A Yeast Artificial Chromosome Telomere Clone Spanning a Possible Location of the Huntington Disease Gene

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Summary

The Huntington disease (HD) gene has been mapped to the most distal subband of chromosome 4p. Analysis of recombination events has not provided an unequivocal location of the HD gene, but it indicates a position very close to the telomere as one possibility. We have constructed a yeast artificial chromosome (YAC) vector (containing a rare-cutter polylinker) for the cloning of mammalian telomeres, used it to prepare a BssHII-telomere library with DNA from an individual homozygous for HD, and have identified a 115-kb clone containing the telomere of 4p. One probable recombinant would confine the telomeric candidate location for the gene to the region covered by the YAC, which makes it possible that the clone described here contains the HD locus in its mutant form.

Introduction

Huntington disease (HD) is a progressive neurological disorder that typically produces emotional disturbances, lack of motor coordination, and intellectual deterioration. The onset of symptoms generally occurs in mid-life, followed by progression of the disease to death over 15–20 years (Martin and Gusella 1986). It is characterized pathologically by an extensive loss of specific neuronal classes, occurring primarily within the caudate nucleus and putamen and with the relative sparing of neurons in the rest of the brain. The biochemical defect underlying the mechanism of this cell death is unknown, and there is no existing treatment that will arrest the course of the disease.

The inheritance of HD is well documented; it is caused by an autosomal dominant gene with complete penetrance and low mutation rate (Martin and Gusella 1986). Genetic linkage to the anonymous DNA marker

D4S10 has localized the mutation to the short arm of chromosome 4 (Gusella et al. 1983), and multipoint linkage analysis has indicated that the gene is distal to this marker within the most telomeric subband, 4p16.3 (Gilliam et al. 1987b). The discovery of linked markers has allowed the development of a test for both the presymptomatic and prenatal identification of probable gene carriers (Meisson et al. 1988). It has also led to the identification of individuals with a high probability of being homozygous for HD, and it is interesting that the progression of HD in these individuals is indistinguishable from that in typical HD heterozygotes (Wexler et al. 1987; Myers et al. 1989). Recently, an intense effort has been employed to isolate closer and especially flanking markers to precisely define the position of the gene (Gilliam et al. 1987a; MacDonald et al. 1987; Pohl et al. 1988; Richards et al. 1988; Smith et al. 1988; Wasmuth et al. 1988; Whaley et al. 1988; Youngman et al. 1988; MacDonald et al. 1989a; Pritchard et al. 1989).

A long-range restriction map has been constructed with more than 20 independently derived DNA probes that map distal to D4S10. The map consists of three as yet unlinked segments that together cover 5 million bp (Mbp) and extends, at a minimum, 4 Mbp distal from D4S10 (Bucan et al. 1990) to a position charac-

terized as either a cluster of well-cut restriction sites or the telomere of 4p. Order and orientation of the segments have been determined by genetic linkage studies (Richards et al. 1988; Wasmuth et al. 1988; Whaley et al. 1988; MacDonald et al. 1989b; Youngman et al. 1989) and by somatic cell genetics (MacDonald et al. 1987; Smith et al. 1988). Analysis of recombination events in HD families (MacDonald et al. 1989b; Robbins et al. 1989) suggests that a possible location for HD is telomeric to the most distal published marker D4S90 (defined by probe D5) situated 300 kb from the end of the map (Bucan et al. 1990). Despite intense analysis, a definitive flanking marker has not yet been identified.

A 4p telomere clone would, at a minimum, set physical limits to the position of the gene and provide a definitive flanking marker to the mutation. We have set out to delineate and clone the telomeric candidate region and have used *Saccharomyces cerevisiae* as a direct route to selectively clone the telomere of the short arm of chromosome 4. Similar approaches using yeast artificial chromosome (YAC) vectors to clone mammalian telomeres by complementation have been described elsewhere (Brown 1989; Cheng et al. 1989; Cross et al. 1989; Riethman et al. 1989). YAC vectors contain a yeast centromere (CEN4) and replicating sequence (ARS1), selectable markers (URA3 and TRP1), and two cassettes of the telomeric repeat from Tetrahymena (TEL) (Burke et al. 1987). The vector telomeres are maintained by the addition of *Saccharomyces* telomeric repeats by the yeast telomerase (Shampay et al. 1984), a process that is template independent (Greider and Blackburn 1985). Telomeric repeats, which consist of tandem arrays of short (6–8-bp) G-T-rich sequences, are highly conserved, seemingly across all eukaryotic species (Blackburn 1984; Meyne et al. 1989). The human telomeric repeat differs by only one base from that of Tetrahymena (Moyzis et al. 1988) and is similarly functional in yeast. We have prepared a YAC vector (YAC-t2) for the selection of mammalian telomeres that contains a rare-cutter polylinker at the cloning site.

In the present paper we describe the isolation of overlapping cosmids, 2R88 and B31 (defining locus D4S142), which on further analysis of the distal region of 4p16.3 by pulsed-field gel electrophoresis (PFGE) were found to map approximately 200 kb distal to D4S90. An *Msp*I RFLP detected by p88-18 (a subclone from 2R88) suggests that the HD mutation may be yet distal to this locus. Several lines of evidence indicated that the end of the pulsed-field gel map is in fact the end of the chromosome. The YAC-t2 vector allowed

the isolation of the most distal 115-kb *Bss*HII fragment of 4p16.3 by the construction of a *Bss*HII-telomere library and its subsequent screening with a probe from 2R88. The construction of the library from an individual homozygous for *HD* guarantees that the clone described here derives from an HD chromosome.

Material and Methods

DNA and Cell Lines

Lymphoblastoid cell lines were as follows: GM1416B (karyotype 48 XXXX) (NIGMS, Human Genetic Cell Repository, Camden, NJ); GUSHMI is an individual from the Venezuela pedigree likely to be homozygous for *HD* (Wexler et al. 1987). Somatic cell hybrids included the following: HHW693, a human-hamster hybrid containing human 4p15.1-4pter translocated onto a fragment of the short arm of human chromosome 5 (Wasmuth et al. 1986); HHW842, a human-hamster hybrid containing human chromosome 5 and human chromosome 4 with an interstitial deletion of 4p14-4p16.3, retaining the terminal portion of 4p16.3 (Smith et al. 1988); HHW847, a human-hamster hybrid containing several human chromosomes, including human chromosome 5, and a t(4:21) chromosome in which 4p16.2-4pter is absent (Smith et al. 1988).

DNA Preparation, Digestion, Fractionation, Transfer, and Hybridization

DNA for conventional Southern blot analysis was extracted from peripheral blood leukocytes and lymphoblastoid cell lines by standard techniques. Isolation of high-molecular-weight DNA in agarose blocks from lymphoblastoid cell lines for both PFGE (7.5×10^5 cells/block) and cloning into YAC vectors (3×10^6 cells/block) were according to methods previously published (Herrmann et al. 1987). High-molecular-weight DNA from blood was prepared by the selective lysis of erythrocytes (Herrmann and Frischauf 1987), with modifications as described by Bucan et al. (1990). Chromosomes from *Saccharomyces cerevisiae*, for use as molecular-weight standards on pulsed-field gels (strain YP148) and for the analysis of YAC recombinants, were prepared according to a method described by Carle and Olson (1985). Lambda multimers were purchased from FMC Bioproducts.

