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Genetics

A cloned DNA segment from the telomeric region of human chromosome 4p is not detectably rearranged in Huntington disease patients

(radiation hybrids/pulsed-field gel electrophoresis/DNA rearrangements/saturation cloning)

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ABSTRACT **Genetic linkage studies have mapped the Huntington disease (HD) mutation to the distal region of the short arm of human chromosome 4. Analysis of recombination events in this region has produced contradictory locations for *HD*. One possible location is in the region distal to the *D4S90* marker, which is located within 300 kilobases of the telomere. Other crossover events predict a more centromeric position for *HD*. Here we analyze the telomeric region of 4p in detail. Cloned DNA segments were derived from this region by utilizing a radiation-induced somatic cell hybrid as a source of DNA combined with preparative pulsed-field gel electrophoresis to enrich for the telomeric fraction. Additional DNA was obtained by using the cloned segments as multiple start points for cosmid walks. This strategy proved to be an effective method for cloning 250 kilobases of DNA in the region telomeric to *D4S90*. Hybridization analysis with the cloned DNA did not provide any evidence for the presence of rearrangements of 100 base pairs or greater in the DNA of individuals affected with HD. We also found no change in the size or structure of the 4p telomere in these samples.**

Huntington disease (HD) is a fatal neurodegenerative disorder of humans that is inherited as an autosomal dominant and highly penetrant trait (1). The disease usually begins between the ages of 30 and 45 years and progresses unremittingly for 15–20 years. Little is known about the biochemical basis of the disease. However, significant progress toward understanding HD has been achieved through genetic studies. In 1983, linkage analysis of two HD pedigrees mapped the mutation to the end of the short arm of chromosome 4, close to the polymorphic marker *D4S10* (2). All subsequent families tested showed linkage to this region (3), suggesting that a single locus is mutated in all individuals affected by the disease. As *HD* also possesses a low mutation rate (1), the same mutation is generally believed to be present in all patients. This view is substantiated by genealogical studies that trace the disease to a single geographical origin (1) and by linkage disequilibrium studies that show that the disease probably arose from a single founder individual (4, 5). An additional feature of HD is that likely homozygotes show no more severe symptoms than heterozygotes, demonstrating that the disease is inherited in a completely dominant fashion (6).

More recent meiotic linkage studies have mapped *HD* to the region between *D4S10* and the 4p telomere (7) in a segment of DNA spanning ≈6000 kilobases (kb) (Fig. 1). Many new DNA markers have been isolated from this region (8–10), several of which have been used to identify recombination events with respect to *HD*. Of the crossover events identified, three suggest a telomeric location for the mutation (9, 11, 12). One of these crossover events also locates *HD* to a 300-kb region between the telomere and *D4S90* (Fig. 1; ref. 12), the most terminal marker yet described for 4p (13, 14). However, an inconsistency in the family data has arisen through the discovery of other crossover events that assign *HD* a centromeric location with respect to *D4S90* (Fig. 1; refs. 11, 15). Such a location is supported by the finding that markers in this more centromeric region show significant linkage disequilibrium with *HD*, whereas telomeric markers, such as *D4S90*, do not (4, 5).

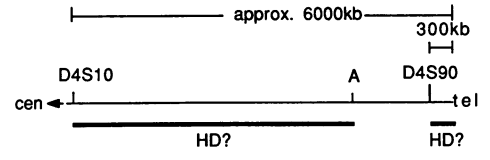

FIG. 1. Possible locations for *HD* within the *D4S10*-telomere region. The 6000-kb region extending from *D4S10* to the 4p telomere is shown. The two likely positions for *HD* are indicated by the black bars; boundaries of the proximal location are presently defined by *D4S10* on the centromeric side and marker A on the telomeric side, which represents the cluster of loci *D4S96/D4S97/D4S115*.

In this report, we present an analysis of one of the regions predicted to contain *HD*: the 4p telomeric region. DNA was cloned from this region by utilizing a radiation-induced somatic cell hybrid as an enriched cloning resource. Additional enrichment was achieved by using pulsed-field gel electrophoresis (PFGE) to fractionate *Not* I-digested DNA of the hybrid, followed by gel elution of the 4p telomeric fraction. DNA segments obtained from this procedure, in combination with cosmid clones derived from chromosome walks, were used to compile a contiguous series of overlapping clones ("contig") extending from *D4S90* for 250 kb in the direction of the 4p telomere.

