GENOMICS 9, 104–112 (1991)

Increased Recombination Adjacent to the Huntington Disease-Linked *D4S10* Marker

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Received April 30, 1990; revised August 17, 1990

Huntington disease (HD) is caused by a genetic defect distal to the anonymous DNA marker *D4S10* in the terminal cytogenetic subband of the short arm of chromosome 4 (4p16.3). The effort to identify new markers linked to *HD* has concentrated on the use of somatic cell hybrid panels that split 4p16.3 into proximal and distal portions. Here we report two new polymorphic markers in the proximal portion of 4p16.3, distal to *D4S10*. Both loci, *D4S126* and *D4S127*, are defined by cosmids isolated from a library enriched for sequences in the 4pter–4p15.1 region. Physical mapping by pulsed-field gel electrophoresis places *D4S126* 200 kb telomeric to *D4S10*, while *D4S127* is located near the more distal marker *D4S95*. Typing of a reference pedigree for *D4S126* and *D4S127* and for the recently described VNTR marker *D4S125* has firmly placed these loci on the existing linkage map of 4p16.3. This genetic analysis has revealed that the region immediately distal to *D4S10* shows a dramatically higher rate of recombination than would be expected based on its physical size. *D4S10–D4S126–D4S125* span 3.5 cM, but only 300–400 kb of DNA. Consequently, this small region accounts for most of the reported genetic distance between *D4S10* and *HD*. By contrast, it was not possible to connect *D4S127* to *D4S125* by physical mapping, although they are only 0.3 cM apart. A more detailed analysis of recombination sites within the immediate vicinity of *D4S10* could potentially reveal the molecular basis for this phenomenon; however, it is clear that the rate of recombination is not continuously increased with progress toward the telomere of 4p.

INTRODUCTION

Huntington disease (HD) is a late-onset neurodegenerative disorder involving progressive choreic movements, cognitive decline, and dementia as a result of specific neuronal cell death (Martin and Gusella, 1986). The dominant genetic defect causing HD has been linked to the DNA marker *D4S10* at an estimated distance of 3–4 cM (Gusella *et al.*, 1983; Conneally *et al.*, 1989). Mapping of *D4S10* by dosage studies in Wolf–Hirschhorn syndrome (Gusella *et al.*, 1985), by *in situ* hybridization (Zabel *et al.*, 1986; Magenis *et al.*, 1986; Wang *et al.*, 1986; Landegent *et al.*, 1986), and by somatic cell hybrid analysis (MacDonald *et al.*, 1987; Smith *et al.*, 1988) has placed the marker in 4p16, most likely in the proximal portion of 4p16.3, the terminal cytogenetic subband of the chromosome. Multipoint linkage mapping using a proximal marker has established that the *HD* gene is located distal to *D4S10* within 4p16.3, which constitutes only 3% of the cytogenetic length of chromosome 4 or approximately 0.2% of the genome (Gilliam *et al.*, 1987a). Somatic cell hybrid panels that allow DNA probes to be mapped to either the proximal or the distal portions of 4p16.3 have been constructed (MacDonald *et al.*, 1987; Smith *et al.*, 1988). These have been used to generate several new markers, including *D4S43* (Gilliam *et al.*, 1987b), *D4S81* (Richards *et al.*, 1988), *D4S95* (Wasmuth *et al.*, 1988), and *D4S125* (Nakamura *et al.*, 1988; MacDonald *et al.*, 1989a) in proximal 4p16.3 and *D4S90* (Youngman *et al.*, 1988, 1989), *D4S98/S114/S113* (Smith *et al.*, 1988; Whaley *et al.*, 1988), *D4S111* (Pohl *et al.*, 1988; MacDonald *et al.*, 1989a), and *D4S115/S96* (Smith *et al.*, 1988; Pohl *et al.*, 1988; MacDonald *et al.*, 1989a) in the distal region. Recent evidence suggests that the *HD* gene is in the terminal half of 4p16.3, although the recombination events detected do not permit an unambiguous localization (MacDonald *et al.*, 1989b; Robbins *et al.*, 1989).

