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Defined Physical Limits of the Huntington Disease Gene Candidate Region

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Summary

The Huntington disease (HD) gene has been mapped 4 cM distal to D4S10 within the telomeric chromosome band, 4p16.3. The published physical map of this region extends from D4S10 to the telomere but contains two gaps of unknown size. Recombination events have been used to position the HD mutation with respect to genetic markers within this region, and one such event places the gene proximal to D4S168, excluding the distal gap as a possible location for the defect. One previously published recombination event appeared to have excluded the proximal gap. We have reassessed this event and have moved the proximal boundary for the *HD* candidate region centromeric to the gap within a "hot spot" for recombination between D4S10 and D4S125. We have closed the proximal gap and report here the complete physical map spanning the *HD* candidate region from D4S10 to D4S168, the maximum size of which can now be placed accurately at 2.5 Mb.

Introduction

Huntington disease (HD) is a late-onset, dominantly inherited, neurodegenerative disorder. The symptoms include progressive chorea, psychological disturbances, and dementia, caused by the premature death of specific neurons primarily in the striatum (Martin and Gusella 1986). The biochemical mechanism underlying the expression of this disease is not understood, and in recent years much energy has been invested in order to clone the HD gene on the basis of its chromosomal location.

The mutation causing HD lies 4 cM telomeric to the DNA marker D4S10 (Gusella et al. 1983; Gilliam et al. 1988*b;* Conneally et al. 1989) within the most terminal cytogenetic subband of chromosome 4p (4p16.3). Recently, complementary genetic (MacDonald et al. 1989*b;* Youngman et al. 1989; Allitto et al. 1991) and physical (Bucan et al. 1990) maps of 4p16.3 have been generated. The long-range restriction map was constructed by using the rare-cutter restriction enzymes *Not*I, *Mlu*I, and *Nru*I. It consisted of three unlinked segments in the following order: centromere–segment I–segment II–segment III–telomere. These segments were approximately 1.5, 2.2., and 1.5 Mb, respectively, and were separated by gaps of unknown size (summarized in fig. 3*A*). D4S10 is located within segment I, and the distal end of segment III has since been shown to correspond to the telomere of 4p (Bates et al. 1990).

Recombination events with *HD* have not decisively positioned the HD gene but suggest two nonoverlapping locations (MacDonald et al. 1989*b*). Three events indicate a location close to the telomere, distal to all known RFLP markers (MacDonald et al. 1989*b;* Robbins et al. 1989). A fourth event predicts a more centromeric location (MacDonald et al. 1989*b;* Whaley et al. 1991), placing the gene proximal to D4S168, a marker situated at the distal end of map segment II (Whaley et al. 1991). This latter recombination event has therefore excluded the more distal gap in the physical map as a possible location for the

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HD gene. The internal location is also supported by the detection of linkage disequilibrium between *HD* and some markers located on map segment II (Snell et al. 1989; Theilmann et al. 1989).

The genetic and physical extent of the telomeric *HD* location is well established (MacDonald et al. 1989*b*; Robbins et al. 1989; Bates et al. 1990; Pritchard et al. 1990). In the present study we have aimed to define a proximal genetic limit to the location of *HD* and to determine the precise physical size of the internal *HD* candidate region. One family has been reported that would appear to place *HD* distal to the gap that separates segments I and II (Youngman et al. 1989). In this case, an incorrect haplotyping of RFLPs at D4S95 and D4S43 appeared to place these markers proximal to the crossover event that occurred between D4S10 and *HD*. We have retyped this family and have found that the recombination event actually occurred between D4S10 and D4S125, in segment I. Analysis of this family has therefore reinstated the gap between segments I and II into the *HD* candidate region, and this prompted us to close the gap and complete the physical map. We have constructed a continuous 3-Mb restriction map which covers the entire 2.5-Mb *HD* candidate location between D4S10 and D4S168.

Material and Methods

Linkage Analysis

The typing of RFLPs was carried out by Southern blot analysis according to a method described elsewhere (Gusella et al. 1983), with the exception that the probes were labeled by random oligonucleotide priming (Feinberg and Vogelstein 1984).

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

DNA extracted from peripheral leukocytes was used for the PFGE analysis. The preparation of both genomic DNA and size markers, as well as restriction-enzyme digestion, size fractionation, transfer to Hybond-N$^+$ (Amersham) membranes, and probe hybridization, were as described elsewhere (Bates et al. 1990). Restriction digests were carried out as recommended by the manufacturer, and enzymes were from New England Biolabs, with the exception of *Spl*I and *Csp*I, which were from Amersham and Promega, respectively. PFGE was performed by contour-clamped homogeneous electric fields in an apparatus similar to that described by Chu et al. (1986) and constructed at the European Molecular Biology Laboratories in Heidelberg. Specific electrophoretic conditions were as described in the figure legends.