Restriction enzymes were from NEB or BRL, and digestions were performed according to manufacturers' recommendations. Restriction of DNA for, and fractionation of DNA by, PFGE was essentially according

to methods described by Herrmann et al. (1987). PFGE was performed by contour-clamped homogeneous electric fields (CHEF) in an apparatus similar to that described by Chu et al. (1986), constructed at the EMBL, Heidelberg. Electrophoresis was at 5 V/cm in 0.25 × TBE at 14°C, with specific gel conditions and pulse times as described in the text and figure legends. DNA was transferred in alkali for 2 h onto Hybond-N+ (Amersham) from conventional agarose gels and for 48 h onto Hybond-N (Amersham) from pulsed-field gels. Probes were labeled in agarose to high specific activity by random oligonucleotide priming (Feinberg and Vogelstein 1984). Hybridizations were performed in 50% formamide at 42°C (Monaco et al. 1985), and filters were washed according to a method described by Church and Gilbert (1984). Probes containing low-copy repeat sequences were prehybridized with cold, sonicated total human DNA (Sealey et al. 1985). The oligomer (CCCTAA)$_4$ was labeled by kinasing and hybridized in 0.5 M Na$_2$HPO$_4$ pH 7.2, 7% SDS, 1 mM EDTA at 42°C for 20 h. The filters were rinsed three times in 3 × SSC, 0.1% SDS (1 × SSC = 0.15 M NaCl, 0.015 M trisodium citrate pH 7.0) at RT and were washed twice for 2 min in 3 × SSC, 0.1% SDS at 55°C.

Restriction Mapping of Cosmids

Restriction mapping of cosmids was done according to the method of Rackwitz et al. (1985) by using a computer program package, described by Zehetner and Lehrach (1986), for restriction-map analysis and manipulation.

Construction of Telomere Cloning Vectors YAC-t1 and YAC-t2

YCp50 (Hieter et al. 1985) is a pBR322-based plasmid which contains ARSI (yeast origin of replication), CEN4 (yeast centromere), and URA3 (selectable marker) while retaining the plasmid origin of replication, amp and tet genes. EcoRI, BamHI, and SalI are among its unique restriction sites. The 0.7-kb BamHI/XhoI fragment from YAC4 (Burke et al. 1987), which contains the TEL sequences, was isolated from an LMP agarose (BRL) gel by treatment with agarase (Burmeister and Lehrach 1989) and ligated into the BamHI/SalI sites of YCp50 to create YAC-t1 (8.38 kb). The correct construct was indicated by tet sensitivity and by the destruction of the SalI site and retention of a BamHI site. Cleavage with BamHI/EcoRI generates a linear telomere cloning vector with TEL sequences at one end and an EcoRI cloning site at the other. Replacement of the BamHI/EcoRI fragment with a rare-cutter polylinker containing NotI, SacII, SalI, MluI, ClaI, and SnaBI sites and flanked by BamHI and EcoRI complementary ends led to construction of YAC-t2 (8.05 kb). This polylinker was analogous to one previously described by Marchuk and Collins (1988) in the YAC-RC vector and, in addition to the unique restriction sites listed above, will accept DNA generated by BssHII, EagI, NaeI, NarI, NruI, SmaI, and XhoI digests because of their ligation compatibility with sites in the polylinker.

Preparation of the YAC Telomere Library

YAC-t2 was cleaved with MluI (NEB) and BamHI (BRL) and phosphatased (Boehringer-Mannheim). High-molecular-weight genomic DNA was prepared from GUSHM1 in agarose blocks at 3 × 10^6 cells/80 μl in 0.6% LMP agarose (BRL). This was digested to completion with BssHII (20 U, NEB) for 3 h. Digestion was terminated by incubation with proteinase K (1 mg/ml) (BDH) and 50 mM EDTA pH 8.0 at 37°C for 30 min. The proteinase K was inactivated with two 30-min incubations in 15 ml of 40 μg phenylmethylsulfonylfluoride (Sigma)/ml in TE (10 mM Tris pH 8.0, 1 mM EDTA) at 37°C. The blocks were equilibrated with NaCl such that the final salt concentration in the ligation reaction was 50 mM and were melted at 68°C for 5 min. Genomic DNA (30 μg) was gently mixed with 60 μg of BamHI/MluI-cut and phosphatased YAC-t2 and ligated overnight at 15°C in a total volume of 200 μl in 1 mM ATP, 40 mM Tris-HCl pH 7.6, 10 mM MgCl$_2$, 1 mM DTT with 5 μl T4 DNA ligase (400 U/μl; NEB). Two ligations of 30 μg of BssHII-cut GUSHM1 to MluI/BamHI-cut and phosphatased YAC-RC were prepared in parallel by the same protocol, with the exception that only 30 μg of vector was used.

The YAC-t2 ligation and one of the YAC-RC ligations were melted at 68°C for 5 min, diluted to 300 μl with 3% LMP agarose (BRL) in TE, and reset into blocks, which were loaded onto a 0.8% agarose gel in TAE. Much of the nonrecombinant vector was electrophoresed out of the ligations for 2 h at 5 V/cm. The gel containing the vector was removed, and the genomic DNA was electrophoresed back into the blocks under the same conditions by using an inverted field. The blocks were removed from the gel and equilibrated, alongside the nontreated YAC-RC ligation, with 50 mM NaCl, 10 mM EDTA and were melted at 68°C for 5 min. All ligations were agarased with 100 U agarase (Calbiochem) at 37°C for 3.5 h. After 1:1 dilution with 2 M sorbitol, they were frozen on dry ice and stored at −70°C. Ligations were thawed and transformed into

AB1380 spheroplasts. The preparation of spheroplasts and the transformation procedure were as described by Burgers and Percival (1987), with the exception that the transformation was performed at 10^9 spheroplasts/ml. 10 ng of YCp50 supercoil DNA was transformed in parallel to provide an estimate of the transformation efficiency. Plates were incubated for 5 d at 30°C in a fan-assisted incubator.

Replication and Screening of Clones

The colonies were replicated in duplicate out of top agar, by using an aluminum plate with 40,000 machined pins (constructed at the EMBL, Heidelberg), onto ura⁻ plates. After 2 d at 30°C they were lifted onto Hybond-N membranes and lysed overnight on Whatman 3MM soaked in 1 mg zymolyase (FMC Bioproducts)/ml in 1 M sorbitol, 0.1 M sodium citrate pH 5.8, 10 mM EDTA pH 7.6, 30 mM 2-mercaptoethanol. Filters were denatured for 10 min on Whatman 3MM soaked in 0.5 M NaOH/1.5 M NaCl, and excess denaturant was removed before neutralization by flotation on 1.5 M NaCl/1 M Tris pH 7.4 (neutralization solution) for 2 min. They were incubated on Whatman 3 MM soaked in 0.1 × neutralization solution containing 200 μg proteinase K (BDH)/ml for 20 min. Cell debris was wiped off with Kleenex tissues soaked in 0.1 × neutralization solution, with which the filters were then rinsed thoroughly. After air-drying, the filters were baked under vacuum for 20 min at 80°C and were UV cross-linked (Church and Gilbert 1984). Hybridizations were as described above, with a probe concentration of 1–2 × 10^6 cpm/ml.

