Physical Maps of 4p16.3, the Area Expected to Contain the Huntington Disease Mutation

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The gene for Huntington disease, a neurodegenerative disorder with autosomal dominant inheritance, has been localized to the terminal portion of the short arm of human chromosome 4 (4p16.3) by linkage analysis. Since eventual isolation of the gene requires the application of high-resolution genetic analysis coupled with long-range DNA mapping and cloning techniques, we have constructed a physical map of the chromosomal region 4p16.3 using more than 20 independently derived probes. We have grouped these markers into three clusters which have been ordered and oriented by genetic and somatic cell genetic mapping information. The mapped region extends from D4S10 (G8) toward the telomere and covers minimally 5 Mb. © 1990 Academic Press, Inc.

INTRODUCTION

Huntington's disease (HD) is an inherited, autosomal dominant disorder affecting the central nervous system. The disease is characterized by progressive abnormal movements, psychological changes, and dementia which result in complete disability and death, usually 15–20 years after the appearance of the first symptoms. Neuropathological changes are caused by premature nerve cell death which is most prominent in the striatum. The disease becomes apparent usually

in the fourth or fifth decade of life; however, the age of onset and severity of symptoms are variable and depend in some cases on the parental origin of the gene (Hayden, 1981). Unexpectedly, individuals with a high probability of being homozygous for the HD mutation do not differ in clinical expression from typical HD heterozygotes (Wexler et al., 1987).

The biochemical defect causing cell death is not understood. However, considerable progress has been made toward localization and possible isolation of the mutant gene. The HD gene was originally mapped by genetic linkage to the D4S10 locus defined by the polymorphic DNA probe G8, which was assigned by somatic cell hybrids (Gusella et al., 1983; MacDonald et al., 1987; Smith et al., 1988), deletion analysis (Gusella et al., 1985), and in situ hybridization (Magenis et al., 1986; Landegent et al., 1986) close to the telomere of the short arm of chromosome 4. Genetic analysis, including multipoint linkage data, demonstrated that HD maps 4 cM distal to D4S10 (Gilliam et al., 1987b). Additional DNA loci, D4S43 (C4H) and D4S95 (BS674), were identified and initially did not show recombination with HD (Gilliam et al., 1987a; Wasmuth et al., 1988). However, genetic analysis using several, more informative DNA probes from the D4S43 locus have identified recombination events between HD and D4S43 placing this locus proximal to the disease gene (MacDonald et al., 1989).

To identify closer and, if possible, flanking markers, a number of new probes defining loci mapping distal to the D4S10 locus were isolated from sorted chromosome libraries and libraries which had been constructed from HHW693, a cell line containing a part of human chromosome 4 (4p15–4pter) translocated onto a fragment of 5p on a hamster background (Was-

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muth et al., 1986). This somatic cell hybrid was used to generate a total lambda library (Smith et al., 1988), a cosmid library (Bućan, unpublished), and a NotI linking library (Pohl et al., 1988), from which human clones were identified by hybridization to species-specific repeat sequences. The loci defined by these probes were mapped relative to each other and, where applicable, relative to the position of the HD mutation by somatic cell genetics (Smith et al., 1988; MacDonald et al., 1987) and linkage analysis (Gusella et al., 1983; Gilliam et al., 1987a; Wasmuth et al., 1988).

The localization of new sequences by genetic linkage analysis can indicate their linear order and their position with respect to the HD gene. Genetic linkage analysis, however, requires highly polymorphic markers and is often incapable of resolving neighboring loci. Pulsed-field gel electrophoresis (PFGE) (Schwartz and Cantor, 1984; Carle et al., 1986; Chu et al., 1986; Barlow and Lehrach, 1987) is a physical mapping technique that provides the resolution required to identify regions short enough to be analyzed by molecular techniques and to assign exact physical distances to the genetically determined intervals. In addition, the establishment of a physical map contributes to the information derived from genetic analysis, since data from physically ordered loci can be easily combined with genetic linkage data. Comparison of the genetic and physical map is necessary for the identification of those DNA fragments which are likely to be closest to the HD gene. The construction of the physical map of the chromosomal region around the HD gene is also of particular interest since it has been suggested that recombination is increased near the telomeres (Laurie and Hulten, 1985a,b).