0888-7543/91 $3.00

The telomeric location of the *HD* defect has raised the possibility that it is in a region of relatively increased recombination. It has been suggested, on the basis of both chiasma counts and linkage information, that the frequency of recombination increases as the telomere is approached (Laurie and Hulten, 1985; Tanzi *et al.*, 1988). Initial data derived from a 200-kb chromosome jump toward the telomere from *D4S10* suggested relatively increased recombination in 4p16.3 (Richards *et al.*, 1988). We have isolated two new DNA markers, *D4S126* and *D4S127*, that map in the proximal portion of 4p16.3 by somatic cell hybrid analysis. Physical mapping of these markers relative to several previously positioned loci and the assessment of their linkage relationships have revealed much higher than average recombination immediately distal to *D4S10*, accounting for much of the genetic separation between this marker and *HD*. However, this increased rate of recombination was not maintained with further progress toward the telomere, suggesting that specific sequence or structural characteristics in the vicinity of *D4S10* may promote recombination.

MATERIALS AND METHODS

Cell Lines

HHW693 is a human–hamster hybrid cell line containing a human translocation chromosome composed of 4pter–4p15.1 and 5p15.1–5cen from which 5q has been deleted (Wasmuth *et al.*, 1986). The cosmid library used in this study was prepared by inserting genomic DNA partially digested with *Sau*3A into the *Bam*HI site of pCOS2 (Poustka *et al.*, 1984). The hybrid cell lines used in the regional mapping panel for 4p have been described previously (MacDonald *et al.*, 1987; Smith *et al.*, 1988). Human lymphoblastoid cell lines from the Venezuela reference pedigree (Tanzi *et al.*, 1988) were established from blood samples by EBV transformation (Anderson and Gusella, 1984). DNA was prepared from all cell lines as previously described (Gusella *et al.*, 1979).

Cosmid Library Screening and Regional Mapping

Individual cosmids from the library were picked into a gridded array on nitrocellulose filters. After incubation overnight at 37°C, replica filters were prepared from the master filter, reincubated, and subsequently processed for hybridization (Gusella *et al.*, 1980). Cosmids containing human inserts were then identified by hybridization of duplicate filters to total human genomic DNA or total hamster genomic DNA labeled with ^{32}P by the random hexamer priming technique (Feinberg and Vogelstein, 1983). The filters were washed to a final stringency of 0.1× SSC at 65°C (Gusella *et al.*, 1980), and cosmids showing stronger hybridization signal with the human probe than with the hamster probe were picked for further characterization. Individual cosmid DNAs were miniprepped by alkaline lysis (Maniatis *et al.*, 1982), labeled with ^{32}P (Feinberg and Vogelstein, 1983), and hybridized directly to regional mapping panels using unlabeled human genomic DNA to suppress repeat sequence hybridization (Sealey *et al.*, 1985).

Linkage Analysis

Typing of restriction fragment length polymorphisms was carried out by Southern blot analysis as previously described (Gusella *et al.*, 1983). Linkage data were analyzed using the MAPMAKER program to define marker order and to estimate recombination frequencies (Lander *et al.*, 1987).

RESULTS

Isolation of Probes from 4p16.3

Thirty-five cosmids containing human DNA were isolated from a library constructed with DNA from the human–hamster hybrid HHW693 (Wasmuth *et al.*, 1986). This line retains 4pter–4p15.1 and 5p15.1–5cen as its only human material. Individual cosmids were labeled with ^{32}P, preannealed to human genomic DNA to minimize the contribution of repeat sequences (Sealey *et al.*, 1985), and then used directly as probes against DNA blots of a somatic cell hybrid panel designed to localize them regionally within the terminal half of 4p (MacDonald *et al.*, 1987; Smith *et al.*, 1988). The results are summarized in Fig. 1. As expected, approximately one-half of the clones derived from chromosome 4 (18 of 35) but most mapped centromeric to 4p16.3. However, two cosmids, BJ14 and BJ56, were assigned to the proximal portion of 4p16.3, the same physical region as *D4S10* (Fig. 1). This regional assignment was later confirmed with single-copy probes derived from the cosmids (see example in Fig. 2) which have been assigned the locus names *D4S126* and *D4S127*, respectively.

Physical Localization of D4S126 and D4S127

A long-range physical map of 4p16.3 is emerging from pulsed-field gel electrophoresis studies (Bucan *et al.*, 1990). The map spans over 5000 kb in the region between *D4S10* and the telomere of 4p, with two gaps of unknown size, one between *D4S125* and *D4S95* and one between *D4S113* and *D4S115* (Fig. 3A). A third gap in the map exists proximal to *D4S10*, which shares no common pulsed-field gel fragments with the *D4S62* locus probably located in 4p16.2.