Results

Characterization of Cosmid Clones 2R88 and B31

2R88 and B31 are two of a series of clones (W. L. Whaley, unpublished data) identified by hybridization, with total human DNA, to a cosmid library constructed from the somatic cell hybrid line HHW693, which contains 4p15.1-4pter translocated onto a fragment of 5p as the only human component on a Chinese hamster cell background (Wasmuth et al. 1986). Figure 1a shows restriction maps of the cosmids, including the positions of sites for the rare-cutter enzymes MluI, SalI, and BssHII which were used in the further analysis. The positions of unique or low-copy fragments that were isolated for use as probes are also indicated. These fragments were mapped to the distal half of 4p16.3 by somatic cell hybrid analysis (data not shown). p2R88-1 and 88BH2.0 detect more than one locus on hybridiza-

tion to genomic DNA, and in each case the fragment size of the strongest-hybridizing locus corresponded to the predicted fragment size as indicated on the cosmid map.

To determine which of the rare-cutter sites identified in the clone are also cleavable in genomic DNA, we hybridized the probes 88BH2.0 and 88E1.8 to Southern blot filters of DNA from the cell line GM1416B, which had been digested either with HindIII only or with both HindIII and a number of rare-cutting enzymes. Figure 1b shows the result of this experiment, indicating cleavage of the rare-cutter sites (BssHII, SalI, and ClaI) in GM1416B DNA. The ClaI site is not present in 2R88 (since this cosmid is derived from HHW693 DNA).

2R88 Is Located in the Most Distal Region of 4p16.3, 100 kb from the End of the Map

Provisional pulsed-field gel mapping data, with the enzymes NotI, MluI, and NruI, suggested that 2R88 maps 60–280 kb distal to D5 on a partial 220-kb MluI fragment. In order to position 2R88 precisely and to determine its orientation, the region was mapped more extensively with BssHII and SalI by using the probe D5 and the probes 88BH4.8, p2R88-1, and 88ES2.3, isolated from 2R88 (fig. 1a). The probes were hybridized to a number of filters containing single and double digests of GM1416B and peripheral leukocyte DNA. Filters were prepared in duplicate with pulse times of 100 and 50 s, which allow a gel resolution of up to 1,500 and 600 kb, respectively. Figure 2 shows the hybridization of p2R88-1 and D5 to one filter illustrating detection of the same 350-kb NruI fragment in leukocyte DNA and the same partial 300-kb MluI fragment in GM1416B DNA. Data of this type, summarized in table 1, allowed the construction of a high-resolution pulsed-field restriction map of the most distal region of 4p16.3 (fig. 3) and localized 2R88 200 kb distal to D5 and 100 kb from the end of the map.

The End of the Map Is Likely to Coincide with the Telomere of Chromosome 4p

The cluster of restriction sites at the distal end of the map (italicized in fig. 3) could represent either a CpG island, containing a well-cut site for all enzymes tested, or the 4p telomere. Pulsed-field gel analysis is unable to distinguish between these two possibilities; however, several further lines of evidence suggested that this was indeed the telomere. Attempts to identify DNA beyond these sites by partial digestion were repeatedly unsuccessful.
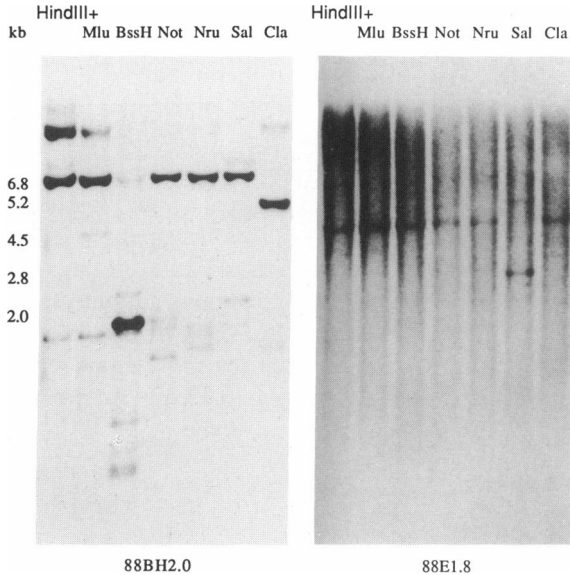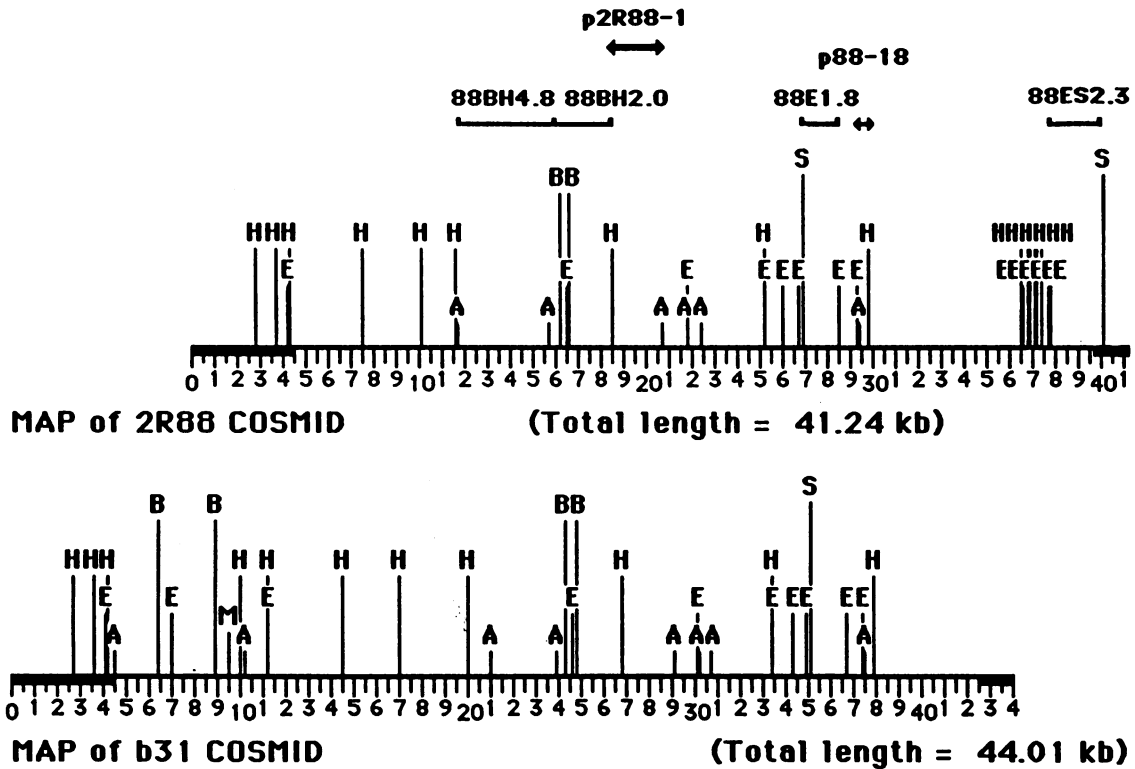
MAP of 2R88 COSMID   (Total length = 41.24 kb)