Table 1

Probes Used in PFGE Analysis

Probe	Locus	Reference
H5.52	D4S10	Skraastad et al. 1989
HDA29	D4S81	Richards et al. 1988
BJ14	D4S126	Allitto et al. 1991
F4ps19	D4S125	C. Lin, unpublished data
L19ps11	D4S180	C. Lin, unpublished data
BJ56	D4S127	Allitto et al. 1991
BS674	D4S95	Wasmuth et al. 1988
L14ps7	D4S181	C. Lin, unpublished data
42RB1.8	D4S43	Bucan et al. 1990
C4H	D4S43	Gilliam et al. 1987*a*
p311	D4S114	Whaley et al. 1988
BS731	D4S98	Smith et al. 1988
62.10KB	D4S113	Bucan et al. 1990
E4ps2	D4S168	Whaley et al. 1991

Probes used in the PFGE analysis are summarized in table 1. L19ps11, L14ps7, and F4ps19 are random *Pst*I/*Sau*3A fragments, subcloned into pGEM4, from the corresponding cosmids L19, L14, and F4. These cosmids were isolated from a library prepared from the human hamster somatic cell hybrid H39-8C10 according to a method described elsewhere (Whaley et al. 1991). L19 and L14 were isolated as random human cosmids mapping to the proximal portion of 4p16.3 (C. Lin, unpublished data), and F4 was isolated as a walk from YNZ32 (Nakamura et al. 1988).

Results

The Proximal Boundary of the Internal HD Region Lies between D4S10 and D4S125

The gap that separates segments I and II on the physical map reported by Bucan et al. occurs between the DNA markers D4S125 and D4S95. Only in a single family has a recombination event between D4S10 and *HD* been reported to occur distal to D4S95 but proximal to the most telomeric marker on the genetic map, D4S90 (Youngman et al. 1989). This event would exclude the gap between segments I and II and would set the limits of the internal *HD* candidate region at D4S95 and D4S168.

We have reassessed this critical crossover family by retyping the markers reported previously and by typing additional markers proximal to the gap. The sum-

mary of our data is presented in figure 1*A*. The *Bgl*I RFLP of D4S10 reveals that a recombination occurred distal to this marker, since the two affected children received opposing alleles from the affected father. The D4S43 *Msp*I RFLP detected by pXP500 indicates that the two affected children received opposite chromosomes from the unaffected mother but the same chromosome from their affected father. D4S180 was informative only for the mother's chromosomes and revealed that on her chromosomes no recombination event occurred between this marker and D4S43. The D4S95 *Taq*I RFLP is heterozygous in both parents, but the two children must have received the same allele from the affected father, since they are known to have received opposite alleles from the mother. Finally, a *Bgl*II RFLP at D4S125 also fails to detect the recombination event (fig. 1*B*). Thus, this crossover event must have occurred between D4S10 and D4S125 and not distal to D4S95 as previously reported. The proximal limit of the internal *HD* candidate region lies between D4S10 and D4S125. The distal limit of this region is defined by a crossover occurring between D4S113 and D4S168 (Whaley et al. 1991).

The Internal Candidate HD *Region Spans as Much as 2.5 Mb*

As published, the physical distance separating D4S10 and D4S168 is unknown, as they are located on pulsed-field map segments I and II, respectively (Bucan et al. 1990; Whaley et al. 1991). In order to place a limit on the size of this *HD* candidate region, we set out to close the gap between the first and second map segments. New rare-cutter enzymes, notably *Spl*I (CGTACG) and *Csp*I (CGGTCCG), were used. These may be predicted to generate large fragments in genomic DNA—*Csp*I by virtue of its 7-bp recognition sequence, *Spl*I because it is more likely to cut at rare nonmethylated sites between CpG islands rather than in the islands themselves (Bird 1989).

Linkage of probes BJ14 (located on segment I), BJ56 (located on segment II), and 42RB1.8 (located on segment II) was demonstrated by hybridization to a common *Spl*I fragment estimated to be approximately 1.6 Mb in length (fig. 2*A*). The size of this fragment, in combination with the established map positions of these markers (Bucan et al. 1990), indicated that the gap separating segments I and II could not be large. In order to position the *Spl*I sites defining this fragment and to determine the extent of the gap, the entire region between D4S10 and D4S168 was mapped more extensively. Probes were hybridized sequentially to filters containing genomic DNA that had been digested to completion both with *Not*I, *Mlu*I, *Nru*I, *Spl*I, and *Csp*I and with all double-digest combinations of these enzymes, as shown in figure 2*B*. The fragment sizes detected were used to compile the restriction map illustrated in figure 3*B*, and the precise placement of probes is discussed in that figure's legend. A table containing all of the primary data used to construct this map will be made available on request.