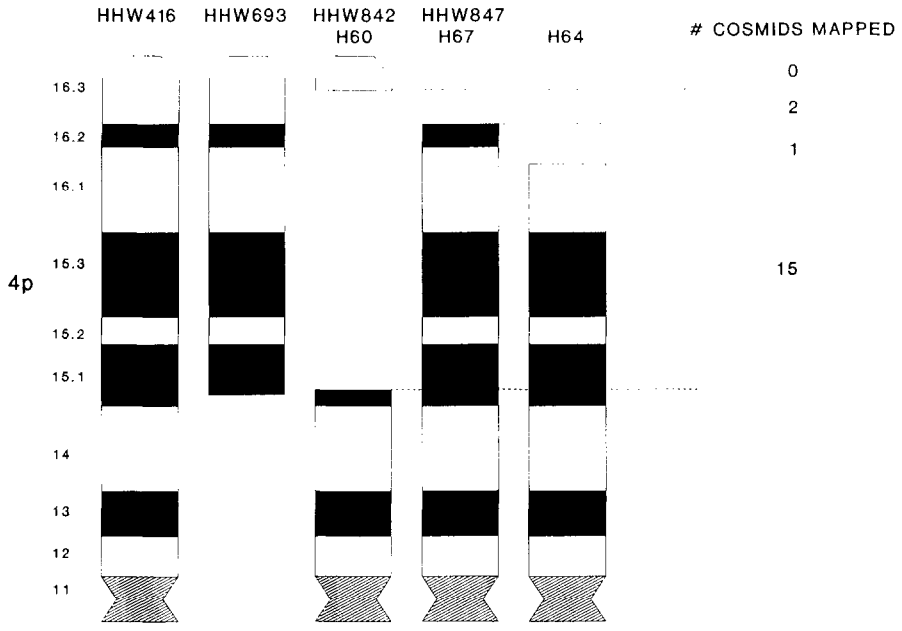

FIG. 1. Regional distribution of chromosome 4p probes. Eighteen cosmids containing human inserts from the HHW693 library were mapped using a regional somatic cell hybrid panel (18, 32). The approximate locations of critical hybrid breakpoints distinguishing regions of 4p16 are shown under the names of the corresponding cell hybrids. In two cases, comparable human × mouse and human × hamster hybrids, containing identical translocation or deletion chromosomes, were available. The numbers of cosmids mapping to each of the four readily distinguishable physical regions are shown.

D4S126 and *D4S127* have been placed on the 4p16.3 physical map by using single-copy subclones (see below) as probes. *D4S126* maps between *D4S10* and the recently described VNTR marker *D4S125*, as shown in Fig. 3A. In the cell line GM1416B, BJ14 (*D4S126*) and YNZ32 (*D4S125*) detect common *Not*I and *Mlu*I fragments, whereas *Nru*I digestion yields a common fragment shared by these probes and G8 (*D4S10*) (Fig. 4). Double digests with these enzymes and mapping with other sources of genomic DNA have produced the map shown in Fig. 3A. *D4S126* is located ~200 kb distal to *D4S10* and within 60 kb of *D4S81*, a locus defined previously on the basis of a chromosome jump from *D4S10* (Richards *et al.*, 1988). *D4S125* maps an additional 100–200 kb telomeric to *D4S126*.

D4S127 maps in a more distal cluster of markers that includes *D4S95* and *D4S43*. Of these three, *D4S127* is proximal, separated from *D4S95* by a single *Nru*I site. It has not yet been possible to connect *D4S125* and *D4S127* by pulsed-field mapping but, in addition to a gap of unknown size, they are separated by a minimum of 700 kb of DNA.

Identification of Restriction Fragment Length Polymorphisms

Single-copy fragments were isolated from BJ14 and BJ56 cosmids by random subcloning using double digests with either *Hin*dIII or *Pst*I and *Sau*3AI designed to produce numerous small subclones of <1.5 kb. Five to ten subclones from each cosmid were screened for RFLPs by hybridization to Southern blots of genomic DNA from five unrelated individuals, each DNA digested with 35–40 restriction enzymes (Gilliam *et al.*, 1987c). In some cases, larger restriction fragments excised directly from the cosmid were also used for RFLP screening.

