block with interruptions of some unrelated DNA in the 153-E7BX breakpoint region. This unrelated DNA does not belong to the 340 bp EcoRI family, which was shown above not to be intermixed with the 550 bp HindIII family (Figure 13; Table I).

Alphoid clusters organization

A recent study done by Palamidis-Bourtsos (1989) determined the number of copies in the 340 bp EcoRI and 550 bp HindIII alphoid families on chromosome 21. From these results a rough estimate of the amount of DNA occupied by these families on chromosome 21 was made. This was done by multiplying the copy number of a family by its repeat size. The estimated values range from 2700 to 2800 kb for the 340-680 bp EcoRI family and from 1350 to 1400 kb for the 550 bp HindIII family. In the present study, the fragment sizes obtained with each restriction enzyme were added up to obtain the total amount of DNA occupied by the 340 bp EcoRI family. The unresolved fraction could not be taken into account. The totals obtained with various restriction enzymes ranged from approximately 2000 to 2800 kb. Out of these estimates those obtained with HindIII and PstI digests were chosen as the most accurate, since they have a small amount of DNA left unresolved and therefore most of the alphoid sequence is present in the fragments. The estimate of the amount of DNA occupied by the 340 bp EcoRI family, obtained from these enzyme digests ranges from 2300 to 2700 kb. Enzymes like ClaI and KpnI are rare cutters and therefore have a relatively greater amount of DNA in the unresolved region of the gels. Since the enzyme BamHI has sites within the family repeat a number of small fragments are generated, that run off the gel in PFGE (Palamidis-Bourtsos, 1989). Therefore an accurate

estimate of the region occupied cannot be obtained with the latter enzymes (ClaI, KpnI, and BamHI).

In the case of the 550 bp HindIII family too, the fragment sizes obtained with each restriction enzyme were added to get an estimate of the total amount of DNA occupied by this family. The range obtained in this case was from 2000 to 3000 kb. For best estimates enzyme digests of PstI, PvuII or BamHI were chosen, as in these digests a relatively small amount of DNA was left unresolved. The values obtained with these restriction enzymes ranged from 2300 to 3000 kb (Table I). Again the enzymes ClaI and KpnI were not chosen for the same reason as that for pHE340-9. HindIII in the case of the 550 bp HindIII family is not suitable as it would give a number of 550 bp fragments which are not seen on the PFGE gel (Doering et al., 1988).

The amount of DNA occupied on chromosome 21 by the two alphoid families together, calculated from the restriction fragment sizes, is estimated to be approximately 4600-5700 kb, indicating that both families together occupy at least 25% of the short arm of chromosome 21 (about 20,000-25,000 kb). The amount of DNA occupied by the 340 bp EcoRI family, estimated from the fragment sizes (2300-2700 kb) is very similar to the amount estimated for it, from the copy number (2700-2800 kb). From this it can be concluded that the 340 bp Eco RI family is not significantly intermixed with any other DNA including pHH550 (confirmed by PFGE) on chromosome 21. On the other

hand, the estimated values of the amount of DNA occupied by the 550 bp HindIII family, obtained from the restriction fragments (2300-3000 kb) are far in excess of those obtained from the copy number of the family (1350-1400 kb). This indicates that the 550 bp HindIII family is interspersed with some DNA other than 340 bp EcoRI. This interspersed DNA may be some other alphoid sequence or some non-alphoid sequence that is still to be determined.

The presence of unrelated DNA sequences in the region occupied by the pHH550 family can be further confirmed by double digests. If there are no unrelated sequences present then the fragments created by a double digest would add up in size to the total size obtained from fragments in a single digest. On the other hand if there are some unrelated sequences present then in a double digest the second enzyme may create a fragment that does not contain the pHH550 family. In this case the total size obtained for the fragments in a double digest would be less than that obtained from their respective single digests. The double digest with BamHI and ClaI when probed with pHH550 revealed a banding pattern similar to that seen in a single digest with BamHI, with one 700 kb fragment missing. This indicated the presence of some other sequence(s), non-pHH550, in that 700 kb fragment or cluster. Cutting with ClaI in the double digest resulted in fragmenting the 700 kb into a small segment (that runs off the PFGE gel) containing the 550 bp HindIII sequences and a big

one containing one or more other sequences other than pHH550. (Figure 15; Table III). If there were not a significant amount of non-pHH550 sequences in the region then a new fragment might have been seen, which along with the small fragment would add up in size to give 700 kb.

One of the possible sequences that might be interspersed with the pHH550 family is the Kpn LINES-1 sequence (Shafit-Zagardo et al., 1982). It is known to be an interspersed repetitive sequence and on chromosome 21 it has been found to be present only on the short arm region (Doering et al., 1988b). Thus it would likely be present among the alphoid families which, as shown above, occupy approximately 25% of the short arm. If this family were intermixed with either of the two alphoid families then some of the same bands would be observed on hybridization with the LINES-1 and the alphoid family probes. Hence a number of previously used blots were hybridized to the probe pHK1.8-44, belonging to the Kpn family (Shafit-Zagardo et al., 1982). The banding patterns obtained with this probe were distinctly different from those obtained with the probes pHE550-31 and pHE340-9 (Figure 16; Table II). Since no common bands were obtained for the three families, it was concluded that the Kpn family is not interspersed with either of the two alphoid families being studied, i.e., it was not present on at least 25% of the short arm of chromosome 21. From this it can be concluded that the Kpn family is not randomly distributed or equally dispersed on the short arm,

but has a more limited organization pattern.

A double digest was also done for the 340 bp EcoRI family to further analyze its pattern of organization. The double digest of BamHI and ClaI when probed with the pHE340-9 probe reveals only the BamHI fragments of sizes 110 and 200 kb. The two high molecular weight ClaI fragments disappear. This indicates that the ClaI fragments have a number of BamHI sites in them. Similar results were obtained with a double digest of BamHI and KpnI, where a 1355 kb KpnI fragment is reduced to the low molecular weight BamHI fragments of sizes 200 and 110 kb, the rest of the fragment is not detected (Figure 17; Table III). Hence, the KpnI fragment also seems to have a number of BamHI sites in it (Figure 17; Table III).

Figure 1. Two models showing possible arrangements of alphoid DNA on a single chromosome.

(A) The alphoid families are interspersed with other alphoid sequences or non-alphoid sequences in multiple blocks.
(B) The alphoid families exist in separate independent clusters. Sequences from one family are not intermixed with sequences from another family or any other unrelated DNA.

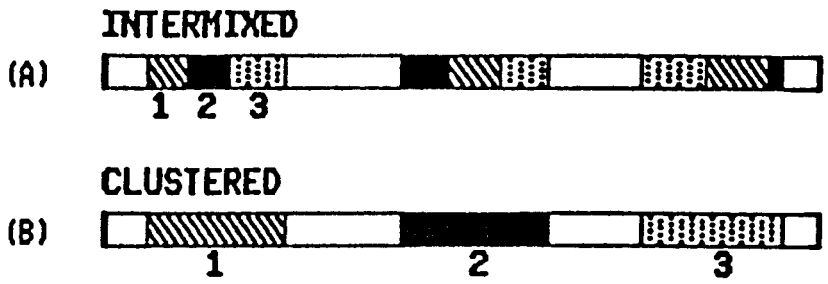

INTERMIXED
(A)
1 2 3
CLUSTERED
(B)
1
2
3

Figure 2. Out-of register alignment of two chromosomes with either of the two possible arrangements of alphoid DNA

(A) Two chromosomes containing alphoid families that exist in separate independent clusters are aligned out-of-register. (B) Two chromosomes containing alphoid families that are interspersed with other alphoid sequences or non-alphoid sequences in multiple blocks are aligned out-of-register.