MAP of b31 COSMID   (Total length = 44.01 kb)

Figure 1   Characterization of the 2R88 and B31 cosmids *a,* Restriction maps of the 2R88 and B31 cosmids, indicating the position of the rare-cutter restriction sites *Bss*HII (B), *Mlu*I (M), and *Sal*I (S). Fragments that were used as probes against Southern filters are outlined above the map. These were as follows: 88BH2.0 and 88BH4.8 are 2.0-kb and 4.8-kb *Hin*dIII (H)*Bss*HII fragments that flank the *Bss*HII sites, 88E1.8 is the 1.8-kb *Eco*RI fragment containing the *Sal*I site, and 88ES2.3 the 2.3-kb *Eco*R (E) *Sal*I fragment (the *Sal*I site is in the vector). p2R88-1 is an *Sau*3A/*Pst*I fragment of 750 bp in pGEM4 that originates from the 2.2-kb *Bam*HI (A)/*Hin*dIII fragment, and p88-18 contains an *Sau*3A fragment in pGEM4 that maps to the 700-bp *Eco*RI/*Hin*dIII fragment. *b,* Demonstration that the *Bss*HII and *Sal*I sites within the 2R88 cosmid are unmethylated in genomic DNA isolated from the cell line GM1416B. 88BH2.0 and 88E1.8 were hybridized in turn to a filter containing GM1416B DNA that had been digested with *Hin*dIII only and with *Hin*dIII and a rare-cutter restriction enzyme as indicated. The 6.8-kb *Hin*dIII fragment detected by 88BH2.0 is reduced to 2.0 and 5.2 kb by *Bss*HII and *Cla*I, respectively. Fainter bands are indicative of cross-hybridization to other loci. The 4.5-kb *Hin*dIII fragment detected by 88E1.8 is reduced to 2.8 kb by *Sal*I cleavage.

Similarly, we were unable to isolate clones from the potential CpG island by chromosome jumping (Poustka and Lehrach 1986; Poustka et al. 1987; Poustka and Lehrach 1988) in either *Mlu*I or *Bss*HI jumping libraries (A. Poustka, unpublished data), while multiple clones extending to a site proximal to this position were recovered (authors' unpublished data). In combination, this evidence was sufficiently compelling to devise, for the isolation of the 4p telomere, a strategy based on the pulsed-field gel analysis presented here.
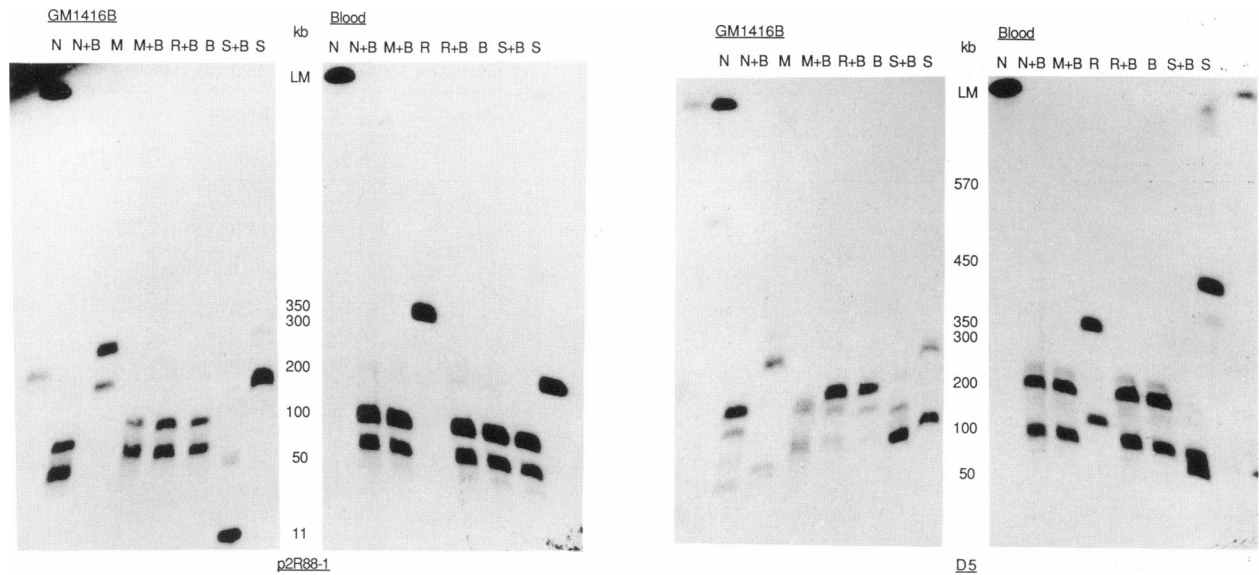
Figure 2 Pulsed-field gel analysis of p2R88-1 (*a*) and D5 (*b*). Hybridization of the probes p2R88-1 and D5 to a pulsed-field filter containing both GM1416B and leukocyte DNA that has been digested with rare-cutting restriction enzymes is indicated. Electrophoresis was for 36 h at 5 V/cm in 0.75% agarose, 0.25 × TBE with a pulse time of 50 s. LM = the region of limiting mobility. N = *Not*I; R = *Nru*I. All other abbreviations are as in fig. 1.

Approach to the Specific Isolation of the 4p Telomere

The similarity of the human telomeric repeat to that of Tetrahymena (functional as a telomere in yeast) has shown that a YAC vector carrying only one Tetrahymena telomere allows the cloning of human telomeres by complementation (Brown 1989; Cheng et al. 1989; Cross et al. 1989; Riethman et al. 1989). To simplify the identification of a clone from 4p and to allow the construction of clones of sufficient length to be tested for possible biological activity (e.g., in a transgenic mouse system), we were especially interested in a telomere library containing long clones. Such large-insert YAC libraries can be constructed by two procedures. One protocol, used by (Burke et al. 1987; Little et al. 1989), relies on the use of very partial digestion with commonly cutting restriction enzymes to generate the large DNA fragments (of the order of hundreds of kilobase pairs) to be cloned. We decided to concentrate on an alternative approach and to use rare-cutter enzymes, enabling us to take advantage of the information in the long-range map of the region.

Choice of Rare-Cutter Restriction Enzyme for the Construction of the Telomere Library

The majority of the clones recovered from the construction of a rare-cutter YAC library, in the absence of prior size selection, will fall within a size range of up to 200 kb. By pulsed-field gel analysis, we have identified a 100-kb *Bss*HII fragment which contains p2R88-1 at its proximal end and extends to the telomere. The preparation of a library from genomic DNA digested with *Bss*HII would therefore generate a clone in a size class for which there is a natural enrichment and allow its identification by hybridization with p2R88-1. Furthermore, hybridization of this probe to DNA, from a number of blood samples and cell lines, that had been digested with *Bss*HII and fractionated by PFGE detected a *Bss*HII polymorphism. In DNA from GUSHM1, an individual from the Venezuela pedigree expected to be homozygous for *HD*, p2R88-1 detects *Bss*HII fragments of approximately 100 and 115 kb. The *Bss*HII fragment of 55 kb previously observed in GM1416B and in leukocyte DNA is not present, indicating either complete methylation or absence of the internal site. Therefore, the construction of a *Bss*HII-telomere clone library from GUSHM1 would provide an increased chance of recovery of a telomeric clone and would allow access to the mutant form of the region.