The RFLPs identified are presented in Fig. 5 and Table 1. Two useful RFLPs were found with probes from the BJ14 cosmid. The subclone p309 detected a very informative two-allele system with *Sac*I, while a two-allele *Ssp*I RFLP was visualized using a 2.5-kb *Bgl*I fragment taken directly from the cosmid. Three subclones from BJ56 detected RFLP. p358 reveals a three-allele RFLP with the restriction enzyme *Pvu*II. p359 detects a two-allele *Pvu*II RFLP that is in linkage disequilibrium with the *Pvu*II RFLP of p358 and a second two-allele RFLP with *Sca*I. A third subclone, p363, detects a two-allele RFLP with *Stu*I. Except for the *Pvu*II RFLPs detected with p358 and p359, none of the other polymorphisms are in strong disequilibrium with each other, thereby increasing the informativeness of these loci as genetic markers.

Genetic Linkage Relationships of the 4p16.3 Markers

The Venezuela reference pedigree, derived from the large HD kindred responsible for the discovery of link-

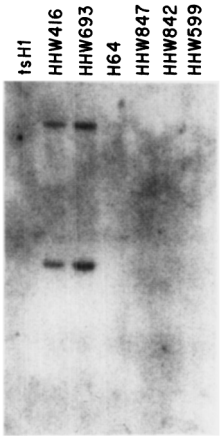

FIG. 2. BJ14 maps to 4p16.3. Each lane contains 5 μg of *Hin*dIII-digested DNA from hamster or hybrid cell lines fractionated by agarose gel electrophoresis, transferred to nylon filters, and hybridized to probe p309. tsH1 is the parent hamster cell line, HHW416 retains an intact human chromosome 4 as its only human material, and HHW599 contains chromosome 5 as its only human material. The other hybrids contain the chromosome 4p regions shown in Fig. 1. Since the p309 probe was isolated by random subcloning using *Sau*3A+ *Hin*dIII, the detection of two *Hin*dIII fragments indicates the presence of two small noncontiguous fragments of the cosmid in this subclone.

age between the disorder and *D4S10,* has previously been used to construct a linkage map of 4p16.3 (MacDonald *et al.,* 1989b). This map extended from *D4S10* in proximal 4p16.3 to *D4S90* within 250 kb of the 4p telomere, and spanned 5.6 cM. We have typed the reference pedigree with the RFLPs for *D4S125, D4S126,* and *D4S127* to confirm the physical map order and to determine the genetic distance between these loci. Two-point analysis demonstrated these loci to be highly informative in the data set, giving lod scores for linkage to *D4S10* of 92.78 at $\hat{\theta} = 0.04$, 70.2 at $\hat{\theta} = 0.02$, and 67.3 at $\hat{\theta} = 0.03$, respectively. When the loci were tested by multipoint analysis, they fit cleanly into the linkage map without disrupting the previously established marker order. The updated genetic map is given in Fig. 3B. In agreement with the physical map, *D4S125* fell between *D4S10* and *D4S43/S95,* with odds of 10^7:1 over the next most likely order (*D4S10–D4S43/S95–D4S125*). Similarly, *D4S126* mapped between *D4S10* and *D4S125,* with odds of 10^7:1 over the next best location, proximal to *D4S10. D4S126* showed no crossovers with *D4S81,* which maps physically to the same region, but is much less informative in this data set. Finally, placement of *D4S127* between *D4S125* and *D4S43/S95* was $10^{2.3}$ times more likely than that between *D4S10* and *D4S125,* the second-best location.

Surprisingly, the recombination frequencies for the intervals *D4S10* to *D4S126* and *D4S126* to *D4S125* were 2.4 and 1.1%, respectively, despite the relatively small physical size of this region, which encompasses only 300 to 400 kb of DNA. By contrast, the physical region of 600–700 kb separating *D4S95* from *D4S43* contained no recombination events in this data set, although both loci are highly informative (pairwise lod $z = 62$, $\hat{\theta} = 0.0$). It should be noted that only one or two recombinants would have been expected for this interval (assuming 1 cM = 10^6 bp) so that the absence of any recombinants does not constitute significant evidence for a "cold spot" in this region. The physical distance between *D4S10* and the telomere of 4p cannot be estimated with certainty because of the two remaining gaps in the long-range restriction map, but must contain a minimum of 4.4×10^6 bp of DNA. Overall, the map distance from *D4S10* to *D4S90* increased to 6.1 cM with the addition of the three new loci, but more than half of this genetic distance is compressed into 300 to 400 kb of the physical map. The remaining 4×10^6 or more base pairs between *D4S125* and the telomere constitutes only 2.6 cM of genetic distance, more in line with the 1 cM = 10^6 bp average expected based on the entire genome. The statistical significance of the apparent increase in recombination near *D4S10* was tested by using a likelihood ratio test (which approximates a χ^2) and the difference in the ratio of genetic to physical distance was found to be highly significant ($P < 0.001$).