As the contig is derived from one of the mapped intervals thought to contain *HD*, it provides a useful resource with which to search for the HD mutation. To begin the search we considered the low mutation rate and complete dominance of *HD*. These are unusual characteristics for a genetic trait and suggest that the disease may have arisen from a very specific DNA rearrangement rather than from a point mutation, which would be expected to occur more than once. By analogy to dominant mutations described in *Drosophila* (16), such rearrangements are likely to arise from the insertion of transposable elements or from larger rearrangements such as chromosomal translocations or deletions. In the present

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Abbreviations: HD, Huntington disease; PFGE, pulsed-field gel electrophoresis; FIGE, field-inversion gel electrophoresis; CHEF, contour-clamped homogenous electric field; contig, group of clones with contiguous nucleotide sequence.

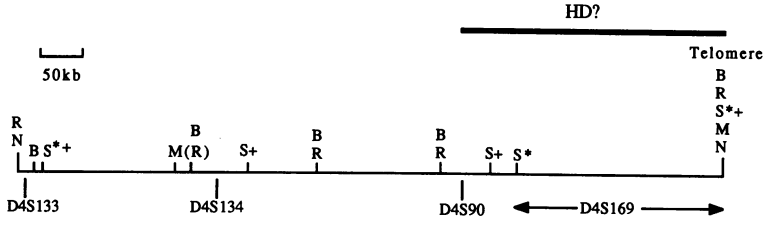

FIG. 2. Long-range restriction map of the four loci located in the 4p telomeric region. Restriction enzymes: *Not* I (N), *Mlu* I (M), *Nru* I (R), *Bss*HII (B), *Sal* I sites in 9TK (S+), *Sal* I sites in blood (S*). The partially cleaved *Nru* I site that links *D4S133* with *D4S134* is shown in parentheses. The possible location of *HD*, between *D4S90* and the 4p telomere, is represented by the black bar.

study we perform a test of the "rearrangement hypothesis" for the telomeric region by analysing HD patient DNA under conditions that can resolve DNA size changes of at least 100 base pairs (bp). Our investigations do not detect a rearrangement associated with HD, suggesting that the HD mutation must be small if it is located within the 4p telomeric region.

MATERIALS AND METHODS

Cell Lines. C25 is a radiation-induced hamster–human hybrid retaining ≈15,000 kb of human DNA from chromosome 4 and little other human DNA (17). 9TK is a human–hamster somatic cell hybrid containing chromosome 4 as its only detectable human component. Lymphoblastoid cell lines from U.S. and Venezuelan HD families and from non-HD individuals were purchased from the NIGMS Human Cell Repository (Camden, NJ).

PFGE. DNA-agarose blocks were prepared from the lymphoblastoid cell lines, C25, 9TK, and human blood as described (10) and were digested with 40 units of restriction enzyme for 12 hr as recommended by New England Biolabs. Field-inversion gel electrophoresis (FIGE) was performed with a switch apparatus from MJ Research (Cambridge, MA; ref. 10). Electrophoresis in a contour-clamped homogenous electric field (CHEF) was performed in a unit constructed according to Chu *et al.* (18).

Gel Electrophoresis and Southern Analysis. Genomic DNA was isolated from HD and non-HD cell lines and cleaved with the following restriction enzymes in single digestions: *Eco*RI, *Hin*dIII, *Hin*cII, *Pst* I, *Pvu* II, and *Sac* I. The resulting restriction fragments were fractionated by electrophoresis through 25-cm 1% agarose gels at 40 V for 18 hr or until fragments of 200 bp in size had reached the ends of the gels. The DNA was transferred to GeneScreen*Plus* nylon membranes (New England Nuclear) and hybridized as described in ref. 10. In the case of whole phage and cosmid DNA samples, probes were prehybridized in 200 μl of 0.1 M sodium phosphate (pH 7.0) containing 250 μg of sheared and denatured human placental DNA.