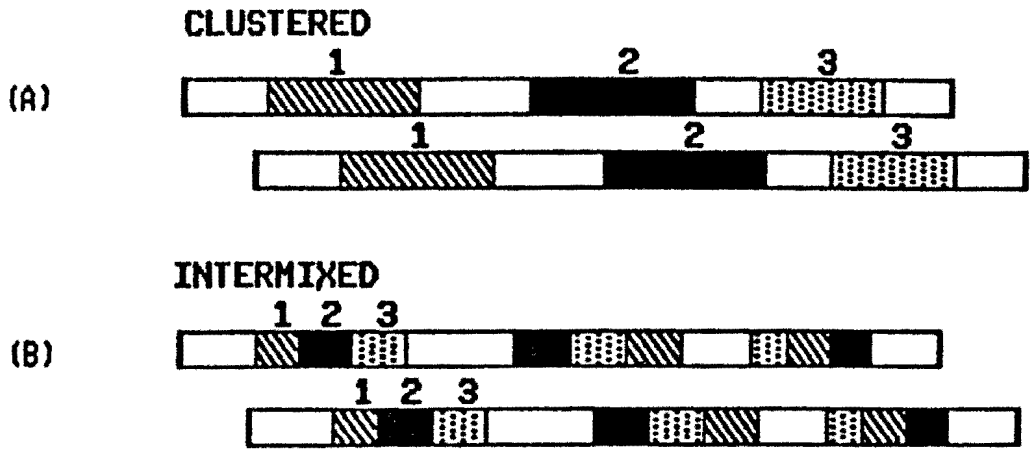

CLUSTERED
(A)
1
2
3
1
2
3
INTERMIXED
(B)
1 2 3
1 2 3

Figure 3. Restriction enzyme digests and a calibration marker on a pulsed field gel.

DNA from human Fibroblast cells (Lanes 6 and 7) and 153-E9A (Lanes 4 and 5) was digested with the restriction enzymes SfiI and PstI and subjected to PFGE. The digested samples are run parallel to control samples containing enzyme buffer but no enzyme and control samples containing no buffer and no enzyme (Lane 1). The sizes of the hybridizing fragments obtained in the digested samples are calibrated using yeast (Saccharomyces cerevisiae) chromosome markers.

Sizes of selected chromosome markers in kb are indicated.

Lane 1: Genomic DNA control, no enzyme buffer or enzyme.
Lanes 2 and 3: 153-E9A DNA controls, containing enzyme buffer but no enzyme.
Lanes 4 and 5: PstI digests of 153-E9A.
Lane 6: PstI digest of genomic DNA.
Lane 7: SfiI digest of genomic DNA.
Lane 8: Genomic DNA control, containing enzyme buffer but no enzyme.
Lane 9: Yeast chromosome markers

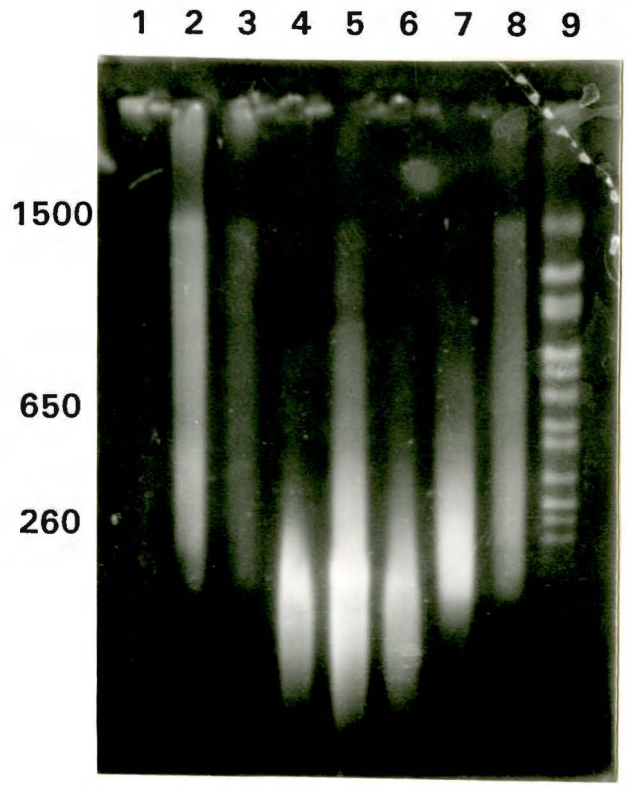
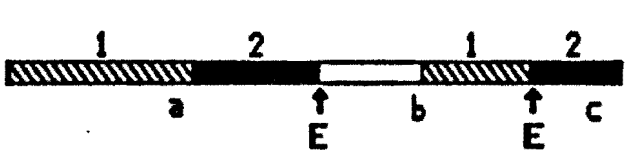

1 2 3 4 5 6 7 8 9
1500
650
260

Figure 4. Schematic showing detection of linked families on a chromosome by hybridization

(A) Sketch of a chromosome showing two different alphoid families 1 and 2. Restriction enzyme sites are shown by E. Fragments created by the restriction enzyme are a, b,and c.

(B) Fragments hybridizing with probe of family 1 are shown in column 1 (fragments a and b). Fragments hybridizing with probe of family 2 are shown in column 2 (fragments a and c).

(A)



(B)

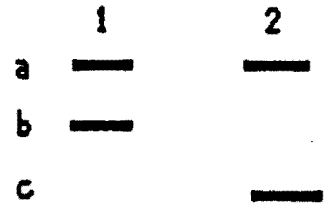


Figure 5. Organization of the 340 bp EcoRI, 550 bp HindIII and 2 kb BamHI families in the genome

DNA from human Fibroblast cells was digested with the restriction enzymes MluI (Lane1), ClaI (Lane 2), PvuII (Lane 3), SstII (Lane 4), BamHI (Lane 5), PstI (Lane 6) and HindIII (Lane 7). The DNA was subjected to PFGE, blotted on Gene Screen Plus and probed with the alphoid sequences shown below. Comparable exposure times were used for the probes pHE340-9 and pHH550-31. Longer exposure times were required for pXBRI which is present in lower copy numbers (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1, 2 and 3: pHE340-9
Lanes 4,5 and 6: pHH550-31
Lanes 6 and 7: pXBRI