The centromeric end of the *Spl*I fragment that links segments I and II maps between HDA29 and BJ14, thereby orienting these markers, and the distal end was found to lie within a cluster of rare-cutter sites between 42RB1.8 and C4H. Partial digestion products were studied in the hope of detecting restriction sites beyond those defining the ends of the map segments. F4ps19 (located 300 kb from the distal end of map segment I) and L19ps11 (located within the most proximal 100 kb of map segment II) hybridize to *Not*I fragments of 420 and 100 kb, respectively, and each detects a fainter partial *Not*I band of 520 kb (fig. 2*B*). In each case, double digestion with *Spl*I reduces the 520-kb *Not*I fragment, by 40 kb, to 480 kb. This is consistent with F4ps19 and L19ps11 detecting a common partial *Not*I fragment which is cleaved at the *Spl*I site situated between HDA29 and BJ14. Therefore, segments I and II overlap, and the large *Spl*I band can be sized at 1.55 Mb on the basis of the sum of its smaller component restriction fragments. The completed map, which extends for 3 Mb and links D4S10 with D4S168, is illustrated in figure 3*B*. Although this region is relatively rich in rare-cutter sequences, *Spl*I has fulfilled the expectation of generating comparatively large fragments. However, the frequency with which *Csp*I sites occur is comparable with that observed for *Mlu*I and *Nru*I. Gaps between map segments in physical maps can be difficult to close, and such closure was greatly helped in this case by the isolation of the new marker L19ps11 located at the proximal end of map segment II.

The centromeric and telomeric boundaries of the internal *HD* candidate region are defined by recombination events occurring between D4S10 and D4S125 and between D4S113 and D4S168, respectively. Therefore, the maximum size of the internal *HD* region has been set at 2.5 Mb.

Discussion

It is well established that the mutation causing HD lies 4 cM distal to D4S10 within 4p16.3 (Gusella et

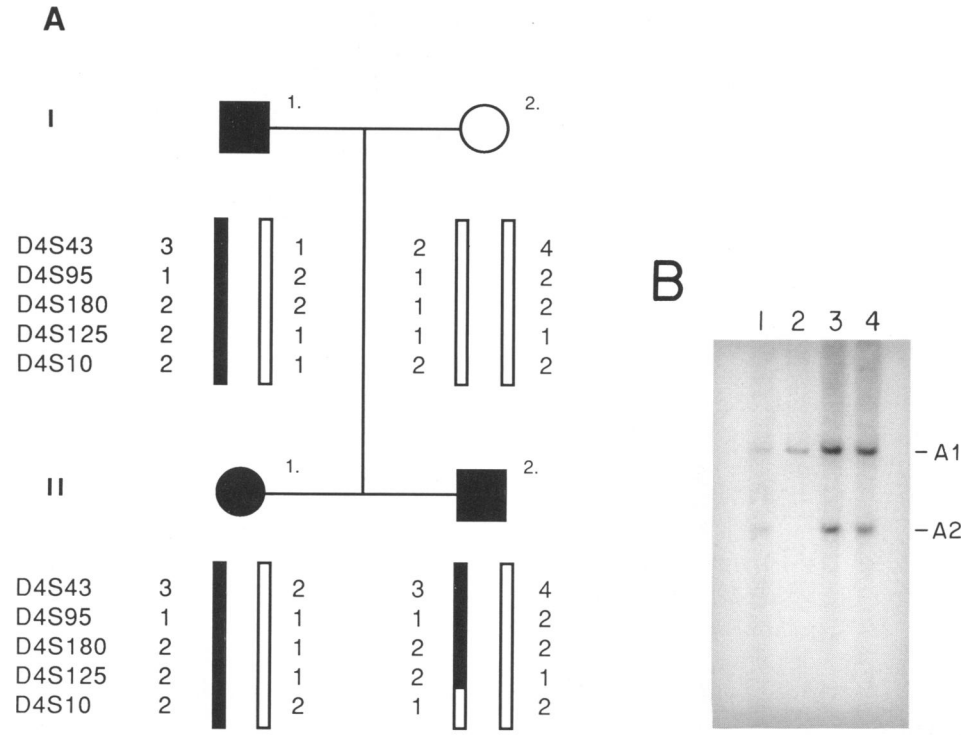
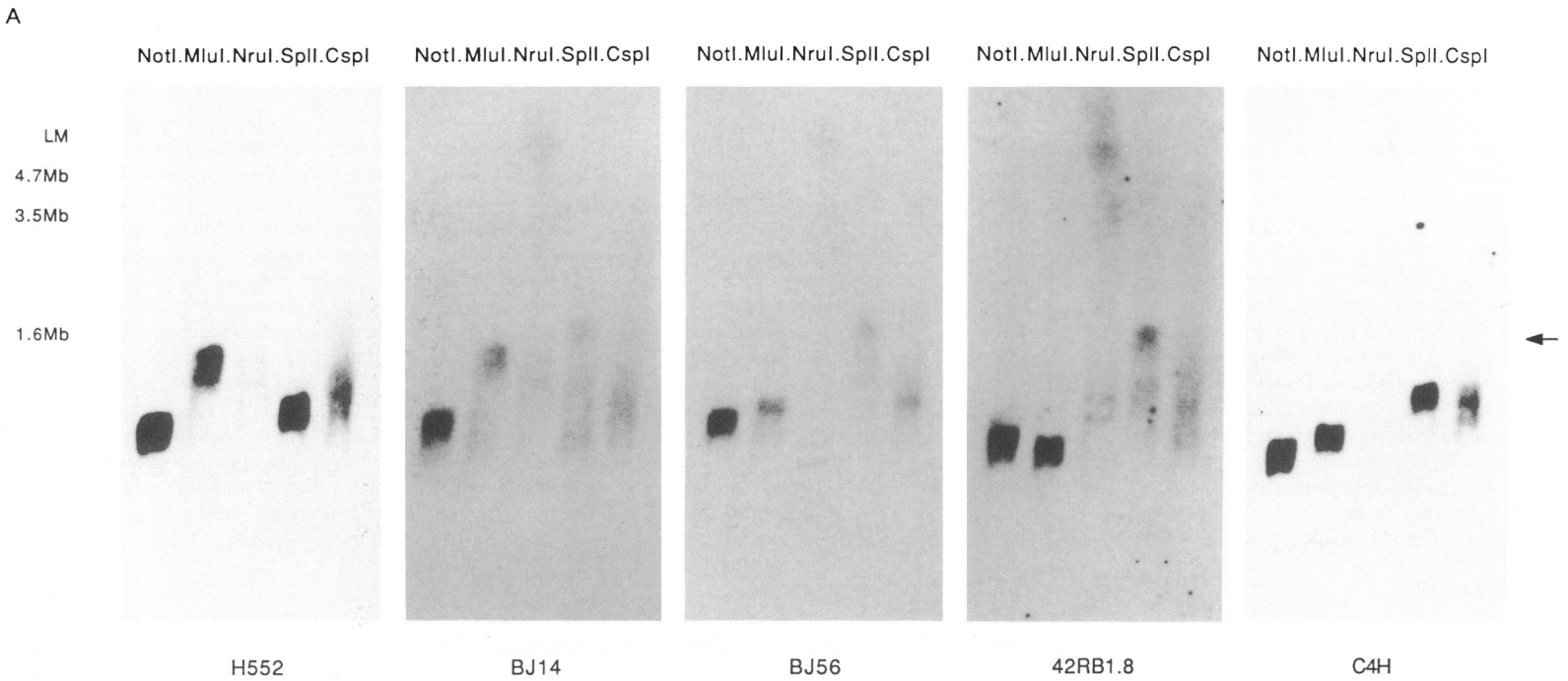