DISCUSSION

The use of DNA markers for human linkage analysis has demonstrated that considerable variation exists throughout the human genome in rates of recombination. Many chromosomal linkage mapping studies have documented dramatically different rates of crossing-over for particular regions in male versus female meioses. Similarly, where physical maps have been available, differences have been observed in the relationship between rate of recombination and physical distance along the chromosomal DNA. For example, on chromosome 21q, fully 40% of the crossovers occur in the terminal 10% of the chromosome arm (Tanzi *et al.,* 1988). This relative increase in recombination in the distal region agrees with predictions from the distribution of chiasmata, which also occur most frequently toward the telomere on other chromosomes (Laurie and Hulten, 1985).

The mapping of the *HD*-linked marker, *D4S10,* near the telomere of 4p has created the impetus for constructing detailed genetic and physical maps of the terminal segment of 4p. Comparison of these maps reveals that there is no continuous increase in the rate of recombination as the telomere is approached. Rather, a striking increase in recombination occurs over a relatively small region of 300 to 400 kb near *D4S10,* but in the 4 to 5 $\times$ 10^6 bp of DNA

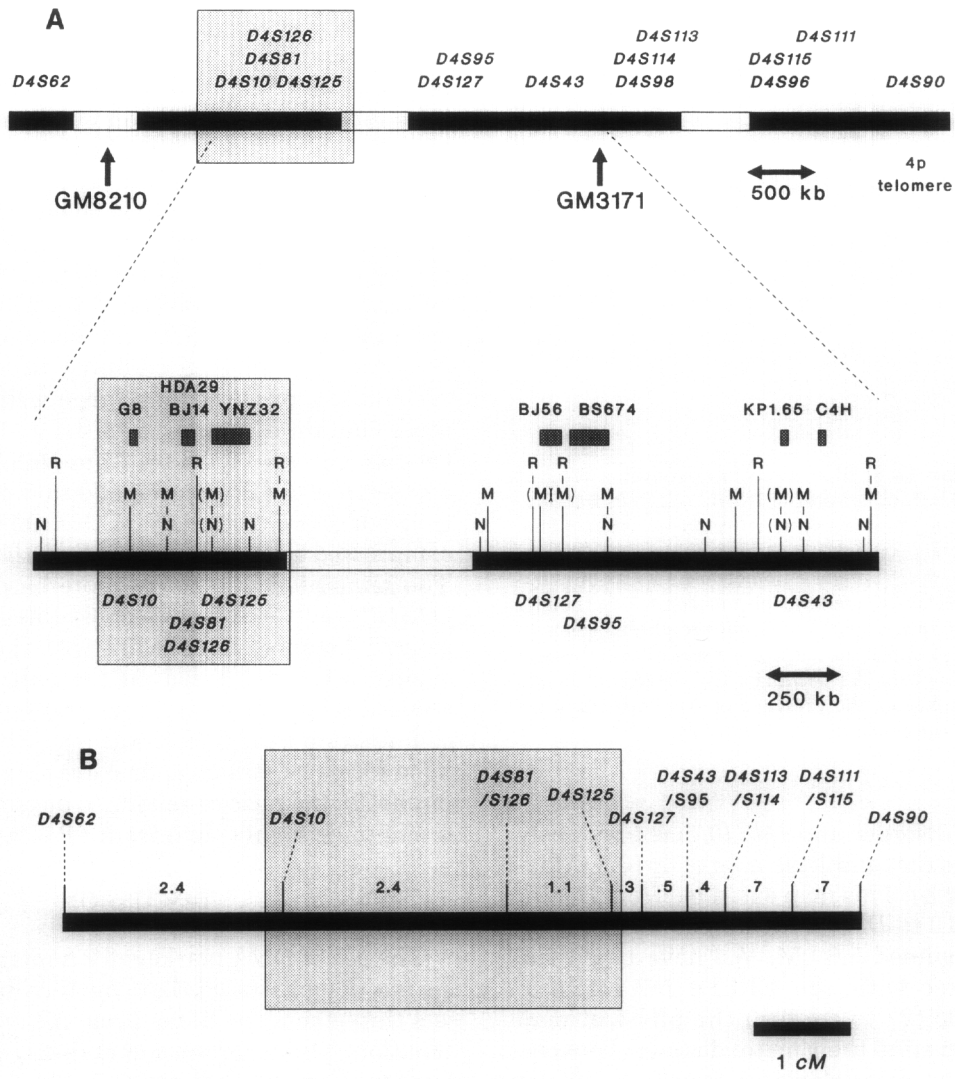

FIG. 3. Positions of *D4S125*, *D4S126*, and *D4S127* in the physical and genetic maps of 4p16.3. (**A**) The long-range restriction map is based on Bucan *et al.* (3). The upper portion represents the map drawn to scale from *D4S62* to the telomere and shows the placement of the chromosome breakpoints used in the somatic cell hybrid panel. The GM3171 deletion chromosome is in hybrids HHW842 and H60. The GM8210 t(4;21) chromosome is in hybrids HHW847 and H67. Three gaps in the PFG map, representing areas that have yet to be visualized, are of unknown length and are therefore each denoted as an arbitrary light-color segment. In the lower portion, the central portion of the map is expanded to show the restriction sites (N = *Not*I, M = *Mlu*I, R = *Nru*I) detected in cell line GM1416B. Sites shown in parentheses are only partially digested in this cell line. The positions of the three markers on the visualized (darkened) segments were determined by single and double digests such as those examples shown in Fig. 4, using GM1416B and other DNA sources (3). The boxed area in each part of the figure denotes the region of increased recombination also illustrated on the genetic linkage map in **B.** (**B**) The genetic linkage map shown represents a complete reanalysis of that reported in MacDonald *et al.* (20), with the addition of the three new markers (*D4S125*, *D4S126*, and *D4S127*) along with the proximal marker *D4S62* for comparison of recombination frequency. There was no significant difference in recombination frequency between male and female meioses. All marker loci that showed no recombination with each other are shown as appended symbols (e.g., *D4S43/S95*). The region of increased recombination is boxed as in **A** for comparison. The order of all marker loci was established without reference to physical map data and was supported by odds of at least 1000:1 with the single exception of *D4S127*, which was placed with odds of about 200:1.

distal to this point, recombination is at least 10-fold lower and is closer to that expected based on the average for the human genome. It is likely that at least a portion of the DNA that defines *D4S10* must be included in the region of increased recombination, since several cases of intralocus crossing-over between individual RFLP sites have previously been reported (Gilliam *et al.*, 1987a; Ikonen *et al.*, 1990; Curtis *et al.*, 1989; Skraastad *et al.*, 1989). *HD* is located 3–4 cM distal to *D4S10* (Conneally *et al.*, 1989). Consequently, the bulk of the crossovers reported between this marker and the genetic defect occur within the *D4S10-D4S125* segment. Beyond this region, relatively fewer crossovers with *HD* can be expected, and those events that have been observed have not permitted unequivocal positioning of the *HD* gene, al-