Construction of a BssHII-Telomere Clone Library from an Individual Homozygous for HD

Vectors YAC-t1 and YAC-t2, which permit the cloning of mammalian telomeres in yeast, were constructed as described in Material and Methods. YAC-t2 con-

Table I

Summary of Rare-Cutter Restriction Fragments (in kilobase sizes ± 10%) Detected by D5 and by Probes Isolated from 2R88

PROBE	GM1416B					BLOOD				
	NotI	NruI	MluI	BssHII	SalI	NotI	NruI	MluI	BssHII	SalI
D5	850 570	350 (315) 170ᵃ	(400)ᵃ (350)ᵃ 300 80	225 170ᵃ (100)ᵃ	(290)ᵃ (270) 110	850	350 170ᵃ	1000 (570)	(240) 225 (170)ᵃ 100ᵃ	(700) 450 (400)ᵃ
p2R88-1	850 280	350 (315)	300 220	100 55	(270) 160	850	(570) 350	1000 (570)	100 55	250
88ES2.3	850 280	350 (315)	300 220	NT	90	850	350	1000	NT	250
88BH4.8	850 280	NT	300 220	15	(270) 160	850	(570) 350	NT	15	250

PROBE	NotI/ NruI	NotI/ MluI	NotI/ BssHII	NotI/ SalI	NruI/ MluI	NotI/ NruI	NotI/ MluI	NotI/ BssHII	NotI/ SalI	NruI/ MluI
D5	350 (315) 170ᵃ 80 35	300 80 20	225 170ᵃ (100)ᵃ 60	(290)ᵃ (270) 110 80	300 170ᵃ 110ᵃ 80	350 170ᵃ	850 (570)	(240) 225 (170)ᵃ 100ᵃ	(700) 450 (400)ᵃ	(570) (400) 350 170ᵃ
p2R88-1	350 (315) 280	300 220	100 55	(270) 160	300 220	350	850 570	100 55	250	(570) 350
88ES2.3	NT	NT	NT	90	NT	NT	NT	NT	250	NT
88BH4.8	NT	NT	15	NT	NT	NT	NT	15	NT	NT

PROBE	NruI/ BssHII	NruI/ SalI	MluI/ BssHII	MluI/ SalI	BssHII SalI	NruI/ BssHII	NruI/ SalI	MluI/ BssHII	MluI/ SalI	BssHII SalI
D5'	225 170ᵃ (100)ᵃ	(260) 100 (60)	185 170ᵃ (100)ᵃ 80	(290)ᵃ (270) (110) 50	(225) 170ᵃ 90	(245) 225 (170)ᵃ 100ᵃ	(170)ᵃ 100	(240) 225 (170)ᵃ 100ᵃ	450 (400)ᵃ	(240) (225) (170)ᵃ 100ᵃ 90
p2R88-1	100 55	(270) 160	100 55	160 130	11	100 55	(350) 250	100 55	250	100 55
88ES2.3	NT	90	NT	90	NT	NT	250	NT	250	NT
88BH4.8	15	NT	15	NT	15	15	NT	15	NT	15

NOTE.—Weakly detected bands, generated by partial digestion of a restriction site are indicated in parentheses. NT = not tested.
ᵃ Weak band detected by cross-hybridization to another locus.

tains CEN4, ARS1, URA3, a single cassette of TEL sequences, and a rare-cutter polylinker to provide NotI, SacII, SalI, MluI, ClaI, and SnaBI as cloning sites. The steps involved in the preparation of the telomere library are summarized in figure 4. High-molecular-weight genomic DNA from GUSHM1 was prepared in agarose blocks, digested to completion with BssHII, and ligated to an excess of YAC-t2 which had been both cleaved with BamHI and MluI and treated with alkaline phosphatase to reduce vector background. To further reduce the background of clones originating from vector alone,

most of the unligated vector was then removed by electrophoresis. As a control, a fraction of the BssHII digest was ligated to MluI-cleaved YAC-RC, a YAC cloning vector containing a rare-cutter polylinker (Marchuk and Collins 1988). Losses incurred during vector removal were estimated by transforming fractions of this ligation both before and after the removal of the vector, a removal which had been carried out in parallel with that for the telomere cloning experiment. Libraries were constructed by transformation of AB1380 spheroplasts. Spheroplast transformation efficiency, tested with
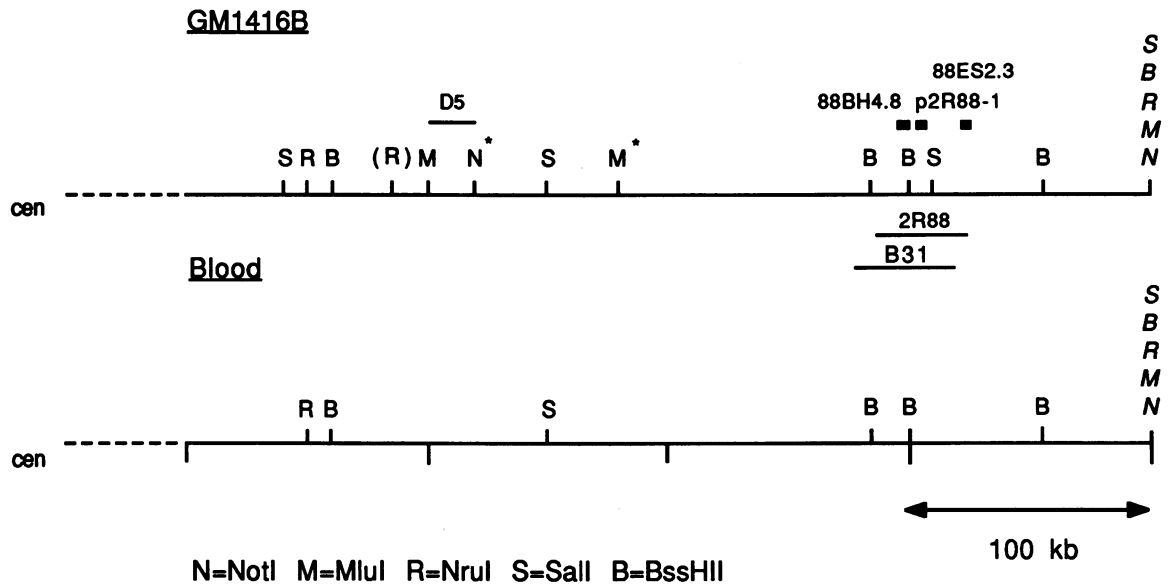
Figure 3 Long-range restriction map spanning 350 kb and illustrating the physical linkage between the D5 (D4S90) and 2R88/B31 (D4S142) loci and the position of the 4p telomere (indicated as italicized restriction sites). Linkage between D5 and 2R88 is demonstrated by hybridization to the same 350-kb NruI, 1,000-kb MluI (table 1), and 850-kb NotI (table 1) fragments in leukocyte DNA and to the same 350-kb NruI fragment and partial 300-kb MluI and 850-kb NotI (table 1) fragments in DNA from GM1416B cells. The SalI fragments detected by D5 in DNA from leukocytes and GM1416B cells are 450 and 90 kb, respectively, and the SalI sites that flank this fragment in GM1416B DNA can be positioned by double digestion with NotI, MluI, and NruI. This places a SalI site 250 kb from the distal end of the map which is the size of the SalI fragment detected by p2R88-1 in leukocyte DNA. It is also the sum of the 160- and 90-kb SalI fragments that hybridize with p2R88-1 and 88ES2.3, respectively (probes that flank the well-cleaved SalI site in the 2R88 cosmid that is not cut in leukocyte DNA). This position of 2R88 is confirmed by the 130-kb SalI/MluI double-digestion product detected by p2R88-1 in GM1416B DNA. The orientation of the cosmid is indicated by the hybridization of p2R88-1 and D5 to GM1416B DNA that has been digested with SalI and BssHII, positioning the BssHII sites flanking the D5 BssHII fragment. Consideration of these data, along with the predetermined position of the SalI site within 2R88, allows only one orientation of the 2R88 cosmid. Parentheses indicate that a site is very partially cleaved. The asterisks denote sites that are available for restriction on one chromosome only and that are situated on opposite chromosomes.