Figure 1 Setting lower boundary for *HD* region. *A*, Pedigree previously published by Youngman et al. (1989) as family 18 and typed with RFLP markers: D4S10 *BglI* RFLP detected by probe R7 (Gusella et al. 1985); D4S125 *BglII* RFLP detected by probe F4ps19 (C. Lin, unpublished data), D4S180 *Bam*HI RLFP detected by L19ps11 (C. Lin, unpublished data), D4S95 *TaqI* RFLP detected by pBS674-D (Smith et al. 1988), and D4S43 *MspI* RFLP detected by probe pXP500 (Gilliam et al. 1987*a*; MacDonald et al. 1989*a*). A recombination event with *HD* is detected only by D4S10, but the phase of this marker relative to the other RFLPs cannot be determined. For illustrative purposes, a phase has been chosen arbitrarily, and the chromosomes of the affected father have been distinguished from each other by being represented by either an unfilled or a filled rectangle. It should be noted that choosing the alternative phase would reverse which child would represent a recombination event but would not alter the conclusion. *B*, Typing of family members from panel *A*, shown for D4S125 *BglII* RFLP that definitely places crossover event proximal to this marker in segment I of physical map reported by Bucan et al. (1990). Lane 1, affected father (I-1). Lane 2, unaffected mother (I-2). Lane 3, affected child (II-1). Lane 4, affected child (II-2).

Figure 2 *A*, Physical linkage of BJ14, BJ56, and 42RB1.8 by hybridization to same *SplI* fragment. Hybridization of H5.52, BJ14, BJ56, 42RB1.8, and C4H to a filter containing genomic DNA digested to completion with *NotI*, *MulI*, *NruI*, *CspI*, and *SplI* and fractionated up to approximately 5 Mb. BJ14, BJ56, and 42RB1.8 hybridize to a common *SplI* fragment of approximately 1.6 Mb, as indicated. Pulsed-field gel conditions were as follows: samples were electrophoresed through a 0.75% agarose gel in 0.5 × TBE at 50 V. The pulse time was initially 50 min for 116 h, followed by 35 min for 97 h. Chromosomes prepared from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* were used as size markers. The sizes indicated correspond to the approximate positions of the two smaller of the three *S. pombe* chromosomes and to the 1.6-Mb *S. cerevisiae* chromosome. *B*, Physical linkage of map segments I and II. Hybridization of (*Bi*) F4ps19 and (*Bii*) L19ps11 to pulsed-field gel filters containing genomic DNA that had been digested to completion with the enzyme combinations indicated. Linkage between F4ps19 and L19ps11—and hence between map segments I and II—is demonstrated by the detection of a common 520-kb *NotI* partial digestion product that is reduced to 480 kb on double digestion with *SplI*. Electrophoresis was in 0.9% agarose in 0.25 × TBE at 170 V with a pulse time of 100 s for 40 h. The size markers were chromosomes prepared from *S. cerevisiae* and lambda multimers. N = *NotI*; M = *MluI*; R = *NruI*; S = *SplI*, and C = *CspI*.