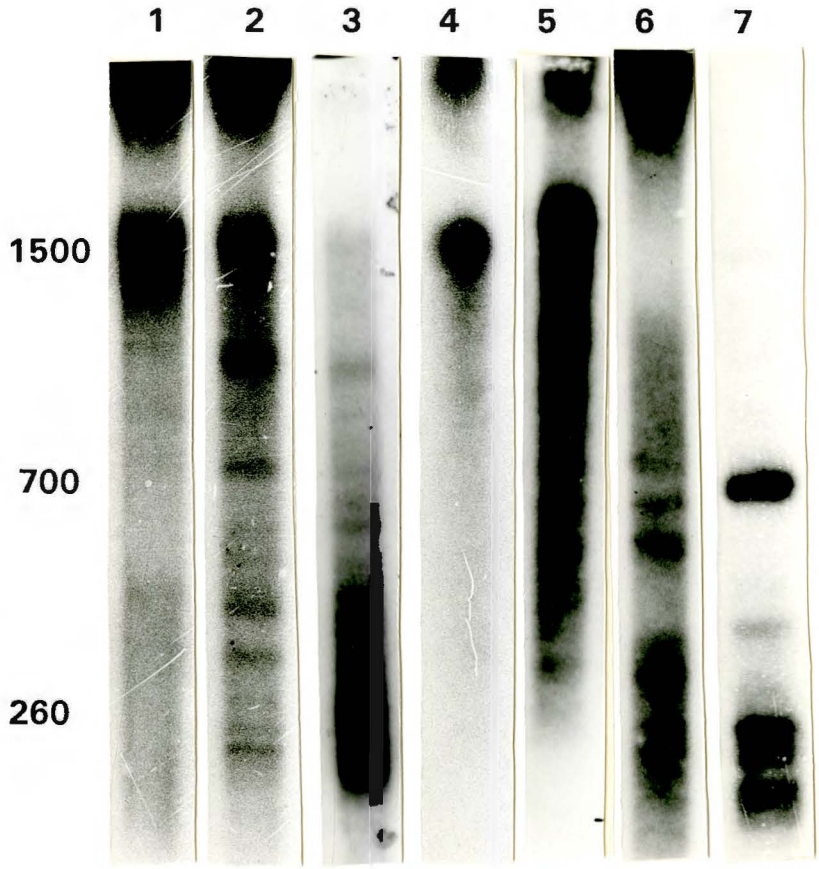

1
2
3
4
5
6
7
1500
700
260

Figure 6. BamHI digest of genomic and chromosome 21 DNA

DNA from human Fibroblast cells (Lanes 1 and 4) and 153-E9A (Lanes 2 and 3) was digested with the restriction enzyme BamHI. The DNA was subjected to PFGE, blotted on Gene Screen Plus and probed with the two indicated alphoid sequences (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1 and 2: pHE340-9
Lane 3 and 4: pHH550-31

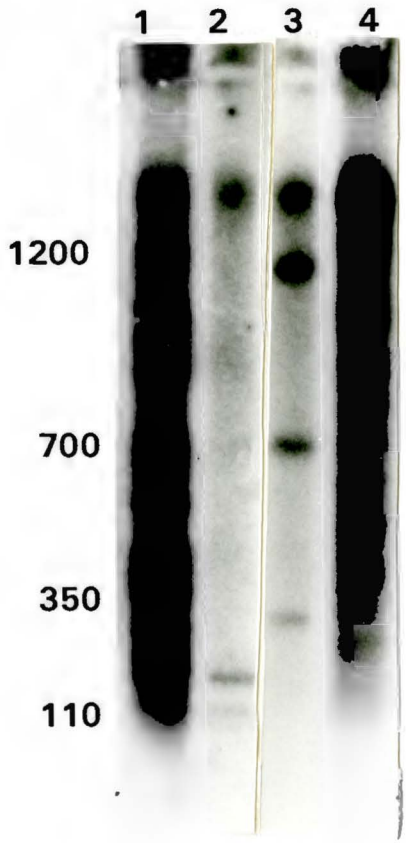

1
2
3
4
1200
700
350
110

Figure 7. PvuII digest of genomic and chromosome 21 DNA

DNA from human Fibroblast cells (Lanes 1 and 5), 153-E9A (Lanes 2 and 6), 153E7BX (Lanes 3 and 7) and 2Fu'1 (Lanes 4 and 8) was digested with the restriction enzyme PvuII. The DNA was subjected to PFGE, blotted on Gene Screen Plus and probed with the two alphoid sequences indicated below (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lanes 1 to 4: pHE340-9
Lanes 5 to 8: pHH550-31

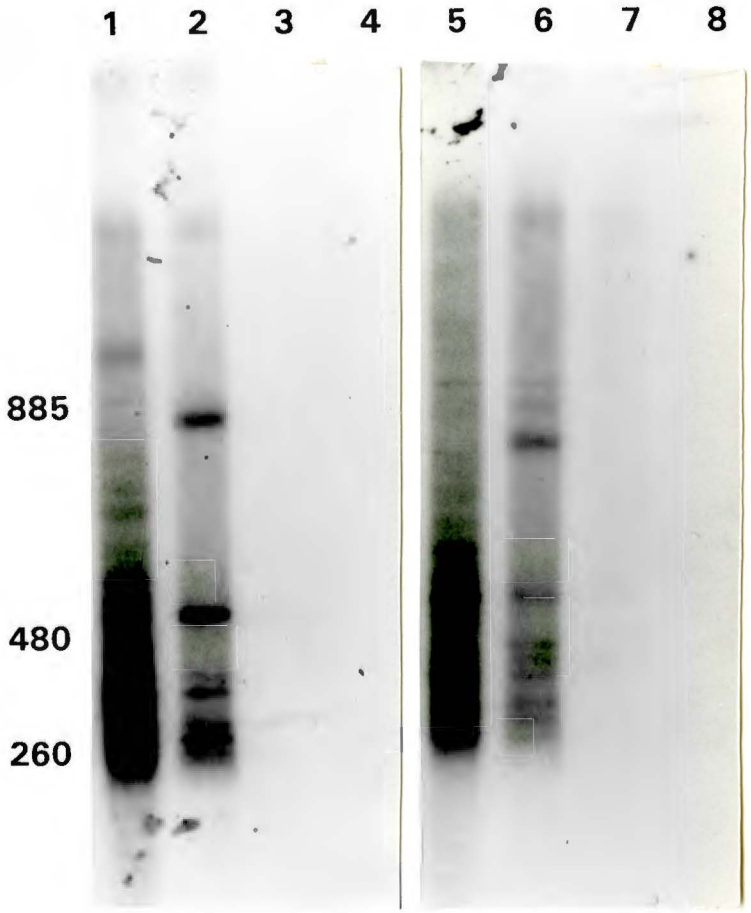

1
2
3
4
5
6
7
8
885
480
260

Figure 8. HindIII digest of genomic and chromosome 21 DNA

DNA from human Fibroblast cells (Lanes 1,2 and 5) and 153-E9A (Lanes 3 and 4) was digested with the restriction enzyme HindIII. The DNA was subjected to PFGE, blotted on Gene Screen Plus and probed with the three indicated alphoid sequences (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1: pXBRI
Lanes 2 and 3: pHE340-9
Lane 4 and 5: pHH550-31

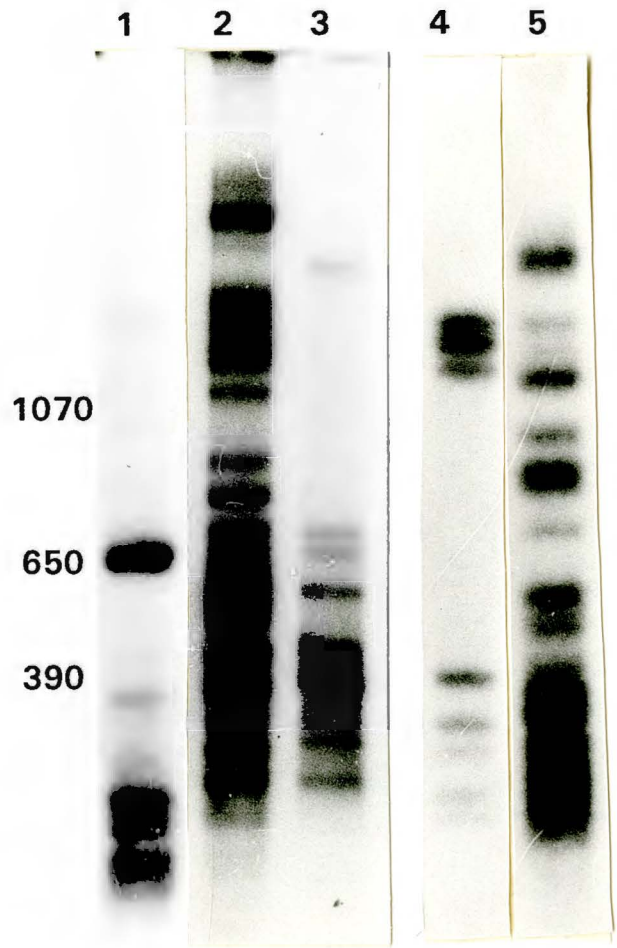

1
2
3
4
5
1070
650
390

Figure 9. HindIII and BamHI digests of genomic DNA from cells of culture passages 13 to 18.