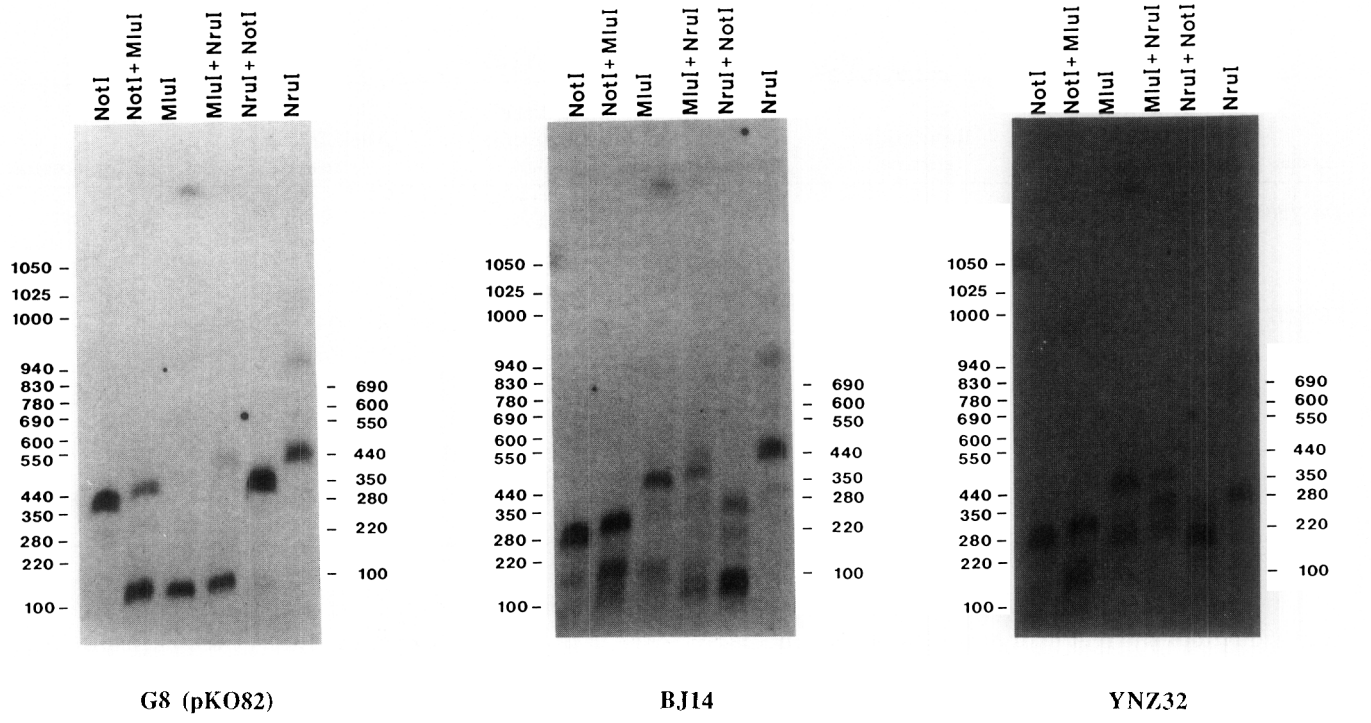

FIG. 4. Pulsed-field gel blots showing common fragments detected using single-copy probes at *D4S10*(pK082), *D4S126*(p309), and *D4S125*(pYNZ32). Pulsed-field gel electrophoresis using DNA from the human lymphoblastoid cell line GM1416B was carried out as described in Bucan *et al.* (3), and the same filter (published for pk082 in (3)) was hybridized sequentially to each of the three probes.

though a position very close to the telomere appears favored (MacDonald *et al.*, 1989b; Bates *et al.*, 1990).

Interestingly, the relative increase in recombination for the 200 kb between *D4S10* and *D4S126* is observed equally in males and females. The increase might be due to the overall structure of the chromosome in the region, or could be the result of specific sequences that promote recombination. It has been reported that minisatellite sequences may mark frequent crossover sites, and it is conceivable that one or more of these will be found within this small segment. However, several "variable number of tandem repeat" markers (VNTRs), including *D4S125, D4S95, D4S115,* and *D4S111,* have already been located within the more distal region where normal to reduced recombination rates are observed (Pohl *et al.*, 1988; MacDonald *et al.*, 1989a). Thus, not all tandemly repeated segments may act to promote recombination.

With a collection of recombination events already defined in the Venezuela reference pedigree, it should be possible to determine whether these crossovers oc-