A
NotI.MluI.NruI.SpII.CspI
NotI.MluI.NruI.SpII.CspI
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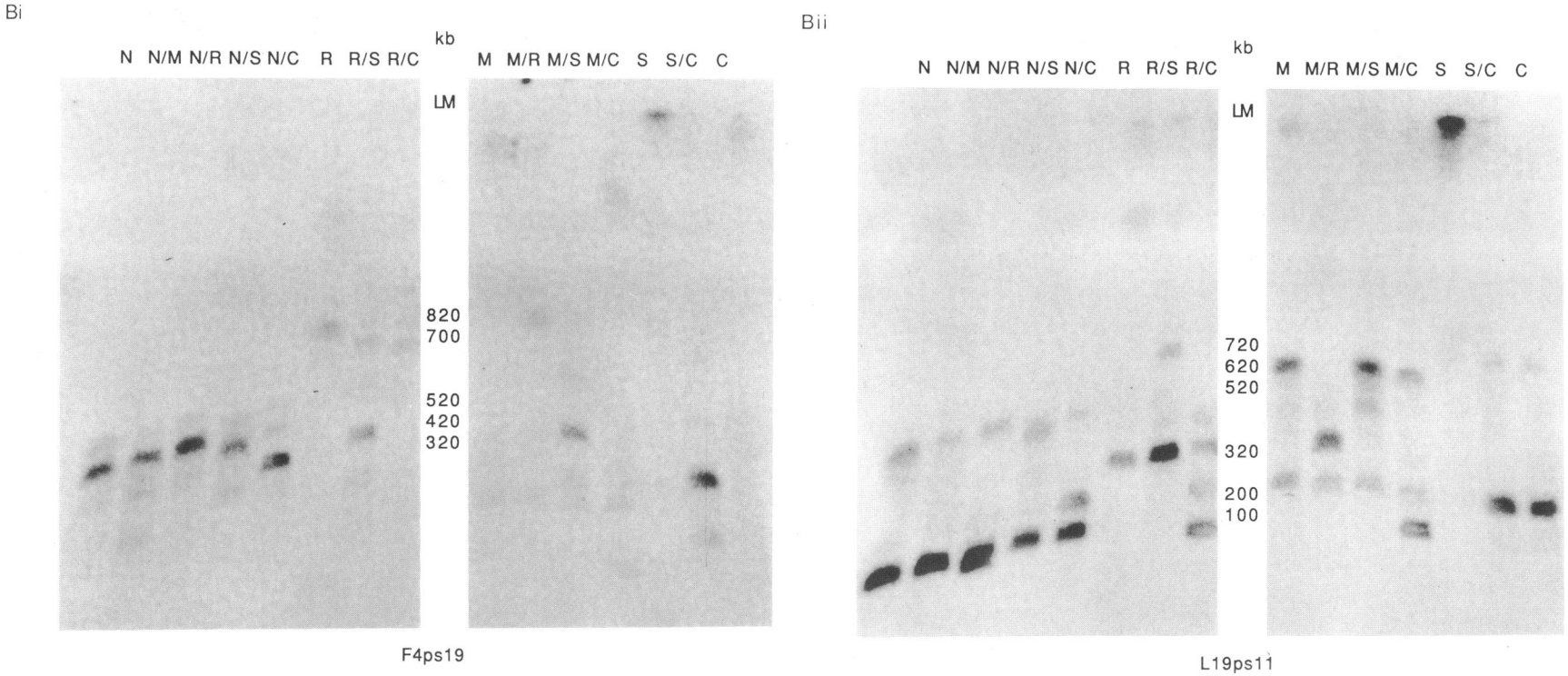
NotI.MluI.NruI.SpII.CspI
LM
4.7Mb