DNA from human fibroblast cell line 3348B at the various passage numbers indicated was digested with the restriction enzymes HindIII (Lanes 1 and 2) and BamHI (Lanes 3 and 4). The DNA was subjected to PFGE, blotted on Gene Screen Plus and probed with the alphoid sequence indicated below (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1: Passage 13
Lane 2: Passage 18
Lane 3: Passage 14
Lane 4: Passage 18

Probes used:
Lanes 1 and 2: pHH550-31
Lanes 3 and 4: pHE340-9

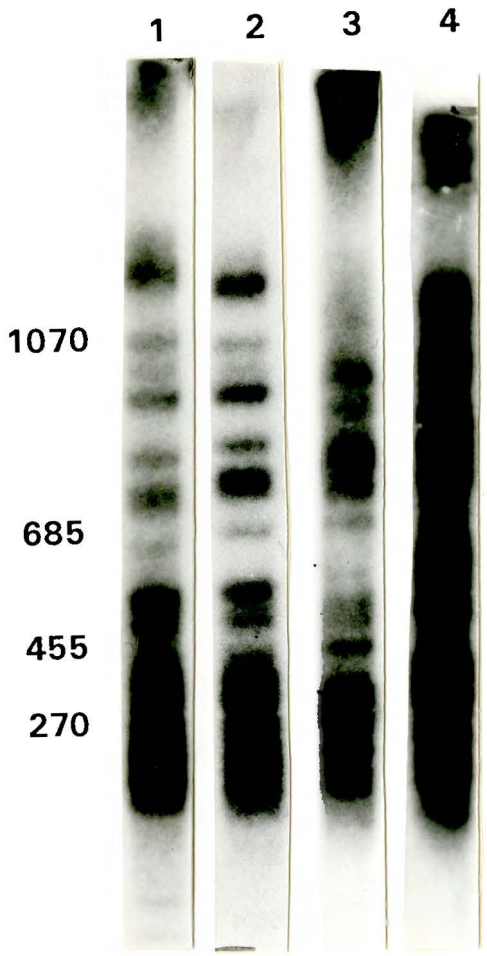

1
2
3
4
1070
685
455
270

Figure 10. PstI digest of genomic and chromosome 21 DNA

DNA from human Fibroblast cells (Lanes 1 and 4), and 153-E9A (Lanes 2 and 3) was digested with the restriction enzyme PstI. The DNA was subjected to PFGE, blotted on Gene Screen Plus and probed with the two alphoid sequences indicated below (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1 and 2: pHE340-9
Lane 3 and 4: pHH550-31

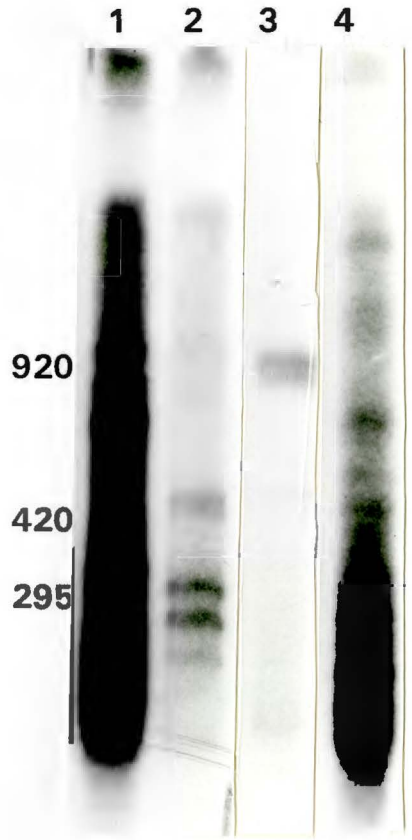

1
2
3
4
920
420
295

Figure 11. Organization of 340 bp EcoRI family on chromosome 21

DNA from 153-E9A was digested with the restriction enzymes indicated below. The DNA was subjected to PFGE, blotted on Gene Screen Plus and probed with the alphoid sequence pHE340-9 (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1: PvuII
Lane 2: ClaI control containing enzyme buffer but no enzyme
Lane 3: ClaI
Lane 4: KpnI control containing enzyme buffer but no enzyme
Lane 5: KpnI

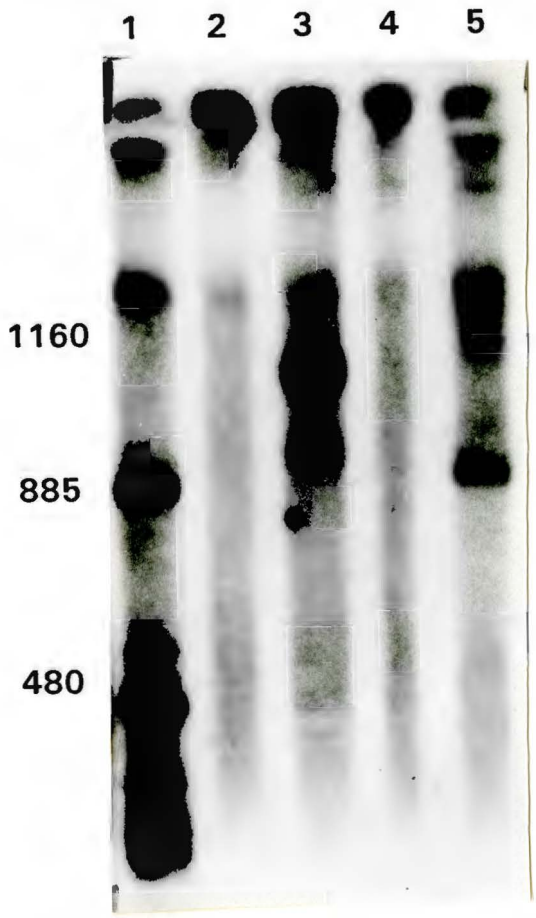

1
2
3
4
5
1160
885
480

Figure 12. Organization of 340 bp EcoRI and 550 bp HindIII families on chromosome 21

DNA from 153-E9A was digested with the restriction enzymes ClaI (lanes 1 and 2) and KpnI (lanes 3 and 4). The DNA was subjected to PFGE, blotted on Gene Screen Plus and probed with the two indicated alphoid sequences (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1 and 3: pHE340-9
Lane 2 and 4: pHH550-31

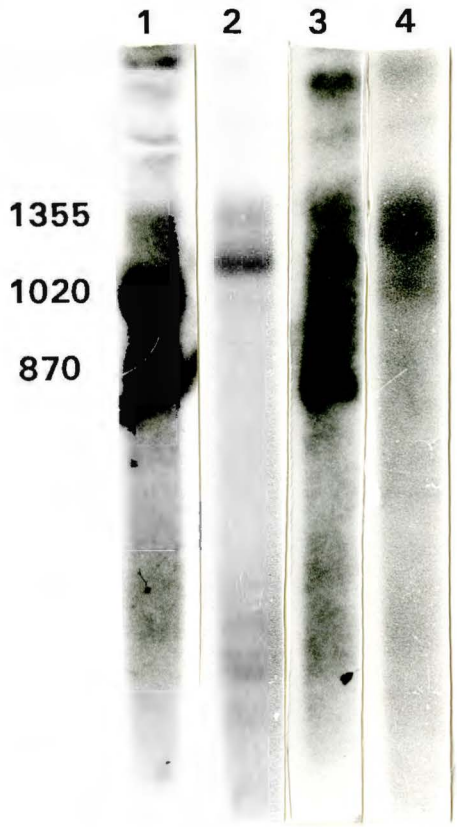

1
2
3
4
1355
1020
870

Figure 13. Localization of the 340 bp EcoRI and 550 bp HindIII families on chromosome 21

DNA from hamster-human hybrid cell lines (indicated below) was digested with the restriction enzyme BamHI. The DNA was subjected to PFGE, blotted on Gene Screen Plus and probed with the two alphoid sequences, pHE340-9 (lanes 4-6) and pHH550-31 (lanes 1-3) as mentioned in Materials and Methods.