YCp50, was 3.5 × 10⁵ colonies/μg vector. The ligation of BssHII-digested DNA into YAC-RC gave a transformation efficiency of 5 × 10³ colonies/μg insert. Therefore, we transformed ligation mix containing approximately 3 μg of insert, sufficient to give one- to twofold coverage of the clonable BssHII fragments in a standard BssHII cloning experiment.

Library Screen

The 25,000 clones recovered were replicated in duplicate directly from the top agar, containing sorbitol, onto ura⁻ plates. Filter lifts were screened with p2R88-1, and a single positive clone (Y88BT) was identified, in rough agreement with the one- to twofold coverage expected. While most of the other clones recovered appeared to be vector background, because of the incomplete removal of vector sequences, approximately 10% (2,500) of the clones were found to hybridize with a cloned Alu repeat probe. These would be expected to

carry human DNA inserts, either because of the formation of (dicentric) clones with two vector arms or because of the low frequency recognition of broken DNA fragments by the yeast telomerase. Previously, the rate-limiting step in the rapid analysis of a large number of YAC recombinants has been the difficulty in the transference of the clones embedded in top agar to a form in which they can be screened rapidly and in high density without library amplification. The method of direct replication used here has circumvented this technical problem.

Y88BT Carries a Human Telomere Repeat

Chromosomes were prepared from the p2R88-1-positive clone, Y88BT, and were fractionated by PFGE. The recombinant chromosome was approximately 115 kb, the size of the larger BssHII fragment detected by p2R88-1 in GUSHM1 DNA. In order to determine that this clone contains the telomere of human chromosome
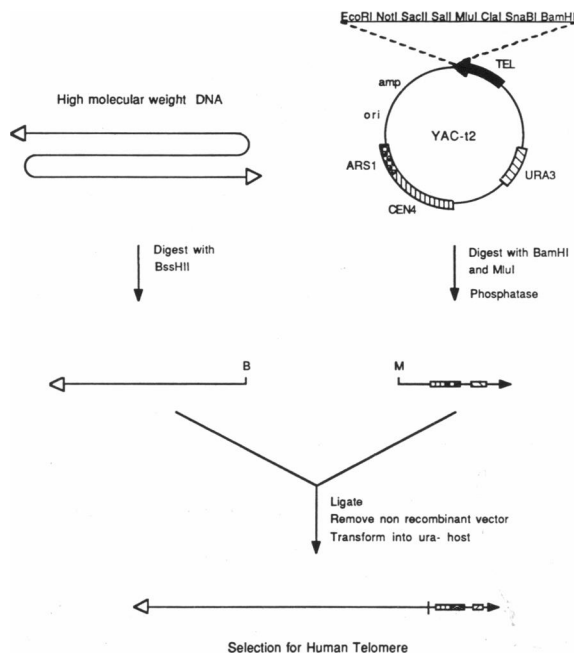
Figure 4   Construction of the BssHII telomere library. High-molecular-weight DNA from GUSHM1 was digested to completion with BssHII (B) and ligated into the MluI (M) site of MluI/BamHI-cut and phosphatased YAC-t2. Recombinant molecules were transformed into AB1380 spheroplasts, and the telomere of human chromosome 4p was subsequently identified by hybridization to p2R88-1.

4p and has not arisen from an internal BssHII fragment, Y88BT was digested with NotI and was fractionated, by PFGE, alongside undigested DNA. NotI digestion separates the vector from the genomic DNA by cleavage within the vector polylinker. The resultant filter was hybridized sequentially with Alu, pBR322, and an oligomer, (CCCTAA)4, that contains the human telomeric repeat (fig. 5). Hybridization with Alu indicated the absence of an internal NotI site within the clone. pBR322 detected the 8-kb vector fragment only, demonstrating that all of the vector DNA has been removed from the artificial chromosome on digestion with NotI. Hybridization with (CCCTAA)4 to the NotI-cleaved recombinant indicates the presence of telomeric sequences on the BssHII fragment. At this stringency the oligomer did not cross-hybridize to the TEL sequences on the vector. It has been shown that there are no detectable nontelomeric tracts of (CCCTAA)n in the human genome (Moyzis et al. 1988) and that if such an event did occur it would be in the immediate vicinity of a telomere (Riethman et al. 1989). The telomeric sequence on Y88BT is human in origin, and this clone therefore should contain the most telomeric 115 kb of chromosome 4p.
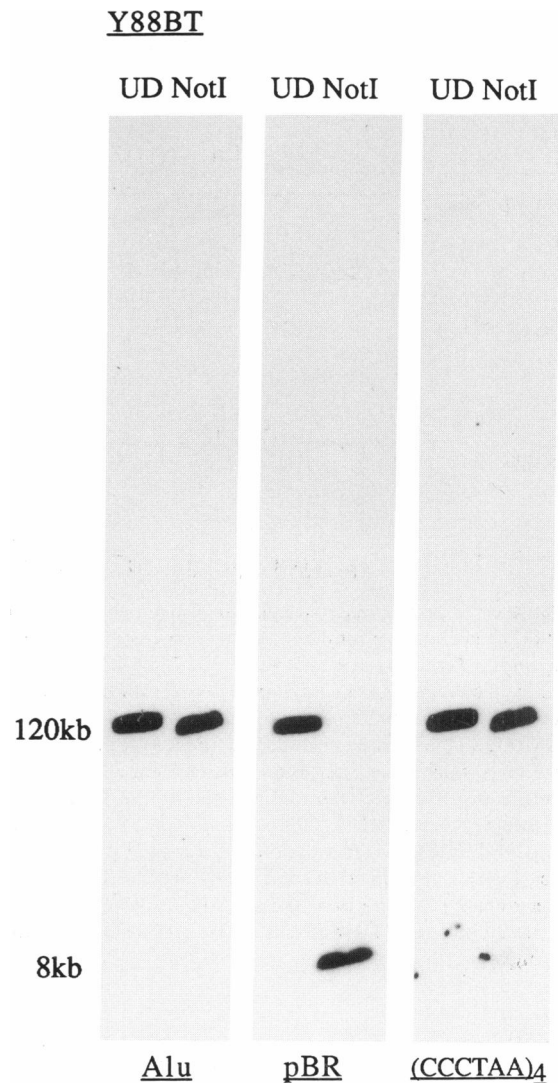

Figure 5   Y88BT shown to contain the human telomeric repeat sequence. Y88BT was digested with NotI and fractionated alongside the undigested clone (UD) in a 0.9% agarose gel with a pulse time of 40 s for 36 h, to give separation up to approximately 500 kb. The resultant filter was hybridized sequentially with Alu, pBR322, and the oligomer (CCCTAA)4.