3.5Mb
1.6Mb
H552
BJ14
BJ56
42RB1.8
C4H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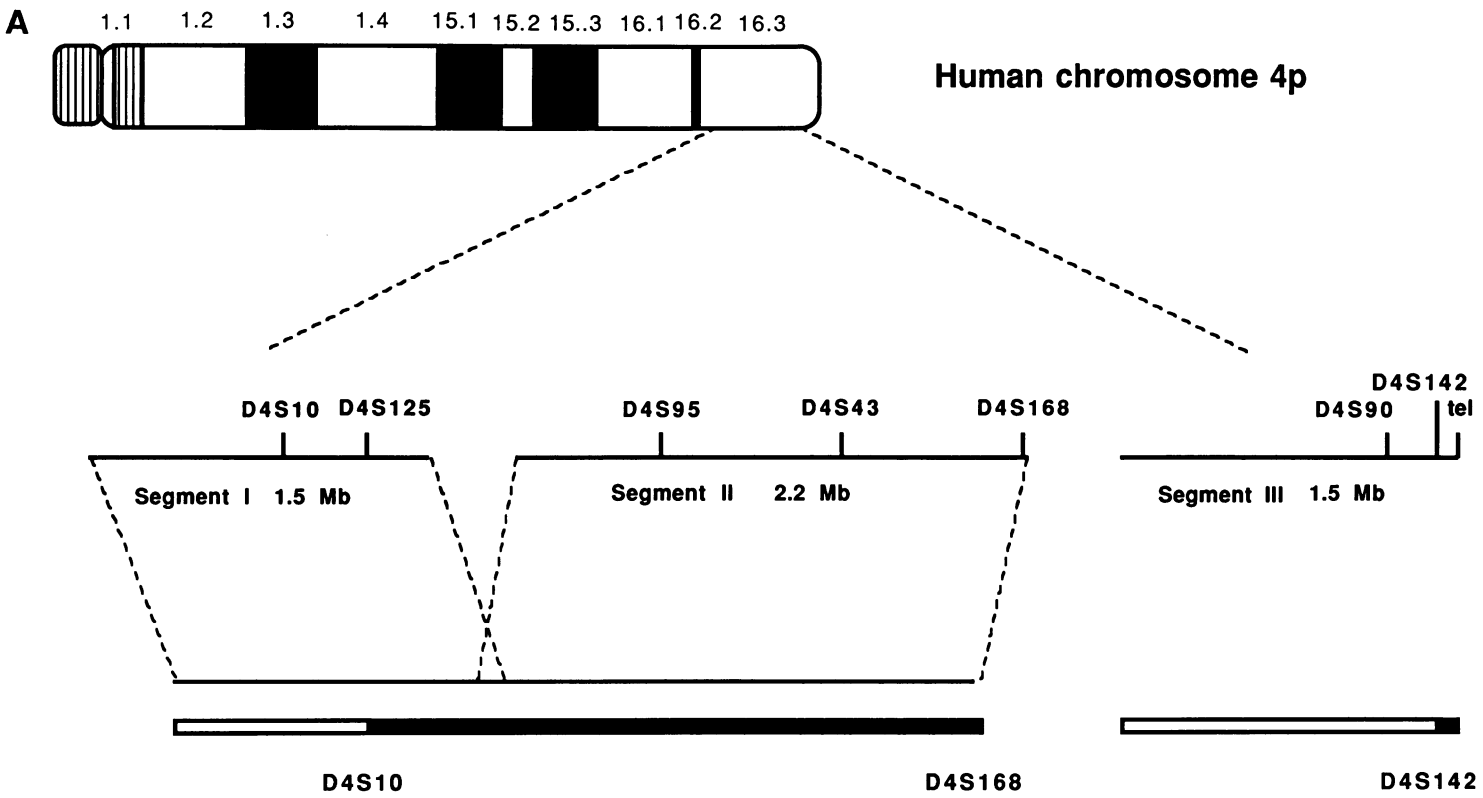
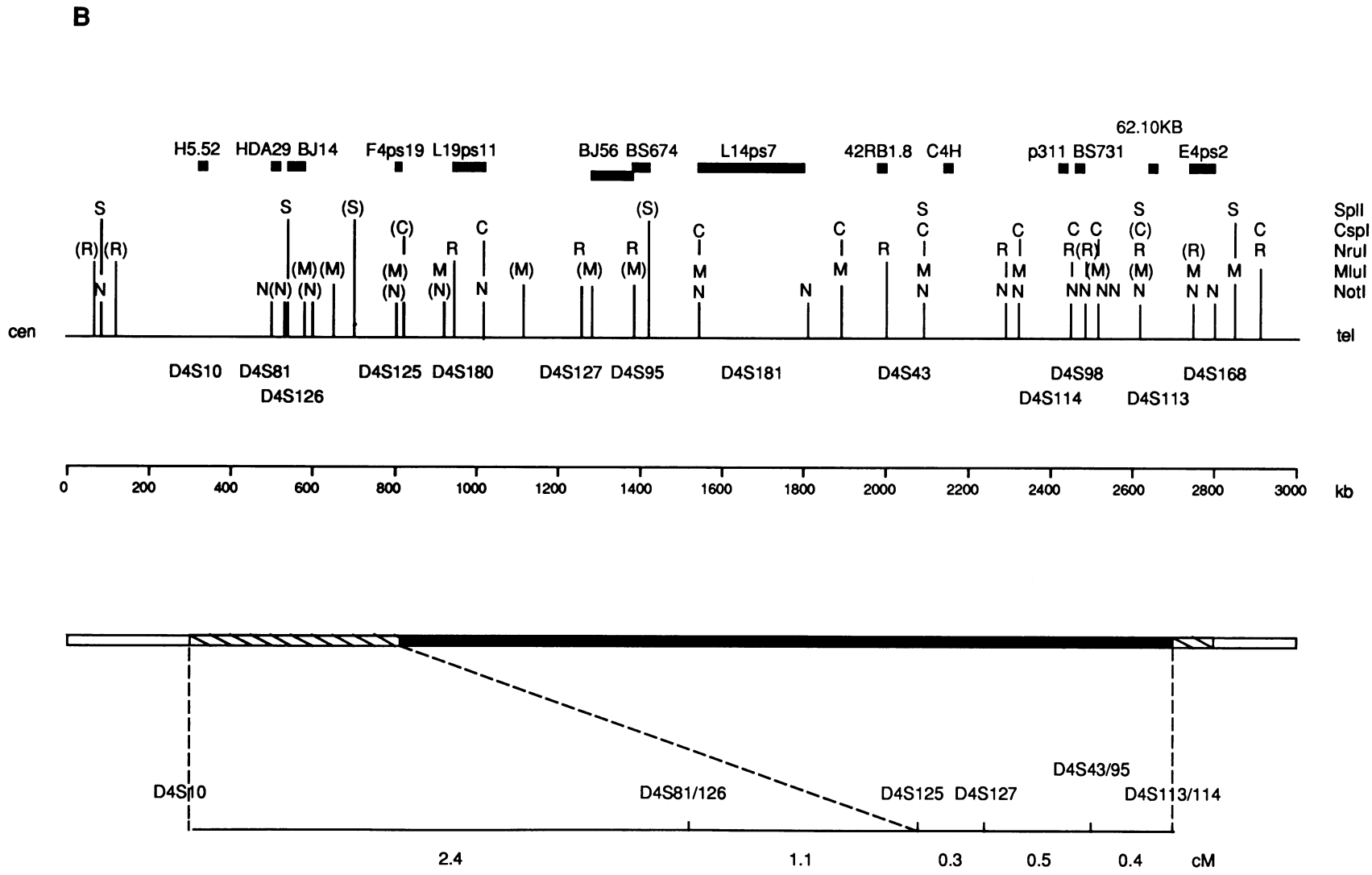