Library Construction. The method used for construction of the library is described in ref. 19. DNA from C25 in 10 agarose blocks was digested overnight with *Not* I and fractionated by FIGE in a 1% low-melting-point agarose gel (BRL Ultrapure). After electrophoresis, 2-mm slices perpendicular to the running direction were cut from the gel. Slices 22 and 23 were identified as the positive fractions by slot-blot analysis with a probe for the telomeric region. DNA was eluted from these fractions by incubation overnight with 100 units of agarase (Calbiochem) at 37°C, extracted with phenol, and precipitated with ethanol. The gel-eluted DNA was digested under partial conditions with *Sau*3AI (4 units of enzyme for 30 min at 37°C in 100 μl) to obtain fragments in the 10- to 20-kb size range, and DNA was treated with 2 units of alkaline phosphatase (Boehringer-Mannheim). After phenol extraction and ethanol precipitation, the DNA was ligated to 2-μg *Bam*HI-cleaved arms of Lambda Dash (Stratagene) in a volume of 65 μl. The ligated DNA was packaged to form a library, which was screened with a human genomic DNA probe (10).

Cosmid Library Screens. Unique-sequence probes derived from the ends of each contig were used to screen a human placenta cosmid library purchased from Stratagene. Conditions for screening the library and for purifying the cosmid clones were as recommended by the manufacturer.

Restriction Mapping of Phage and Cosmid Clones. Restriction maps were determined by partial digestion of the phage and cosmid DNA clones with *Hin*dIII or *Eco*RI, followed by hybridization with oligonucleotide probes specific for the T3 and T7 promoters in the phage and cosmid vectors as described in ref. 20.

RESULTS

PFGE Analysis of 4p Telomeric Region. We previously reported the use of irradiation/cell fusion procedures to generate a hybrid, designated C25, that contains the terminal 15,000 kb of 4p and little other human DNA (17). This cell line was used to isolate six DNA markers from the region between *D4S10* and the 4p telomere (10). Subsequently, an additional marker, *D4S169*, was identified from the same library and mapped to the *D4S10*-telomere region (unpublished data). PFGE analysis showed that three of the seven markers (*D4S133*, *D4S134*, and *D4S169*) lie on a common *Not* I fragment of 850 kb that is also shared by *D4S90*. Since the region near *D4S90* has been identified as one of the two intervals likely to contain *HD*, we constructed a more detailed physical map for the four loci by using PFGE (Fig. 2 and Table 1). The long-range restriction map was constructed

Table 1. Fragment sizes for the four loci

	Enzyme												
Locus	*Not* I	*Mlu* I	*Nru* I	*Sal* I (BL)	*Sal* I (9TK)	*Bss*HII	*Not* I + *Mlu* I	*Not* I + *Nru* I	*Not* I + *Sal* I	*Not* I + *Bss*HII	*Nru* I + *Bss*HII	*Nru* I + *Sal* I (BL)	*Nru* I + *Sal* I (9TK)
D4S133	850	360	210 (370)	340	340	90	190	210 (370)	30	20	20	30	30
D4S134	850	660	150 (370)	570	260 (580)	150 (370)	660	150 (370)	As *Sal* I	150 (370)	150 (370)	150 (370)	70
D4S90	850	660	340 (560)	570	290 (580)	340 (560)	660	340 (560)	As *Sal* I	340 (560)	340 (560)	80	70
D4S169	850	660	340 (560)	250	280 (580)	340 (560)	660	340 (560)	As *Sal* I	340 (560)	340 (560)	250	280

Sizes are given in kb. Weakly hybridizing bands arising from partial cleavage are shown in parentheses. To avoid confusion in the interpretation of the table, the homology bands detected by probes for *D4S134* and *D4S90* are not included.

with human DNA from two different sources: human blood and the hybrid 9TK, which retains human chromosome 4 in a background of hamster chromosomes. The restriction map is essentially the same for both sources of DNA, except for the presence of distinct *Sal* I sites, reflecting possible methylation differences in the two cell types.