A Rare-Cutter Restriction Map of Y88BT

In order to identify and position rare-cutter restriction sites within Y88BT, a restriction map was constructed using a combination of complete and partial digests (table 2 and fig. 6b). This demonstrated that the proximal end of Y88BT starts at the BssHII site in B31, a site that is adjacent to an MluI site. The exact correlation between the position of rare-cutter sites within Y88BT and those in 2R88 and B31 argues against any rearrangements in the most proximal 40

Table 2

Summary of Restriction Fragments (in kilobase sizes) Generated by Complete Digestion of Y88BT with Rare-Cutter Enzymes

Probe	BssHII	EagI	SalI
pBR322..........	23	8	8
p2R88-1	45	55	25
Alu	45	55	90
	23	45	25
	11	15	
(CCCTAA)₄	45	45	100

kb of the clone. The presence of the *Bss*HII site 55 kb from the telomere indicates that the differential cleavage of this site between individuals and cell lines in genomic DNA is the result of a methylation polymorphism rather than the result of the absence of a restriction site. The human telomeric repeat was found to hybridize to the distal end of the YAC clone, as expected.

Genetic Evidence Suggests That the Most Likely Position for the HD Mutation Is within Y88BT

HD has been localized to the 4p16.3 band by linkage analysis with numerous DNA markers (Gusella et al. 1983; Gilliam et al. 1987*b;* Wasmuth et al. 1988; MacDonald et al. 1989*a*). However, its precise position within this small segment of the genome can only be established by a relatively small number of cases in which recombination events occur between the disease locus and the closest DNA markers. Such landmark crossovers have not provided an unequivocal placement of *HD,* but they do suggest two possible locations: distal to D4S90, within 300 kb of the telomere, or proximal to D4S115, which is located 1,500 kb from the telomere (MacDonald et al. 1989*b;* Robbins et al. 1989; Youngman et al. 1989; Bucan et al. 1990).

Probe p88-18, from 2R88, was found to detect a rare RFLP when hybridized to *Msp*I-digested human genomic DNA. The alleles, corresponding to fragments of 2.0 and 1.8 kb, displayed frequencies of .98 and .02, respectively ($N = 100$, where N is the number of chro-
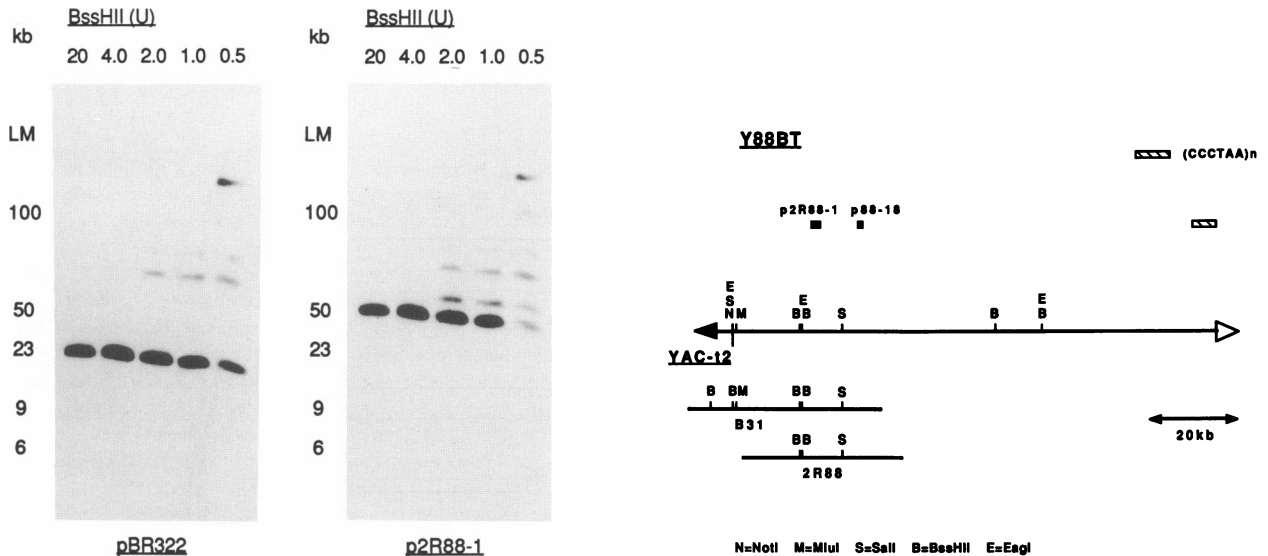

Figure 6 Rare-cutter restriction map of Y88BT. *a,* Hybridization of pBR322 and p2R88-1 to a Southern blot of *Bss*HII partial digests of Y88BT. Agarose blocks containing approximately 1 µg of Y88BT DNA were digested with decreasing amounts of enzyme (as indicated) in 200 µl for 3 h. PFGE was in 1% agarose with a pulse time of 5 s for 24 h, to provide a gel resolution of 100–150 kb. Lambda multimers and lambda DNA digested with *Hind*III were used as size markers. LM = region of limiting mobility. *b,* Rare-cutter restriction map of the Y88BT clone with the enzymes *Mlu*I (M), *Sal*I (S), *Eag*I (E), and *Bss*HII (B) (*Not*I *Eag*I, and *Sal*I cut within the polylinker). The map was constructed from a combination of data generated by PFGE analysis of complete (summarized in table 2) and partial digests by using the conditions described in *a.* The resultant filters were hybridized sequentially with p2R88-1, Alu, the human telomeric repeat oligomer, (CCCTAA)₄, and with pBR322. The *Sal*I, *Eag*I, and *Mlu*I sites were positioned on the basis of information gained from complete digestion of the YAC. The number and relative positions of the *Bss*HI sites were determined from the fragments observed by hybridization, with pBR322 and p2R88-1, to the partial digests as shown in *a.* The distances between sites (estimated to be ± 10%) were judged on the basis of the size of complete digest bands detected by Alu.

mosomes screened). Although not informative in the bulk of the recombination events used to assign a telomeric position for *HD*, this RFLP was heterozygous in a critical meiosis from the extended Venezuela HD pedigree (fig. 7), where *HD* segregates with the rare allele. In the mating shown, several DNA markers from 4p16.3, including D4S115, D4S111, and D4S90, have previously been ascertained to recombine with *HD* (MacDonald et al. 1989*b*), a result of which, if it is interpreted as a single recombinant, would suggest a location for *HD* proximal to these markers. However, this is incompatible with other recombination events, which favor an assignment to the telomeric segment distal to D4S90 (MacDonald et al. 1989*b*; Robbins et al. 1989). If the terminal location is correct, the *Msp*I RFLP at D4S142 also shows recombination with *HD*, indicating that the event described above may be a double recombinant with a second recombination event located distal to the *Msp*I RFLP at D4S142. Since the polymorphic *Msp*I site detected by p88-18 is located within the Y88BT clone, this clone would span the entire telomeric *HD* candidate region.