Figure 3 *A,* Summary of physical maps of 4p16.3. The physical map of 4p16.3, as previously published (Bucan et al. 1990), is illustrated below the ideogram of 4p, and the positions of markers used in the analysis of recombination events are indicated above the map. In this analysis, we have demonstrated that segments I and II overlap as shown. The filled-in bars at the bottom of the figure illustrate the extent of the internal and telomeric *HD* candidate regions. The physical and genetic extent of the internal region is described in detail in panel 3*B,* and the telomeric region as defined by D4S142 and the telomere has been previously published (Bates et al. 1990). *B,* Physical map of internal *HD* candidate region. The map was constructed in the blood of one individual by using the restriction enzymes *Not*I, *Mlu*I, *Nru*I, *Csp*I, and *Spl*I, and partially cleaved sites are indicated in parentheses. The minimal regions to which probes could be assigned are indicated above the map, with their corresponding locus names placed below. The new probes L19ps11 and L14ps7 were positioned as follows: L19ps11 was initially placed within the most proximal 100 kb of map segment II. It hybridizes to the same 620-kb *Mlu*I fragment as do BJ56 and BS674, and the *Not*I fragments detected by L19ps11 (100 kb) and BS674 (520 kb) lie within this *Mlu*I fragment. L14ps7 lies on a 270-kb *Not*I fragment between BS674 and 42RB1.8. All three probes hybridize to a common *Nru*I fragment of 620 kb, and the *Not*I sites defining the 270-kb *Not*I band were positioned on the basis of the sizes of the *Not*I/*Nru*I double-digest products detected by BS674 and 42RB1.8. The locations of the remaining markers with respect to *Not*I, *Mlu*I, and *Nru*I sites has been established elsewhere (Bucan et al. 1990; Whaley et al. 1991). The present study allowed the more precise positioning of HDA29 (on a 30-kb *Not*I partial fragment), BJ14 (within a 60-kb *Not*I/*Spl*I double-digest product), F4ps19 (on a 20-kb *Mlu*I/*Csp*I double-digest product), and BS674 (within a 40-kb *Spl*I/*Nru*I double-digest product). The existence of many *Not*I sites between p311 and 62.10KB has been comprehensively documented (Pohl et al. 1988; Bucan et al. 1990; Whaley et al. 1991). The hybridizations required to determine exactly how many of these sites are cut in this particular blood sample were not repeated, and, therefore, the *Not*I site distribution between these two probes, as illustrated here, is merely representative. The bar below the map illustrates the extent of the *HD* candidate region which is bordered by D4S10 and D4S168. The diagonally hatched sections between D4S10 and D4S125 and between D4S113 and D4S168 indicate the location of the crossover events that define the proximal and distal boundaries. Below this is shown this region's complementary genetic map, (MacDonald et al. 1989*b*; Allitto et al. 1991) which, in combination with the physical map, shows the striking variation in recombination frequency between the loci indicated.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H5.52
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cen
tel
D4S10
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D4S126
D4S125
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D4S95
D4S181
D4S43
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D4S114
D4S113
D4S168
0
200
400
600
800
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2200
2400
2600
2800
3000
kb
D4S10
D4S81/126
D4S125
D4S127
D4S43/95
D4S113/114
2.4
1.1
0.3
0.5
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cM