Probes for *D4S90* and *D4S169* detect common *Bss*HII and *Nru* I fragments of 340 kb and 560 kb, indicating their close physical linkage in the genome (Fig. 2 and Table 1). Similarly, *D4S134* and *D4S133* are close to each other, located on a partial *Nru* I fragment of 370 kb. Digestions with *Mlu* I place *D4S134* closer than *D4S133* to *D4S90* and *D4S169*. In addition, *Sal* I digestions of blood DNA show that *D4S134* and *D4S90* lie on the same fragment of 570 kb, establishing the order *D4S133-D4S134-D4S90-D4S169* for the four markers (Fig. 2). *D4S90* is located within 350 kb of one end of the *Not* I fragment; this end is now known to represent the 4p telomere (14, 21). A major difficulty encountered during the construction of the restriction map was cross-hybridization of the probes for *D4S134* and *D4S90* to sequences located elsewhere on the 850-kb *Not* I fragment. To distinguish between cross-homology and partial digestion, we used other probes derived from regions close to *D4S134* and *D4S90*, which do not show the cross-homology, to confirm the PFGE map (data not shown; the probes were isolated as described below).

Isolation of New Markers from the 4p Telomeric Region. Analysis of DNA from the C25 radiation hybrid demonstrated that the 850-kb *Not* I band is present intact in this cell line. Hybridization with a probe for the *Alu* family of repeats also showed that C25 contains few, if any, other human *Not* I fragments of 850 kb (data not shown). Therefore, by using C25 in combination with PFGE fractionation and gel-elution techniques, DNA highly enriched for sequences in the distal end of 4p could be obtained. Although greater enrichment could, in theory, be achieved by use of other rare-cutter enzymes such as *Mlu* I or *Nru* I, analysis of C25 with the *Alu* probe showed that many other human fragments comigrated with the telomeric fragments generated by these enzymes. Thus, the strategy we followed was to digest C25 DNA with *Not* I, subject the DNA to PFGE, and elute fragments in the 850-kb size range. A λ library constructed with the eluted DNA was screened with a human DNA probe. Phage DNA preparations were made for 77 positive human clones, each of which was used as a hybridization probe against a mapping panel of somatic cell hybrids containing breaks in the *D4S10*-telomere region (10). Of the 77 clones analyzed, 26 were found to lie in the telomeric region of 4p, indicating that approximately one-third of the sequences in the eluted DNA was derived from the 850-kb *Not* I fragment. The remainder of the human DNA probably comprises contaminating sequences from other fragments in the 850-kb size range.

Saturation Cloning of a 250-kb Region Distal to *D4S90*. By comparing the *Eco*RI and *Hin*dIII restriction maps of the 26 human phage, overlaps were detected in several cases. When the overlaps were accounted for, 13 independent contigs could be defined. To determine the locations of these contigs, unique-sequence probes were derived from each and used for PFGE analysis. Surprisingly, 10 of the 13 contigs mapped to the terminal *Nru* I and *Bss*HII fragments of 340 kb, upon which *D4S90* and *D4S169* also reside (Fig. 2). Such a bias in the distribution of clones may reflect a greater cloning efficiency of sequences located close to the telomere. Alternatively, the sequences in this region may possess a greater number of repeated sequences than those in the more proximal part of the *Not* I fragment.

To link the 10 contigs from the telomeric region into a single segment, probes from the ends of each contig were used to screen a cosmid library. The cosmids obtained from the screens allowed most of the contigs to be linked into a single segment (Fig. 3). However, two gaps existed in the cloned DNA. Further screens of genomic libraries failed to obtain the DNA spanning these gaps, suggesting that the missing DNA does not clone efficiently in bacteria. Thus, to complete the restriction map of the large contig, human genomic DNA was cleaved with *Eco*RI or *Hin*dIII and hybridized with probes on both sides of the gaps. From this analysis, the two missing segments were both found to be <2 kb in size. The complete restriction map of the contig is shown in Fig. 3. The position of the *Sal* I site that separates *D4S90* from *D4S169* and generates a telomeric *Sal* I fragment of 250 kb in human blood DNA is shown on the contig. The contig extends for 200 kb from this site and is estimated to end within 50 kb of the 4p telomere. This distance from the telomere is only an approximate value, however, since the size of the *Sal* I