Sizes of selected fragments in kb are indicated.

Lanes 1 and 6: 153-E9A
Lanes 2 and 5: 153-E7BX
Lanes 3 and 4: 2Fu'1

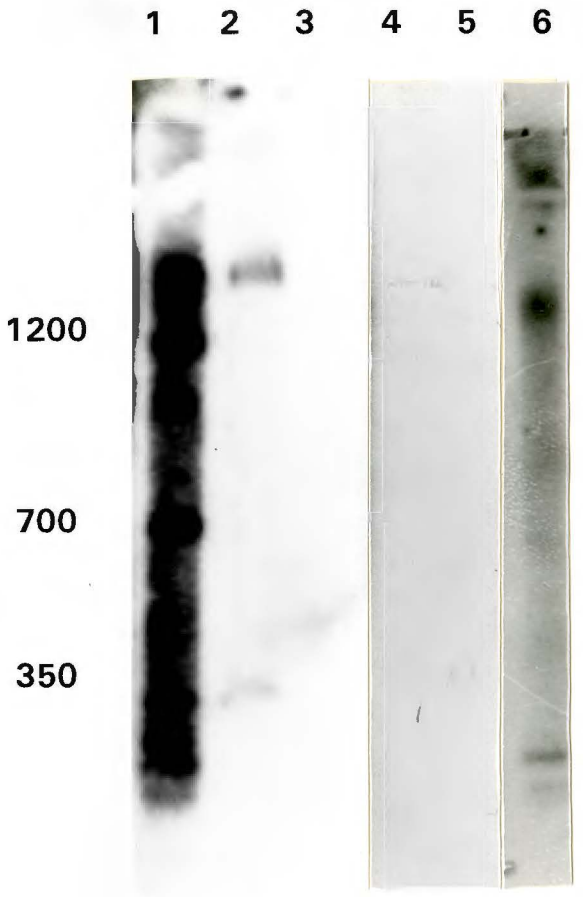

1
2
3
4
5
6
1200
700
350

Figure 14. Localization of the 550 bp HindIII family on chromosome 21

DNA from hamster-human hybrid cell lines (indicated below) was digested with the restriction enzymes HindIII. The DNA is subjected to PFGE, blotted on Gene Screen Plus and probed with pHH550-31 (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1: 153-E9A
Lane 2: 153-E7BX
Lane 3: 2Fu^{r}1

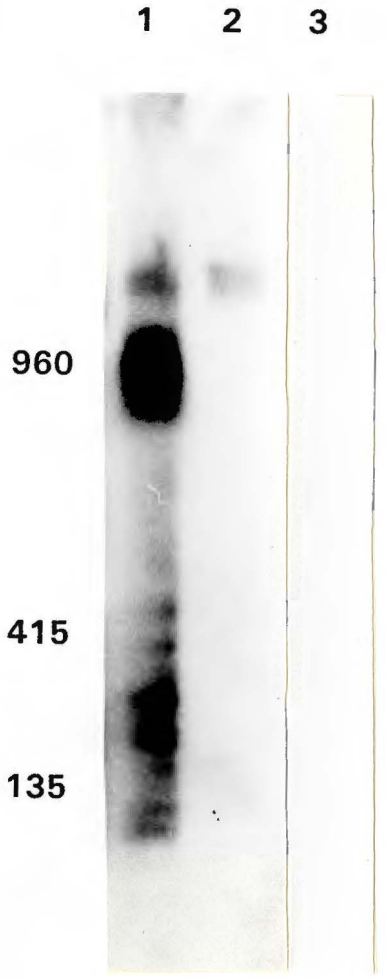

1
2
3
960
415
135

Figure 15. Double digest of the 550 bp HindIII family on chromosome 21

DNA from 153-E9A was digested with restriction enzymes indicated below, subjected to PFGE, blotted on Gene Screen Plus and probed with pHH550-31 (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1: BamHI
Lane 2: BamHI + ClaI
Lane 3: Cla

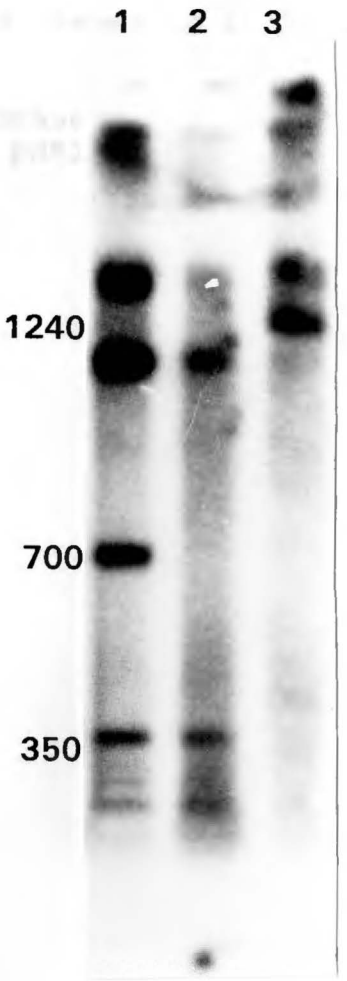

1
2
3
1240
700
350

Figure 16. Organization of a non-alphoid sequence (LINES-1) and two alphoid sequences (340 bp EcoRI and 550 bp HindIII) on chromosome 21

DNA from 153-E9A was digested with the restriction enzymes BamHI (lanes 1 and 2), ClaI (lanes 3 and 4) and HindIII (lanes 5 and 6). The DNA was subjected to PFGE, blotted on Gene Screen Plus and hybridized with the probes indicated below (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1: pHE340-9
Lanes 3 and 5: pHH550-31
Lanes 2,4 and 6: pHK1.8-44

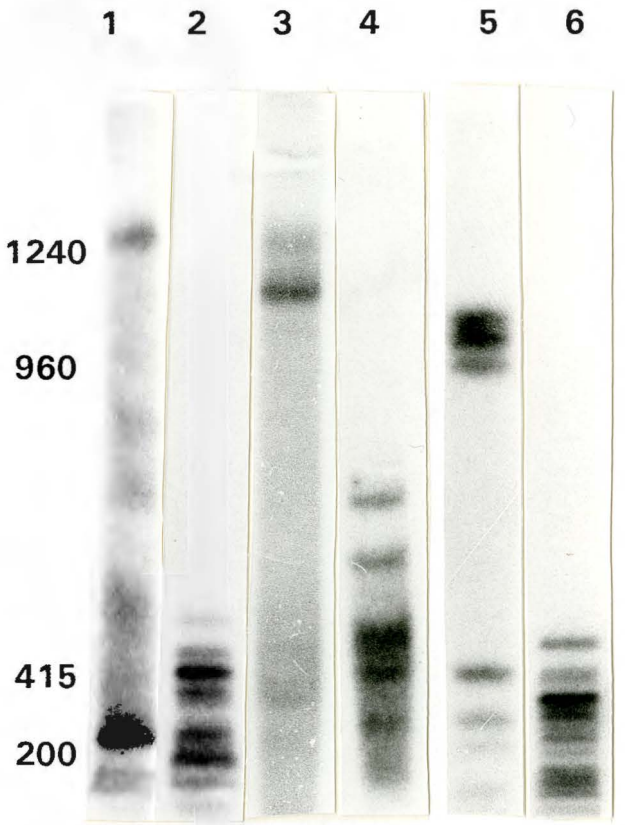

1
2
3
4
5
6
1240
960
415
200

Figure 17. Double digest of the 340 bp EcoRI family on chromosome 21

DNA from 153-E9A was digested with restriction enzymes indicated below, subjected to PFGE, blotted on Gene Screen Plus and probed with pHE340-9 (see Materials and Methods).