We have placed all available DNA probes from the 4p16.3 region in a physical map that spans at least 5 Mb. Data from linkage analysis in reference and HD families (MacDonald et al., 1989; Youngman et al., 1989) allow the three map segments to be ordered and oriented relative to each other and to the HD gene. Several lines of evidence indicate that most of the DNA present in the telomeric region of chromosome 4 is represented on the map.

MATERIALS AND METHODS

Cells and DNA

DNA for PFGE analysis was prepared from lymphoblastoid cell lines and peripheral blood leukocytes. Lymphoblastoid cell lines used were GM1416B (karyotype 48,XXXX; NIGMS Human Genetic Cell Repository, Camden, NJ); the cell lines GusHM1, GusHM2, and GusHM3 isolated from patients with a high probability of being homozygous for HD; and the non-Huntington patient-derived cell lines AH148 and AH150 (kindly provided by M. Siniscalco), EJ167T12

(kindly provided by P. Goodfellow and I. Pickford), Gus115, and Gus641.

High-molecular-weight DNA from lymphoblastoid cell lines was prepared in agarose blocks according to published protocols (Herrmann et al., 1987). High-molecular-weight DNA from blood was prepared by selective lysis of the erythrocytes (Herrmann and Frischauf, 1987). The leukocyte pellet was washed three times in lysis buffer and once with phosphate-buffered saline (PBS), and the cells were resuspended in the same buffer at a concentration of 7×10^5 cells per 40 μl PBS and used similarly in the preparation of agarose blocks.

DNA Probes

The probes used in PFGE analysis are listed and described in Table 1. Insert DNA was purified by electrophoresis on low-melting-point (LMP) agarose gels, followed by digestion of the agarose with agarase (Bućan et al., 1986; Michiels et al., 1987). Hybridization probes were radiolabeled to a specific activity of 3×10^9 cpm/μg with [^{32}P]dATP using the Klenow fragment of DNA polymerase I and random hexamer priming (Feinberg and Vogelstein, 1983). To obtain longer hybridization probes we have used a reduced concentration of random hexamers (1/16th of the amount recommended by Feinberg and Vogelstein).

Pulsed-Field Gel Electrophoresis

The methods of pulsed-field gel electrophoresis, including DNA preparation in agarose blocks, preparation of yeast chromosomes and phage multimers used as size markers, restriction analysis and alkaline blotting onto GeneScreen filters, and the hybridization conditions have been described before (Herrmann et al., 1986, 1987; Church and Gilbert, 1984). Contour clamped homogeneous electric field (CHEF) electrophoresis was carried out in an electrophoresis apparatus similar to the one described by Chu et al. (1986) constructed at the EMBL, Heidelberg, West Germany. Agarose gels (0.75%) were electrophoresed in 0.25× TBE buffer for 40 h at 14°C at a voltage gradient of 5 V/cm, using a switching time of 45 s for the separation of DNA fragments up to 600 kb in length or of 100 s for the separation of fragments up to 1500 kb. Size markers used were chromosomes of Saccharomyces cerevisiae, strain YP148, kindly provided by P. Hieter and multimers of the phage EMBL3A (monomer length 42.5 kb).

Derivation of Clones by Chromosome Jumping

Clones 252 and J252 were derived from the human NotI jumping library which was constructed from cell line GM1416B using BamHI to recut and insert the

DNA into the vector (Poustka *et al.,* 1987; Poustka and Lehrach, 1988).

RESULTS AND DISCUSSION

Probes and Loci

The names of probes and loci, their sources, and a short description of their derivation are given in Table 1.

The sequences corresponding to the probes listed in Table 1 are deleted in cell lines H64 and HHW847, localizing them to the most distal subregion of 4p16.3 (Smith *et al.,* 1988; MacDonald *et al.,* 1987). Their presence or absence in the cell line HHW842, human/ Chinese hamster hybrid, derived from a patient with a large interstitial deletion that appears to involve 4p16.3–4p14, but contains the terminal part of 4p (Smith *et al.,* 1988; MacDonald *et al.,* 1987), allowed further classification into two subregions: the proximal subregion, B, contains 7 loci including *D4S10* (G8), *D4S43* (C4H), and *D4S95* (BS674), while the distal subregion, A, contains 12 additional loci (Table 1).