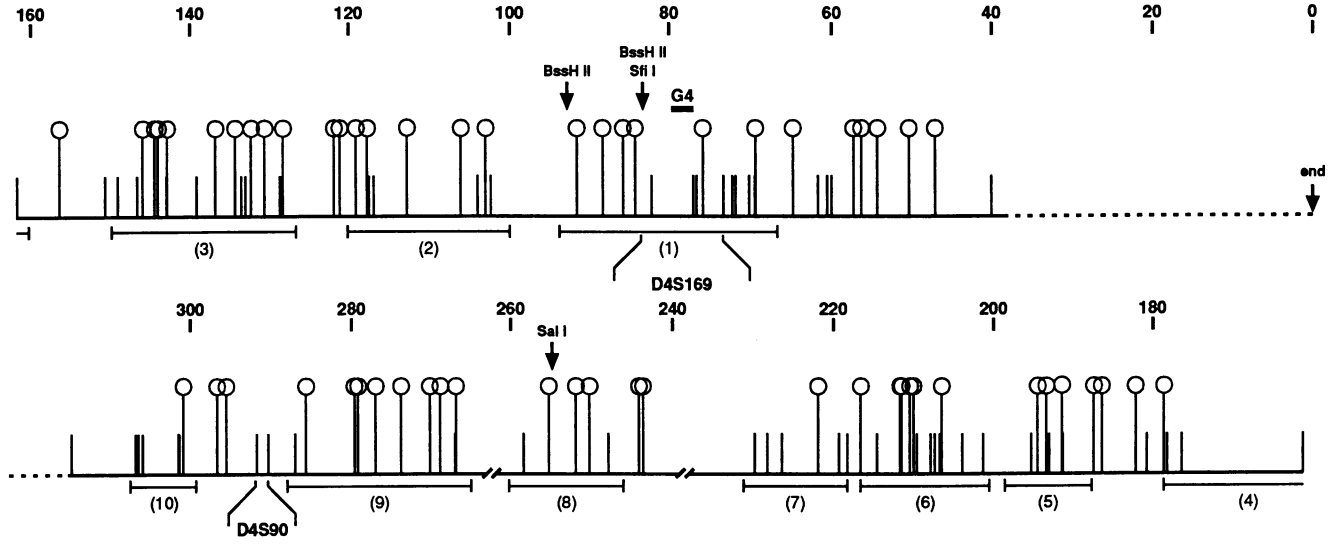

FIG. 3. Detailed restriction map of the cloned DNA. *Eco*RI (short vertical lines) and *Hin*dIII (long vertical lines with circles) sites are shown for the cloned DNA. The scale of the map is indicated in kb; 0 kb indicates the end of the chromosome. The positions of the 10 contigs obtained from the screen of the C25 λ library are labeled 1–10. The locations of the gaps in the contig (regions that we failed to obtain from repeated screens of cosmid and λ genomic libraries) are indicated by double slashes. The G4 probe is indicated by the black bar. A larger and more detailed version of this restriction map can be obtained from C.P. upon request.