Sizes of selected fragments in kb are indicated.

Lane 1: ClaI
Lane 2: ClaI + BamHI
Lane 3: BamHI
Lane 4: BamHI + KpnI
Lane 5: KpnI

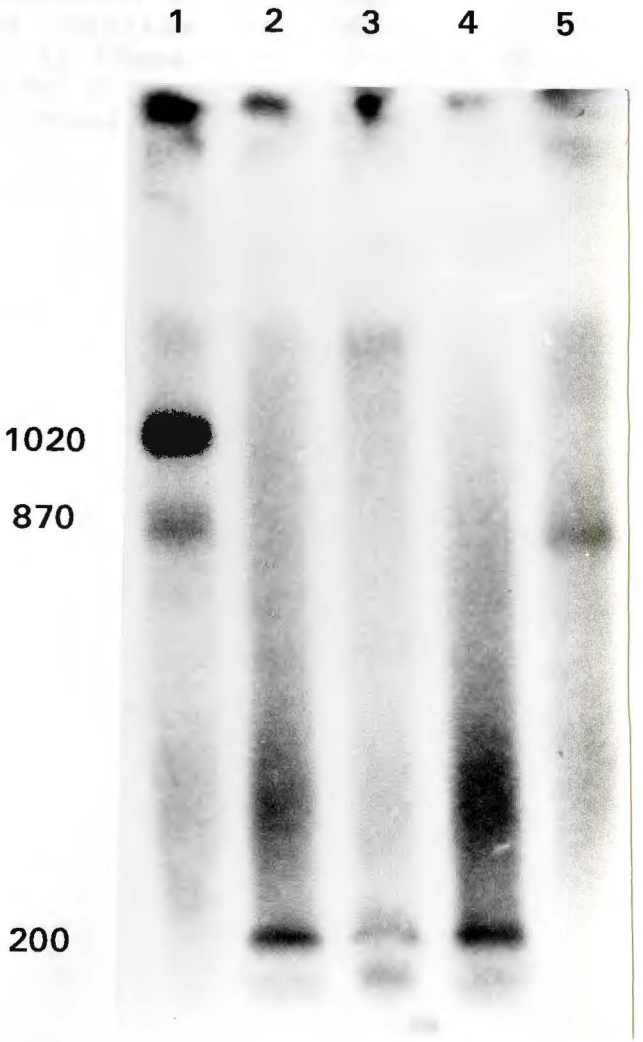
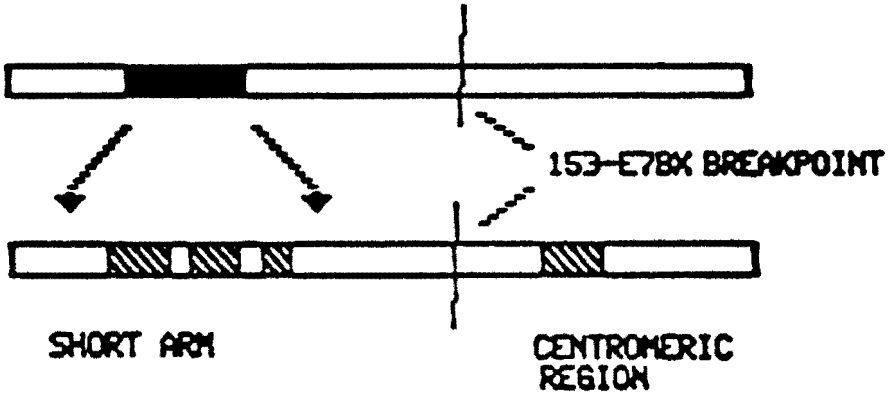

1
2
3
4
5
1020
870
200

Figure 18. Proposed organization of alphoid families on chromosome 21.

The 340 bp EcoRI family (filled box) is present on only the short arm of chromosome 21, as a single continuous block. The 550 bp HindIII family (hatched box) is present on both the short arm and the centromeric regions. In these regions it is present as small multiple blocks, interspersed with some unrelated sequences within these blocks.
Both alphoid families are absent from the long arm region. The location of these families relative to each other is not known (shown by the broken arrows). The broken lines indicate the 153-E7BX breakpoint.



= 340 bp EcoRI = 550 bp HindIII

Table I. Sizes of the restriction fragments obtained with various restriction enzymes when hybridized to three different alphoid sequences.

Sizes (in kilobases) of the restriction fragments obtained with the various restriction enzymes indicated in the table when hybridized with the three alphoid probes pHE340-9, pHH550-31 and pXBRI. Size marker used to calibrate was yeast (Saccharomyces cerevisiae) chromosomes (size range: 260-1500 kb). In some gels, fragments that were smaller than 260 kb could not be calibrated accurately therefore their sizes have not been indicated. All fragments with an asterisk (*) are fragments that were faint and were observed only in autoradiographs that were exposed for longer periods of time.

Probe:	----pHE340-9----		-----pHH550-31-----			-pXBRI-
Cell Lines:	3348B	153-E9A	3348B	153-E9A	153-E7BX	3348B
Enzyme	1480	1480	1480	1480	1500	1480
1. BamHI	1220		1290			1250
	1130		1200	1200		
	1010		1010			1020
	920					
	840					
	685		700	700		
	635		625			620
	580		515			540
	455		400			480
	420					
	375		352	350	350	365
	295		275			260
	245					
	200	200				
	110	110				
2. HindIII	1500				1500	
	1460					
	1430	1390				
	1285		1295*			
	1110		1250			1110*
	1020		1070	1070		
	990		1010	1010		
	940		960	960		970
	845		820			880
	775		785			
	705	705	760			
	655	655	705			640
	615					
	575	575	575			
	550					
	510		525			
	460	460				
	440	440	415	415		
	390	390				
	370		370			370
	335					
	270	270	295	295		270 *
	225	225	225			250 *
			178	170		195
			135	135		150
						115

Probe:	---pHE340-9---		-----pHH550-31-----			-pxBRI-
Cell Lines:	3348B	153-E9A	3348B	153-E9A	153-E7BX	3348B
Enzyme	1490	1490*	1500			
3. PstI	950	950 *	1380*	920		
	860	860 *	1110*	880		
	775	632 *	1040*			
	575 *	612 *	790			
	520 *		685			
	470 *		625	630		
		420	470			
		365	390			
	340		330			
	295	295	260			
	232	232	190	190		
	185	170				
4. KpnI		1500		1500		
		1160		1355		
		880				
5. ClaI	1500	1500	1500	1500	1500	1500
	1020	1020	1240	1240		1390
	870	870				1020
	700					500
	670					450
	565					315
	480					
	370					
	330					
	290					
6. PvuII	1500		1500	1500	1500	
			1440*			
			1165*			
	1065		1065*			
	920		955	955		
	885	885	900	900		
	810		805	820		
	760		720			
	565		615			
	520		540			
	480	480	515	515		
	290	290	450	400		
	265	265	355	355		
			335	335		
			315	315		
			260	260		

Table II. Sizes of the restriction fragments obtained with various restriction enzymes when hybridized to two different alphoid sequences (pHE340-9 and pHH550-31) and to a non-alphoid sequence (LINES-1).

Sizes (in kilobases) of the restriction fragments obtained with the various restriction enzymes indicated in the table when hybridized with the two alphoid probes pHE340-9, pHH550-31 and a non-alphoid probe pHK1.8-44 (LINES-1). Size marker used to calibrate was yeast (Saccharomyces cerevisiae) chromosomes (size range: 260-1500 kb). In some gels, fragments that were smaller than 260 kb could not be calibrated accurately therefore their sizes have not been indicated. All fragments with an asterisk (*) are fragments that were faint and were observed only in autoradiographs that were exposed for longer periods of time.