al. 1983; Gilliam et al. 1987*b;* Conneally et al. 1989). Further delineation of its position depends on the integration of physical and genetic mapping approaches. These currently include the construction of a long-range restriction map and its correlation with the genetic map, the identification of rare recombination events between genetic markers and *HD,* and the detection of linkage disequilibrium.

Putative single recombination events so far identified have suggested two mutually exclusive locations for the gene (MacDonald et al. 1989*b;* Robbins et al. 1989). Those that point to a location close to the telomere would have occurred distal to all available RFLPs. In contrast, the more proximal location is defined at its distal boundary by a discrete recombination event occurring between two markers—namely, D4S113 and D4S168 (MacDonald et al. 1989*b;* Whaley et al. 1991). This controversy in the location of *HD* will be conclusively resolved only by the detection of an informative polymorphic marker at the telomere. Such a marker might be derived from a recently isolated telomere yeast artificial chromosome (YAC) clone that contains the telomeric candidate region (Bates et al. 1990). If the crossovers suggesting the telomeric location appear to be still distal to such a marker, it may be necessary to postulate that the recombinant chromosomes responsible for these observations are the result of double recombination or gene conversion (MacDonald et al. 1989*b*), which would place *HD* within the more proximal candidate region. This hypothesis will only be testable once a polymorphic marker located within the recombined or converted region has been identified (MacDonald et al. 1989*b*).

In this present study we set out to define both genetically and physically the limits of the internal *HD* candidate region. We have established that the size of this region remains considerable, extending from a crossover event occurring between D4S10 and D4S125 to one between D4S113 and D4S168, a distance of as much as 2.5 Mb of DNA. The genetic linkage map that covers the region between D4S10 and D4S168 (MacDonald et al. 1989*b;* Allitto et al. 1991) is reproduced in figure 3*B*. Comparison of the genetic and physical maps reveals that the frequency of recombination across this region varies considerably. The majority of the genetic distance separating D4S10 and D4S168 is contributed by the comparatively high recombination frequency occurring between D4S10 and D4S125, within the proximal 500 kb of the map. It may be expected that the majority of the crossover events that are informative in positioning *HD* will also occur within this "hot spot." In contrast, the recombination frequency distal to D4S125 is considerably lower, and, consequently, the identification of relatively fewer crossovers with *HD* can be expected.

As an alternative to recombination events, linkage disequilibrium—i.e., the nonrandom association of marker alleles with a disease mutation—may be a useful indicator of the position of a gene; and, in the case of cystic fibrosis, it proved to be an extremely informative guide toward predicting the gene's location (Estivill et al. 1987; Kerem et al. 1989). Linkage disequilibrium has been reported between the HD mutation and some RFLPs at the D4S95 and D4S98 loci (Snell et al. 1989; Theilmann et al. 1989). Although these markers are separated by a physical distance of approximately 1 Mb pairs, the recombination frequency between them is comparatively low, and, therefore, it is not necessarily inconsistent that linkage disequilibrium should be detected over a relatively large region. However, the current reports are too preliminary to provide any additional information as to the precise placement of *HD*. In the eventuality that a peak of linkage disequilibrium exists, its detection will require a much more extensive study incorporating many more loci, especially those located between D4S95 and D4S98. In the absence of either further crossovers or a sharp peak in disequilibrium, the prospect of characterizing the potential HD involvement of all of the expressed sequences that reside in this region must be contemplated. The observed high density of rare-cutter sites within this region is likely to be indicative of many CpG islands and, therefore, of many genes (Bird 1986; Gardiner-Garden and Frommer 1987). A similar distribution of rare-cutter sites has been observed for chromosome 21, where the density of CpG islands is highest in the most telomeric subband, 21q22.3. Here there is evidence to confirm that this represents a high gene density and is not the result of chromosomal structure near telomeres (Gardiner et al. 1990). Furthermore, the same study found that some of the rare-cutter CpG islands in this region did not define single genes but gene clusters (Gardiner et al. 1990).

The completion of the physical map provides the basis on which strategies for the cloning of the *HD* candidate region can be designed. The map already contains sufficient markers to permit the isolation of the entire region in overlapping YAC clones (Burke et

al. 1987), thus generating the resource required to systematically study the potential HD involvement of all of the expressed sequences in this region.

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