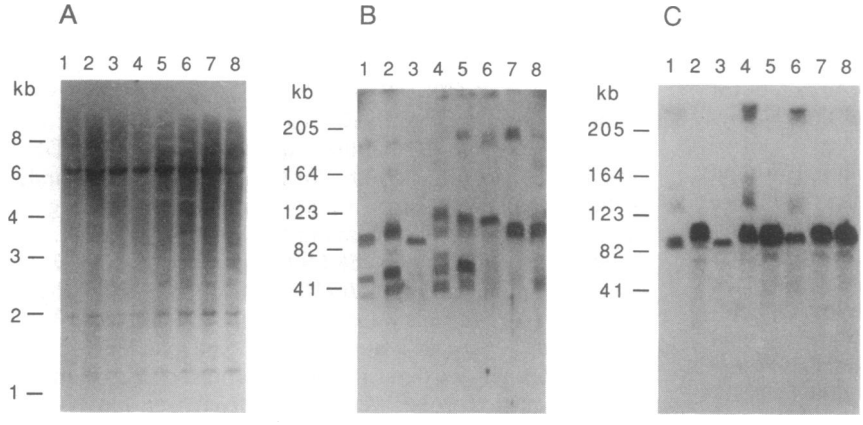

FIG. 4. Analysis of HD and non-HD DNA. Lanes 1 and 2, non-HD (GMO nos. 6316A and 5684B); lanes 3 and 4, HD heterozygotes from a U.S. family (GMO nos. 4219 and 4197); lanes 5 and 6, HD heterozygotes from a Venezuelan family (GMO nos. 6767 and 6651A); lanes 7 and 8, HD samples from members of the Venezuelan pedigree that are probably homozygous for HD (GMO nos. 5542B and 4856A; see ref. 6). (*A*) DNA was digested with *Hinc*II and hybridized with the λ probe for *D4S169*. (*B* and *C*) DNA was digested with *Bss*HII (*B*) or *Sfi* I (*C*) and hybridized with the G4 probe. CHEF electrophoresis was performed for 40 hr at 160 V with a switch time of 20 s. Multimers of the phage Lambda Zap (unit size = 41 kb) were used as size markers.

fragment is estimated from PFGE gels and the estimate may vary by as much as 20 kb.

Hybridization Analysis of HD Patient DNA. To investigate the hypothesis that the HD mutation involves a detectable chromosomal rearrangement in this region, genomic DNA from HD and non-HD individuals was hybridized to phage and cosmid clones from the contig. For this analysis, DNA samples collected from two different HD pedigrees were examined, including samples from individuals that are probably homozygous for *HD* (6). The genomic DNAs were cleaved with six different restriction enzymes that generate fragments <10 kb in size on average. After digestion, the DNA fragments were electrophoresed in high-resolution agarose gels and hybridized to probes spanning the length of the 250-kb contig. From our previous experience with agarose gels, we estimate that the electrophoresis conditions used for the analysis resolve DNA fragments differing by at least 1% of their length. This suggests that rearrangements involving as little as 100 bp of DNA could be detected in a single digest. By performing the analysis with several different restriction enzymes, the likelihood of detecting such a small DNA size change is greatly increased.

Changes in fragment sizes were occasionally observed for some probes. Further analysis showed that these alterations reflected restriction fragment length polymorphisms rather than the HD mutation itself; the polymorphisms were not present in all HD patients and were also observed in some unaffected individuals (data not shown). In no case did we detect a rearrangement consistent with the inheritance of HD (see Fig. 4*A* for an example). Genomic DNA fragments spanning the two gaps existing in the contig were also not rearranged at this level of resolution.

To determine whether structural rearrangement at the telomere is responsible for the disease, HD and non-HD genomic DNA was analyzed by PFGE. In this case, the DNA was digested with *Sfi* I or *Bss*HII, fractionated in a CHEF gel, and hybridized with a unique-sequence probe from contig 1, G4. The locus for G4 lies ≈80 kb from the end of the chromosome, telomeric to an *Sfi* I site and one of the *Bss*HII sites that have been identified within the cloned DNA of contig 1 (Fig. 3). Many small fragments of different sizes were observed when lymphoblastoid cell line DNA was analyzed with *Bss*HII (Fig. 4*B*). Such fragments probably arise through partial cleavage of *Bss*HII sites that are methylated to different degrees in the lymphoblastoid lines. The additional bands may also reflect *Bss*HII site polymorphisms, although this seems unlikely as the smaller fragments are not stoichiometric in intensity. All of the partial fragments are known to extend in the direction of the telomere since no other *Bss*HII sites have been identified in the cloned contig, apart from those located in contig 1 (Fig. 3). However, Bates *et al.* (21) have reported the presence of an additional *Bss*HII site at ≈50 kb from the end of the chromosome. This *Bss*HII site was not observed in the DNA we cloned but probably accounts for the appearance of the 40-kb band detected in some of the DNA samples (Fig. 4*B*). The diffuse appearance of the 80-kb telomeric fragments in *Sfi* I and *Bss*HII digestions (Fig. 4 *B* and *C*) can be attributed to the natural length variability frequently observed at the ends of chromosomes (22). Although considerable heterogeneity was observed with the G4 probe, particularly in the *Bss*HII digestions, we could find no DNA fragment that appeared consistently in the DNA of HD patients and that was not seen in the non-HD DNA.