Probe:	pHE340-9	pHH550-31	LINES-1
Cell Line:	153-E9A		
Enzyme			
1. BamHI	1500	1500	
		1200	
		700	
			490
			400
		350	365
			320
	200		220
	110		
2. ClaI	1500	1500	
		1240	
	1020		
	870		
			380
			280
3. KpnI	1500	1500	
		1355	
	1160		
	880		750
			630
			490
			390
			250
4. HindIII	1390		
		1070	
		1010	
		960	
	705		
	655		
	575		
	460		
	440		515
	390	415	405
	270	295	360
	225	225	350*
		170	320
		135	300
			255
			235
			215
			180

Table III. Sizes of the restriction fragments obtained with double digests of various restriction enzymes when hybridized to two different alphoid sequences (pHE340-9 and pHH550-31)

Sizes (in kilobases) of the restriction fragments obtained with the various restriction enzymes indicated in the table when hybridized with the two alphoid probes pHE340-9 and pHH550-31. Size marker used to calibrate was yeast (Saccharomyces cerevisiae) chromosomes (size range: 260-1500 kb). In some gels, fragments that were smaller than 260 kb could not be calibrated accurately therefore their sizes have not been indicated. All fragments with an asterisk (*) are fragments that were faint and were observed only in autoradiographs that were exposed for longer periods of time.

Enzymes:	ClaI	ClaI+BamHI	BamHI	BamHI+KpnI	KpnI
Probe:					
1. pHE340-9					
	1500		1500		1500
					1160
	1070				
	870				
		200	200	200	
		110	110	110	
2. pHH550-31					
	1500	1500*	1500		
	1240	1200	1200		
			700		
		350	350		

DISCUSSION

In this study, the long range organization of three human alphoid families has been examined. The alphoid families were each found to have a distinct pattern of organization on chromosome 21 and in the total genome. They all showed a simple pattern of organization and were found not to be intermixed with each other, but rather to exist as separate independent clusters. The organization of these alphoid families on chromosome 21 was also found to be a small subset of that present in the total genome, indicating that the pattern of organization of an alphoid family may differ from one chromosome to another.

On chromosome 21, the 340-680 bp EcoRI alphoid family was found to be located only on the short arm, primarily organized in a continuous block and not significantly intermixed with any other sequences. The 550 bp HindIII alphoid family was found to be present both on the short arm and in the centromeric region, and it seems to be periodically interrupted by some unrelated sequences. The two alphoid families are not closely linked to each other and they together comprise at least 25% of the short arm. This is the largest amount of the short arm to be characterized to date.

Simple Pattern of Organization of Alphoid Families in the Human Genome

The three alphoid families examined in the current study are known to be present on a number of different chromosomes in the human genome (Manuelidis, 1978; Doering et al, 1988; Palamidis-Bourtsos, 1989). There are several possibilities for the arrangement of each of these sequences on the different chromosomes. First, a given alphoid family could be present on a small number of chromosomes, each having their own distinct alphoid cluster. Second, a family could be present on many chromosomes whose multiple alphoid clusters are all of approximately the same size. Third, a given family could be present on many chromosomes whose multiple alphoid clusters are all of different sizes. If genomic DNA digests on hybridization with the alphoid probe show a simple banding pattern, then it can be concluded that the family is arranged in one of the first two possible ways. On the other hand, a complex banding pattern containing numerous bands would be indicative of the third possibility.

The results obtained on digesting total genomic DNA revealed a simple banding pattern (as few as 5-6 bands) with all three alphoid probes, indicating a simple organization of these alphoid families in the genome (Figure 4). This could, however, still mean that these alphoid families are arranged in one of the first two ways mentioned above. These possibilities were further distinguished by examining the organi-

zation of alphoid families on a single chromosome, chromosome 21. Since chromosome 21 had only a few of the bands observed in the total genome for each of the two different alphoid probes, it implied that the clusters on chromosome 21 comprise only a small subset of those found in the genome (Figures 5,6,7 and 8). This suggests that the alphoid clusters most likely have a chromosome-specific organization, i.e, one alphoid family may be organized differently on different chromosomes.

There are a number of studies in the literature which emphasize that each human chromosome has its own specific alphoid family (see Literature Review). However, this does not preclude the presence of additional alphoid families on a given chromosome. Indeed, recent studies (including the present study) have shown the presence of multiple alphoid families on a single chromosome (see Literature Review). The 2 kb BamHI family, initially thought to be X chromosome specific (Yang et al., 1982; Willard et al., 1983), has been found to be present on other chromosomes as well (Palamidis-Bourtsos, 1989). The organization of the 2kb BamHI family as 2 kb BamHI repeats has been found to be specific for the X chromosome. Similarly, the 550 bp HindIII family has been found to be present on many human chromosomes, having a chromosome-specific organization (Palamidis-Bourtsos, 1989). On chromosome 21 it is predominantly organized as a 1.1 kb AluI or EcoRI fragment, but on other chromosomes many other

variants have been observed (Palamidis-Bourtsos, 1989). Previous work has not examined the chromosome-specificity of long range organization as done in the present study.

Members of these different alphoid families could be either intermixed with each other or present in separate independent clusters in the genome. In the present study, the restriction enzyme digests of genomic DNA upon hybridization with each of the three different alphoid probes were found to give distinctly different banding patterns (Figures 5,6,7 and 8). Thus, the alphoid families are not significantly intermixed with each other, but rather exist as separate independent clusters.

Organization of Two Alphoid Families On Chromosome 21

From the results obtained with genomic DNA, showing the alphoid families not to be intermixed with each other, it was expected that the same would be true for alphoid families on chromosome 21 (Figures 5,6,7 and 8). On chromosome 21 also, two of the alphoid families were found not to be intermixed with each other but exist as separate independent clusters. This organization for the alphoid sequences would be predicted from the out-of-register recombination models, which suggest that similar sequences should be linked and not intermixed with less similar sequences, as a result of homo-genization mechanisms (Smith, 1976). There is one report in the literature which also indicates that different families lie in separate domains even on the same chromosome. A study by

Willard et al. (1987) showed the presence of at least two independent domains of alphoid DNA on chromosome 7 with no detectable interspersion between them.

Digestion of chromosome 21 DNA with rare cutting restriction enzymes, like ClaI and KpnI, showed the presence of each of the two families on only one or two fragments and in the unresolved fraction (Figure 11). These results indicated that the two families are each restricted to only a few clusters (maybe one or two) on chromosome 21. If the clusters of the two families are linked, then digestion of chromosome 21 DNA, at least with the rare cutting restriction enzymes, would create some fragments that contain both families. The clusters belonging to the two alphoid families in this study cannot be closely linked to each other since none of the restriction enzymes, including the rare cutters, create fragments that contained both the families. This indicates that the two families are far enough apart such that all the enzymes used in the present study have a restriction site in the regions separating them (Figures 5,6,7,8 and 11). This distance between the two families must be at least one million base pairs, since even the biggest fragments, seen in ClaI and KpnI digests, contained only one family.

Alphoid sequences could be all clustered towards one end of the fragment(s) seen with the rare cutters or they could be more evenly spread. For each family, enzymes like PstI and HindIII created substantially more fragments than did the rare

cutting enzymes. Thus family members are likely distributed throughout the length of the large fragments.

Each of the two alphoid family domains could be present either as a continuous block or as multiple interrupted clusters on chromosome 21. To determine their organization, the amount of DNA that might be occupied by each of these families on chromosome 21 was calculated from the copy number of each family on this chromosome (Palamidis-Bourtsos, 1989). This number was compared to the total size of the fragments occupied by the family as seen with restriction enzyme digests (Table I). For the 340 bp EcoRI family these two numbers are in close agreement, indicating that the cluster(s) of this family are not significantly interrupted by unrelated sequences. On the other hand, for the 550 bp HindIII family, the total size of the restriction fragments occupied by the family is far more than the estimated value from the copy numbers. Therefore, it was concluded that the 550 bp HindIII family is interspersed with some other DNA, which could be alphoid or non-alphoid (still unknown). This interspersed DNA is definitely not the 340 bp EcoRI family according to the results of the present study. Double digests confirmed the presence of unrelated sequences in the region of the pHH550 family (Figure 15).