Loci from the two cytogenetically defined subregions were further analyzed by linkage studies, carried out in both reference and HD families. This allowed the establishment of a most "likely order" of the more informative marker loci (Gusella *et al.,* 1983; Gilliam *et*

TABLE 1

List of Molecular Probes Used in PFGE Analysis

Probe	Locus	Source	Region	Description	Ref.
G8 (pK082)	*D4S10*	Random marker	B	*Eco*RI, 5 kb	(14)
HDA29 (HDA RB1.6)	*D4S81*	Jump from G8	B	*Eco*RI–*Bam*HI 1.6 kb	(36)
BJ14	*D4S126*	HHW693 cosmid library	B	*Hind*III, 1.6 kb	Allitto, unpublished
YNZ32	*D4S125*	VNTR marker	B	*Rsa*I, 3.3 kb	(30)
BJ56	*D4S127*	HHW693 cosmid library	B	*Hind*III, 0.3 kb	Allitto, unpublished
BS674	*D4S95*	HHW693 lambda library	B	*Eco*RI, 1 kb	(38)
C4H	*D4S43*	Chromosome 4 sorted library	B	*Hind*III, 3.0 kb	(12)
39S4.8	*D4S43*	Cosmid walk from C4H	B	*Sst*I, 4.8	This paper
42RB1.8	*D4S43*	Cosmid walk from C4H	B	*Eco*RI–*Bam*HI, 1.8 kb	This paper
BS678	*D4S96*	HHW693 lambda library	A	*Eco*RI, 0.8 kb	(38)
BS731	*D4S98*	HHW693 lambda library	A	*Bam*HI, 1.3 kb	(38)
BS385	*D4S99*	HHW693 lambda library	A	*Hind*III, 4 kb	Smith, unpublished
BS854	*D4S97*	HHW693 lambda library	A	*Pst*I, 0.6 kb	(38)
D5	*D4S90*	Chromosome 4 sorted library	A	*Eco*RI, 1.5 kb	(43)
157.1BN1.5	*D4S111*	HHW693 linking library	A	*Bam*HI–*Not*I 1.5 kb	(31)
157.6R8.9	*D4S111*	Cosmid walk from 157	A	*Eco*RI, 8.9 kb	This paper
252BN4.0	*D4S115*	Jump from 157	A	*Bam*HI–*Not*I, 4.0 kb	This paper
J252K0.5	*D4S112*	Jump from 157	A	*Kpn*I, 0.6 kb	This paper
W92	*D4S114*	Cloning of DNA fragments from PFG	A	*Kpn*I–*Hind*III, 0.6 kb	(42)
102BN4.0	*D4S113*	HHW693 linking library	A	*Bam*HI–*Not*I, 4.0 kb	(31)
62.5BN2.8	*D4S113*	HHW693 linking library	A	*Bam*HI–*Not*I, 2.8 kb	(31)
62.10RB3.5	*D4S113*	Cosmid walk from 62	A	*Eco*RI, 3.5 kb	This paper
133S3.5	*D4S113*	HHW693 linking library	A	*Sal*I, 3.5 kb	(31)
133S2.8	*D4S113*	HHW693 linking library	A	*Sal*I, 2.8 kb	(31)
417R2.8		Cosmid walk from 417	A	*Eco*RI, 2.8 kb	This paper
417BN1.5		HHW693 linking library	A	*Bam*HI–*Not*I, 1.5 kb	(31)
107S1.8		HHW693 linking library	A	*Sal*I, 1.8 kb	This paper
107BN0.5		HHW693 linking library	A	*Bam*I–*Not*I, 0.5 kb	This paper
J107S3A0.9		Jump from P107	A	*Sau*3A, 0.9 kb	This paper
cJ107.1BN2.5		Cosmid walk from J107	A	*Bam*HI–*Not*I, 2.5 kb	This paper

al., 1987a; Wasmuth et al., 1988; Whaley et al., 1989; MacDonald et al., 1989; Youngman et al., 1989; Allitto et al., in preparation). Loci from subregion B have been assigned to three groups: the most proximal locus D4S10 (G8); a second locus, D4S81 (HDA29), placed distal to D4S10 (G8) by two recombination events (Richards et al., 1988); and the most distal group, consisting of loci D4S125 (YNZ32), D4S95 (BS674), and D4S43 (C4H). Loci mapping to the terminal subregion A all lie distal to D4S43 (C4H), and can also be assigned to three distinct groups: the group defined by loci D4S113 (P62, P102), D4S114 (W92), and D4S98 (BS731) maps nearest to D4S43 (C4H); a second group, defined by loci D4S115 (P252), D4S111 (P157), and D4S96 (BS678), was placed distal to the first group by recombination events; while the marker D5 (D4S90) defines the most distal locus analyzed.