DISCUSSION

Genetic linkage analysis with probes located on the short arm of human chromosome 4 has placed *HD* in a 6000-kb region between the *D4S10* marker and the 4p telomere (7). The relatively small size of this region has prohibited a finer localization of *HD* by the standard linkage approach, so that further attempts to map the mutation have relied on the direct analysis of a small number of HD families displaying crossover events in the *D4S10*-telomere region. Three of the identified recombination events are interpreted to indicate a telomeric location for *HD* (9, 11, 12). One of these also limits *HD* to a 300-kb region between *D4S90* and the 4p telomere (Fig. 1; ref. 12). Other identified recombination events exclude the telomeric region as a possible location for *HD* and predict, instead, that the mutation is located in a more centromeric region (Fig. 1; refs. 11, 15). Such an inconsistency in the family data has created a problem for studies aimed at cloning *HD*; until it is resolved, attempts to find the mutation must focus on both of the regions defined by the genetic studies. In this report we analyzed the 4p telomeric region in detail.

We first devised a strategy to clone the DNA from the telomeric region. The cloning strategy exploited a somatic cell hybrid, which contains the terminal 15,000 kb of 4p in a hamster cell and little other human DNA, as a cloning resource. Previously, we used this hybrid to construct a genomic library from which 1 of 10 human clones was located to the *D4S10*-telomere region (10). To achieve an additional enrichment for the telomeric region, preparative FIGE was used to fractionate *Not* I fragments in the size range known to contain the telomeric fragment. A library constructed with DNA eluted from this fraction was then screened with a human genomic DNA probe. Of the human clones obtained, 1 of 3 was found to be derived from the telomeric *Not* I fragment. Such a result indicates that we achieved a 3-fold greater enrichment for sequences in the *HD* region than by using total genomic DNA from the hybrid as a library resource. This cloning strategy also achieved a manyfold greater enrichment for sequences near the 4p telomere than any of the previously described cloning methods (8, 23).

The DNA sequences obtained from the enriched library were of great value in providing multiple start points for chromosome walks and for the saturation cloning of a 250-kb region near the 4p telomere. This combined cloning approach can be applied to other regions of the mammalian genome and may serve as an alternative or supplement to cloning large segments of DNA in yeast artificial chromosome (YAC) vectors (24). One problem with the method, however, is that it does not allow the cloning of sequences that do not propagate efficiently in bacteria, as illustrated by the fact that we failed to close two gaps existing in the contig or to isolate any sequences residing within 50 kb of the telomere. In these cases, alternative cloning methods may be required to obtain the missing DNA. Indeed, recent experiments have demonstrated the benefit of using YAC vectors to clone large fragments of DNA extending to the telomeres of human chromosomes (21, 25, 26).

The contig we have mapped extends for 250 kb from *D4S90* and ends within 50 kb of the 4p telomere. To look for *HD* within this DNA, we hybridized probes located throughout the contig to HD and non-HD genomic DNA in search of a chromosomal rearrangement that might be associated with the mutation. Our investigations failed to find such a rearrangement, even though the DNA was analyzed under conditions that we are confident would detect alterations involving as little as 100 bp. DNAs from individuals that are probably homozygous for HD were also not missing or rearranged, thus excluding the possibility that the mutation is represented by a deletion of the entire 250-kb contig. We additionally considered that the mutation could involve an alteration of DNA at the 4p telomere itself. This possibility was investigated by performing PFGE analysis with a distal probe from the contig. Again, however, no chromosomal rearrangement was detected in the HD samples. Thus, we can conclude that, if *HD* is located in this telomeric region, the mutation must be smaller than is detectable by the techniques we used.

An alternative, and perhaps more likely, possibility is that *HD* is not located in the telomeric region. Evidence now exists that the mutation may occupy a more centromeric location on 4p, and, given this possibility, searches for *HD* must now also focus on the other region. To begin the search, DNA located within the more centromeric region first needs to be cloned. Once this is achieved, a rational approach for finding the mutation may be to look for evidence of a chromosomal rearrangement, as has been attempted in this report.

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