The LINES-1 sequence was found not to be interspersed with either the pHH550 family nor the 340 bp EcoRI family (Figure 16). Since these two alphoid families occupy at least

25% of the short arm of chromosome 21, this result indicates that LINES-1 sequences are excluded from a large region of this chromosome. Thus, contrary to the usual situation with interspersed repetitive sequences, the LINES-1 family is not uniformly distributed on the short arm, but has a more limited organization pattern.

Location of these families on chromosome 21, with respect to the centromere, was done using hybrid cell lines containing fragments of chromosome 21. Results obtained in this study confirmed those of a recent study done by Palamidis-Bourtsos (1989). The 340 bp EcoRI alphoid family was found to be located only on the short arm region of chromosome 21, while the 550 bp HindIII alphoid family was found to be present on both the short arm and in the centromeric region of this chromosome. The major portion of the 550 bp HindIII family is present on the short arm (Figures 13 and 14). These studies also confirmed the presence of unrelated sequences interspersed in the pHH550 family, since the breakpoint (153-E7BX) which shows this family to be in two chromosomal regions is itself in a region containing unrelated sequences (Figures 13 and 14). Figure 17 summarizes the proposed organization of the two alphoid families on chromosome 21. They have been found to exist in a simple organizational structure and are seen not to be intermixed with each other. The 340 bp EcoRI family seems to exist in a continuous block on the short arm of chromosome 21 not significantly intermixed with any other

sequences. The 550 bp HindIII family is found to be present on both the short arm and the centromeric regions and is restricted to a few clusters in these regions. These clusters of pHH550 are periodically interrupted by unrelated sequences. The relative location of one family with respect to the other is not presently known. The length of the region over which the 550 bp HindIII family is spread on chromosome 21 is greater than that occupied by the 340 bp EcoRI family. Both the families are completely absent from the long arm of chromosome 21.

The results obtained in this study show that the alphoid families on chromosome 21 are primarily organized in clusters. A number of studies done on the long range organization of other alphoid families, have shown them to also exist as large independent domains. Examples of such studies are on chromosome 3 and chromosome Y (Tyler-Smith and Brown, 1987; Waye and Willard, 1989). A study on chromosome 3 demonstrated that the higher order repeat units of the alphoid sequence are localized in large domains at least 1000 kb in length (Tyler-Smith and Brown, 1987). On chromosome Y, a simple major block of alphoid DNA approximately 475-575 kb in length was found by Waye et. al. (1989). However, studies of other alphoid DNAs have found them to be interspersed with other alphoid and non-alphoid DNA. Examples of such an organization are of alphoid DNA are on chromosomes 1 and 17 (Carine et al., 1989; Waye and Willard, 1989). Carine et. al. (1989) showed that four diff-

erent tandemly repeated sequences are present on chromosome 1, three of which were alphoid sequences. Results in this study indicated that sequences are not found in a single continuous block but exist in multiple clusters interspersed with other sequences. An investigation of chromosome 17, using a series of cosmid clones suggested that the human alphoid DNA arrays are relatively frequently interrupted by other non-alphoid DNA (Waye and Willard, 1989). In the present study both the 340 bp EcoRI and the 550 bp HindIII families have a clustered organization, although the 550 bp HindIII family is interspersed with some unrelated sequences within its clusters. Thus, it seems that there is no conserved pattern of organization for all alphoid families and that one family may be organized differently from another, or perhaps one family may even be organized differently on different chromosomes. In other words, each family may have a specific organization according to its location.

Possible Function of Alphoid DNA

At present the function of alphoid DNA is unknown. However, some theories have been proposed regarding its possible function. Due to the location of alphoid DNA at the centromeric region and its sequence similarity with the functional CEN sequences in yeast S.cerevisiae, it has been proposed to play a role in centromeric function (Fitzgerald-Hayes et al., 1982; Carine et al., 1989). Due to their preferential location also on the short arms of acrocentric

chromosomes, it has been suggested that alphoid DNAs are involved in facilitating the Robertsonian translocations which frequently occur in these regions (Therman, 1986; Jorgensen et al., 1987; Choo et al., 1988). Therefore, the present structural studies can help in clarifying the possible function of the alphoid sequences.

Two basic models for the possible arrangements of the long range organization of these alphoid sequences are shown in Figure 1. They differ in the degree of interspersion and in the redundancy in the system. In one model the different alphoid families are clustered into separate independent blocks/units (Figure 1-a), and in the other the various alphoid families are interspersed with each other in multiple blocks. Assuming that the alphoid sequences do play a role in centromeric function, and if these sequences are organized in separate independent clusters (Figure 1-a) then each block may have an independent role to play in centromere function as a whole or some of these blocks may be nonfunctional or insignificant in centromere function. Alternatively, if the different families are intermixed with each other in multiple blocks (Figure 1-b), then some of these blocks could be redundant from the functional point of view. Results from the present study indicate that the 340 bp EcoRI family most likely does not have any role in centromeric function since it is absent from the centromeric region. The 550 bp HindIII family is present on both the short arm and in the centromeric

region, although restricted to a few clusters/blocks within those regions. If this family does play a role in centromeric function, then each of the blocks may have an independent role to play. The possible role of this family in centromeric function may be better understood once the organization of this family is elucidated in greater detail.

Referring to Figure 1, it is also apparent how the organization of alphoid DNAs might facilitate the recombinations involved in Robertsonian translocations. From Figure 1-a it is clear that if the sequences have a clustered organization, then even when two chromosomes are not aligned and are out-of-register there is a chance of crossover between homologous regions since some overlap between homologous regions is still maintained. On the other hand, if these families are interspersed with each other they are less likely to facilitate out-of-register recombination or Robertsonian translocation (Figure 1-b), since the regions of similarity no longer overlap when the two chromosomes lie out-of-register.

From the proposed model for the organization of the alphoid families on chromosome 21 (Figure 3), one can see that these sequences might be facilitating out-of-register recombination and thus Robertsonian translocations. Since the 340 bp EcoRI family is in a continuous block on the short arm of the chromosome it could facilitate such recombination. The 550 bp HindIII family, which seems to be present in smaller blocks within a defined region, is also more likely to be

facilitating out-of-register recombination in this organization than it would if the blocks were spread over a much bigger region and not confined to an area. The family is more likely to facilitate out-of-register recombination if the pHH550 sequences span long regions within the clusters than if the stretches of alphoid DNA are short and interrupted by other sequences. A more detailed structural analysis of this family's organization will clarify this point.

Since the frequencies of recombination and the formation of dNORs and satellite associations involving chromosome 21 are higher in Down's syndrome patients (Hansson, 1979; Jackson-Cook et al, 1985; Jorgensen et al., 1987), it is possible that there are some structural differences in the short arm and centromeric regions of chromosome 21 between patients and normal individuals. A detailed map for sequences on the short arm of chromosome 21 could help in characterizing any such structural changes. Since other acrocentric chromosomes frequently interact by satellite associations and translocations with chromosome 21, a detailed sequence map of the short arm of chromosome 21 may lead to an understanding of the short arm maps on other acrocentric chromosomes. Thus further studies on the long range organization of alphoid families on the various acrocentric chromosomes are likely to lead to a clearer understanding of the functional role being played by these repetitive sequences.

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APPROVAL SHEET

The thesis submitted by Malini Chawla Gupta has been read and approved by the following committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and the thesis is now given final approval by the committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirement for the degree of Master of Science.

4-17-91
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