Discussion

The extreme telomeric location of *HD* within 4p16.3 has made the search for closer—and, especially, flanking—markers particularly difficult. It has not been possible to identify a proven flanking marker or to define with absolute certainty the region containing *HD* either by the analysis of discrete crossovers (MacDonald et al. 1989*b*; Robbins et al. 1989) or by the observation of linkage disequilibrium with some but not all RFLPs (Snell et al. 1989; Theilmann et al. 1989).

We have isolated overlapping cosmids 2R88 and B31 (D4S142) and have positioned them, by pulsed-field gel analysis, 200 kb distal to D4S90, within a region previously shown to be a possible location for *HD*. An RFLP at this locus, detected by p88-18, although relatively uninformative in the general population, segregates with *HD* in the Venezuela pedigree and in one sibship identifies a recombinant with the mutation. This cross-

Figure 7   Presence of D4S142 in Y88BT and segregation of the locus in an HD recombinant family. *A*, p88-18 probe was hybridized to blots containing 5 µg of the following DNAs digested with *Msp*I: lane 1—HHW842, a human-hamster somatic cell hybrid containing human chromosome 5 and an interstitial deletion human chromosome 4 which retains the terminal portion of 4p16.3; lane 2—hamster cell line tsH1 spiked with 100 ng of DNA from yeast containing Y88BT; lane 3—tsH1; lane 4—HHW847, a human hamster hybrid containing several human chromosomes, including human chromosome 5
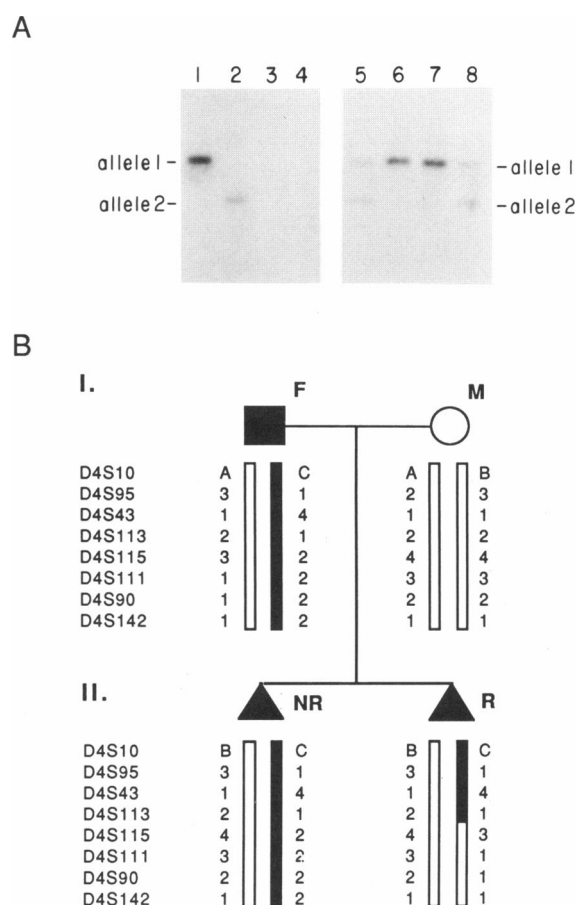
and a t(4;21) chromosome in which 4p16.2-pter is absent; lane 5—father of the nuclear family shown in *B*; lane 6—mother; lane 7—an affected offspring showing recombination between *HD* and the D4S142 *Msp*I RFLP; and lane 8—affected offspring showing no recombination. The presence of the expected allelic fragment in HHW842 but not in HHW847 establishes that the locus maps to the terminal portion of 4p16.9. The fragment representing allele 2 is seen in Y88BT, since this allele segregates with *HD* in the extended Venezuelan pedigree from which the homozygous *HD* cell line, used for library construction, was derived. The results in lanes 5–8 can be interpreted by referring to *B*. *B*, Nuclear family derived from the Venezuelan HD kindred and consisting of an affected father (F), unaffected mother (M), and two affected progeny (NR and R) with differing genotypes for several 4p16.3 markers as reported elsewhere (MacDonald et al. 1989*b*). To protect the confidentiality of the family and to preserve the blinded status of our clinical collaborators, the sex of the children is not given. The normal phase of the various marker alleles with respect to the HD chromosome, shown in individuals F and NR and symbolized by the filled chromosome schematic, is known from other affected sibs and close relatives in the remainder of the Venezuelan pedigree. The affected individual designated R has been reported to display recombination between the markers D4S115, D4S111, and D4S90 and the disease gene. The data shown in *A* indicate that D4S142 also displays recombination, leaving the following two possible locations for the *HD* gene: (1) the region between D4S10 and D4S115 and, in the case of a double recombination event, (2) the region between D4S142 and the telomere.

over suggests an *HD* location proximal to D4S115, a location incompatible with other recombinants, which in combination indicate a location distal to D4S90. Therefore, one class of these recombinant chromosomes must be the product of a mechanism other than a single recombination event. If the event described here originates from a double recombinant, *HD* would be located distal to D4S142.

In order to pinpoint the position of the gene more precisely, it has been imperative that yet more distal and informative markers be identified. In parallel to the work described here, we have used a combination of chromosome jumping (Poustka and Lehrach 1986; Poustka et al. 1987; Poustka and Lehrach 1988) and walking to isolate markers distal to this locus. A jump from the well-cut *Bss*HII site in 2R88, in a *Bss*HII jumping library (A. Poustka, unpublished data) and a subsequent phage walk, has allowed us to reach within 30 kb of the telomere (authors' unpublished data).

The isolation of Y88BT has identified the position of the telomere of 4p and has set an absolute distal limit to the location of *HD*. In addition, this clone should allow the isolation of polymorphic sequences adjacent to the 4p telomere, a step essential to resolve the ambiguity in the placement of *HD*. The telomeric candidate region of *HD* has been reduced to a region spanning 100 kb, and the identification of the p88-18 RFLP within Y88BT indicates that the HD locus may lie within the YAC clone. Since the *Bss*HII-telomere library was prepared from an individual homozygous for *HD*, the locus would be present in its mutant form. Therefore, we have isolated within a single clone one of the candidate regions for the position of the HD gene.

The isolation of a YAC clone that may carry the mutant form of *HD* will allow the development of a potential functional assay in a transgenic mouse system. However, in the case of such a telomeric location, the assumption that the HD mutation directly affects a coding sequence may require reconsideration. In addition to the conventional strategy of searching for expressed or conserved sequences, a strategy based on the assumption of the existence of a cell-specific "killing gene" within the region, less conventional approaches may have to be entertained.

Acknowledgments

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