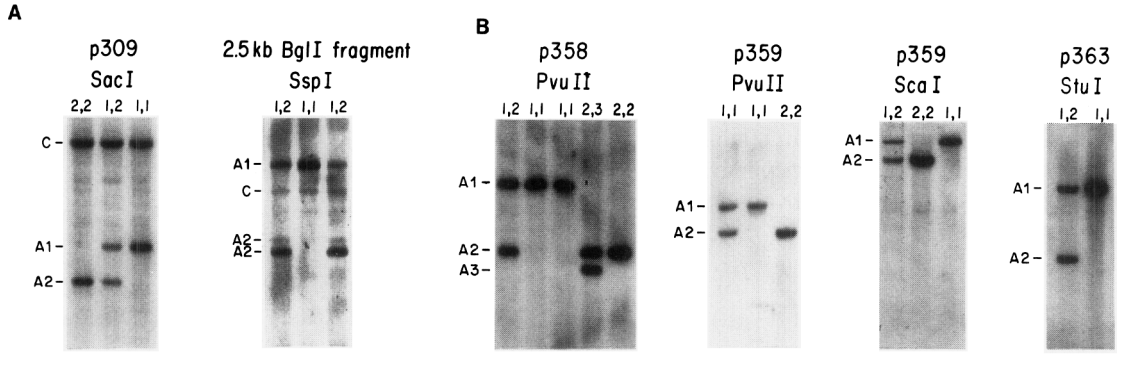

FIG. 5. RFLPs at the *D4S126* and *D4S127* loci. Allelic fragments (A1, A2, A3) are indicated for RFLPs detected with subclones or DNA fragments from the cosmids BJ14 (**A**) and BJ56 (**B**). C denotes constant bands. Genomic DNAs isolated from lymphoblastoid cell lines were used in screening for RFLPs (9) with the following enzymes: *Apa*I, *Ava*I, *Bam*HI, *Ban*I, *Ban*II, *Bcl*I, *Bgl*I, *Bgl*II, *Bgl*III, *Bsp*1286, *Bst*EII, *Bst*NI, *Bst*xI, *Dra*I, *Eco*RI, *Eco*RV, *Hinc*II, *Hin*dIII, *Hin*fI, *Hph*I, *Kpn*I, *Msp*I, *Nci*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I, *Sau*96I, *Sca*I, *Stu*I, *Sty*I, *Taq*I, *Xba*I, and *Xmn*I.

TABLE 1

RFLPs at *D4S126* and *D4S127*

Locus	Probe name	Restriction enzyme	Invariant fragments (kb)	Allelic fragments (kb)	Allele frequency tested	Chromosomes
D4S126	p309	*Sac*I	9.3	4.3	0.46	186
				3.8	0.54	
	2.8-kb *Bgl*I fragment	*Ssp*I	8.4	12.5	0.85	52
				5.6 + 6.2	0.15	
D4S127	p358	*Pvu*II		3.1	0.61	232
				2.0	0.36	
				1.8	0.03	
	p359	*Pvu*II		6.0	0.61	126
				4.5	0.39	
	p359	*Sac*I		26	0.83	110
				20	0.17	
	p363	*Stu*I		10.1	0.40	186
				6.1	0.60	

cur throughout the region or are clustered around particular sites. This will require cloning the region, saturating it with closely spaced DNA polymorphisms, and typing each of these in the critical crossover individuals. If the crossovers occur randomly throughout the *D4S10–D4S125* stretch, then it will be necessary to determine whether the structure of the region differs in some significant way from that of the surrounding regions. Currently, the only atypical characteristic of the immediate *D4S10* segment is that over a stretch of at least 25–30 kb it is essentially devoid of repetitive DNA elements. This is certainly not the case for the original clones defining *D4S81* and *D4S126,* which do contain repetitive elements, indicating that the entire region of high recombination is not single-copy DNA. If it is found that the recombination events are clustered over a relatively small segment, it would then be possible to determine the specific sequence found at the site of high recombination frequency. Analysis of such sequences in the mouse has implicated a distinct molecular organization (Shiroishi *et al.,* 1990). Interestingly, however, the same study presented evidence that sequences promoting recombination in the mouse histocompatibility complex are within the vicinity of, but not at, the "hot spot." Molecular definition of such a hot spot in humans would permit a direct experimental approach to identifying the fundamental mechanisms by which recombination is increased.

ACKNOWLEDGMENTS

We thank the members of the Venezuela Collaborative Huntington's Disease Project for collection of blood samples from the reference pedigree and Dr. Y. Nakamura and Dr. R. White of the Howard Hughes Medical Institute for the pYNZ32 probe. We are grateful to Dr. A. M. Frischauf, Dr. David Housman, and Dr. Allan Tobin, our colleagues in the Hereditary Disease Foundation HD Collaborative Research Group, for their helpful discussions. This work was supported by NINCDS Grants NS16367 (Huntington's Disease Center Without Walls), NS22031, and NS20012 and by grants from the W. M. Keck Foundation, the Hereditary Disease Foundation, and the Huntington's Disease Society of America. B.A. and W.L.W. received postdoctoral fellowships from the Huntington's Disease Society of America and the Wills Foundation.

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