Combination of cytogenetic and linkage data therefore allowed the ordering of loci from 4p16.3: [D4S10 (G8)]–[D4S81 (HDA29)]–[D4S125 (YNZ32), D4S95 (BS674), D4S43 (C4H)]–[D4S113 (P62, P102), D4S114 (W92), D4S98 (BS731)]–[D4S115 (P252), D4S111 (P157), D4S96 (BS678)]–[D4S90 (D5)].

Strategy of Physical Mapping

To construct a physical map of the chromosomal region around the HD gene 21 probes from 4p16.3 were analyzed by PFGE analysis using restriction enzymes which cut infrequently in mammalian DNA. Preliminary experiments had shown that 4p16.3 contains many CpG islands and therefore probes from that region detect relatively small bands in DNA digested with rare cutting enzymes containing only C and G in their recognition sites (BssHII, EagI, and NotI). In addition to the enzyme NotI, we used the enzymes MluI and NruI which contain A and T as well as two CpG sequences in their recognition sites. A major fraction, 73% (Brown and Bird, 1986), of MluI and NruI sites is expected to occur outside CpG islands. MluI and NruI, despite their shorter recognition sequence, often generate larger DNA fragments than NotI in regions rich in CpG islands (Brown, 1988).

Two different sources of DNA were used for the preparation of filters: peripheral blood leukocytes and the lymphoblastoid cell line GM1416B containing four X chromosomes (48,XXXX karyotype). Preliminary analysis suggested that most regions within 4p16.3 are undermethylated in the DNA from the cell line GM1416B in comparison to DNA isolated from peripheral blood leukocytes. It often proved possible to demonstrate physical linkage over longer distances in digests of blood leukocyte DNA, while the hybridization pattern in digests of GM1416B DNA allowed more precise localization and distance determination. In addition, changes in band positions due to differential site methylation were used to confirm or exclude the

detection of the same bands by two different probes. This resulted in two different maps that can be aligned at points corresponding to sites that are cleaved in both DNAs.

Probes were successively hybridized to blood and cell line DNA, digested with MluI, NruI, NotI, and combinations of these three enzymes (Fig. 1). On the basis of fragment sizes detected by each probe (results summarized in Table 2) a map consisting of three continuous segments was constructed (shown as segments I, II, and III in Fig. 2).

Segment I

Map segment I (Fig. 2A) was constructed using three independently isolated clones, G8 (D4S10) (Gusella et al., 1983), YNZ32 (D4S125) (Nakamura et al., 1988), and BJ14 (D4S126) (Allitto et al., in preparation), and the probe HDA29 (D4S81) obtained by chromosome jumping from G8 (Richards et al., 1988). D4S81 is located 200 kb distal to D4S10 and a partial restriction map covering the region surrounding these two probes was reported by Richards et al., (1988). D4S10, D4S125, D4S126, and D4S81 were mapped to the cytogenetic subregion B by a somatic cell hybrid panel (MacDonald et al., 1987, 1989; Allitto et al., in preparation).

The analysis of the hybridization pattern in blood DNA showed that the probes HDA29, BJ14, G8 (subclone pK082), and YNZ32 are in close physical proximity; HDA29, BJ14, and YNZ32 are located on the same 420-kb NotI fragment and G8 is located on an adjacent NotI fragment of the same size (Fig. 2A; Table 2). Hybridization to the less methylated DNA from the cell line GM1416B established the order G8–(HDA29, BJ14)–YNZ32 and allowed more precise localization of the probe sequences (Fig. 2A). The resulting map segment (segment I) covers 1500 kb and is oriented genetically from the observation of recombinant chromosomes. D4S10 (G8) was positioned proximal to the other markers D4S81 (HDA29), D4S126 (BJ14), and D4S125 (YNZ32). Two recombination events separate D4S10 and D4S81 (Richards et al., 1988), reflecting the high recombination frequency in the region between D4S10 and D4S81 (Richards et al., 1988; Allitto et al., in preparation).

Segment II

The map of the next segment (segment II, Fig. 2B) contains two groups of probes, mapping respectively to the proximal and distal subregions of 4p16.3, and therefore straddles the cytogenetically defined boundary between these two subregions. The physical map of part of segment II which is not contained in HHW842, and therefore proximal to the boundary between regions B and regions A, is based on the analysis of three probes from the locus D4S43 (C4H, 39S4.8, and 42RB1.8) and the independently isolated clones
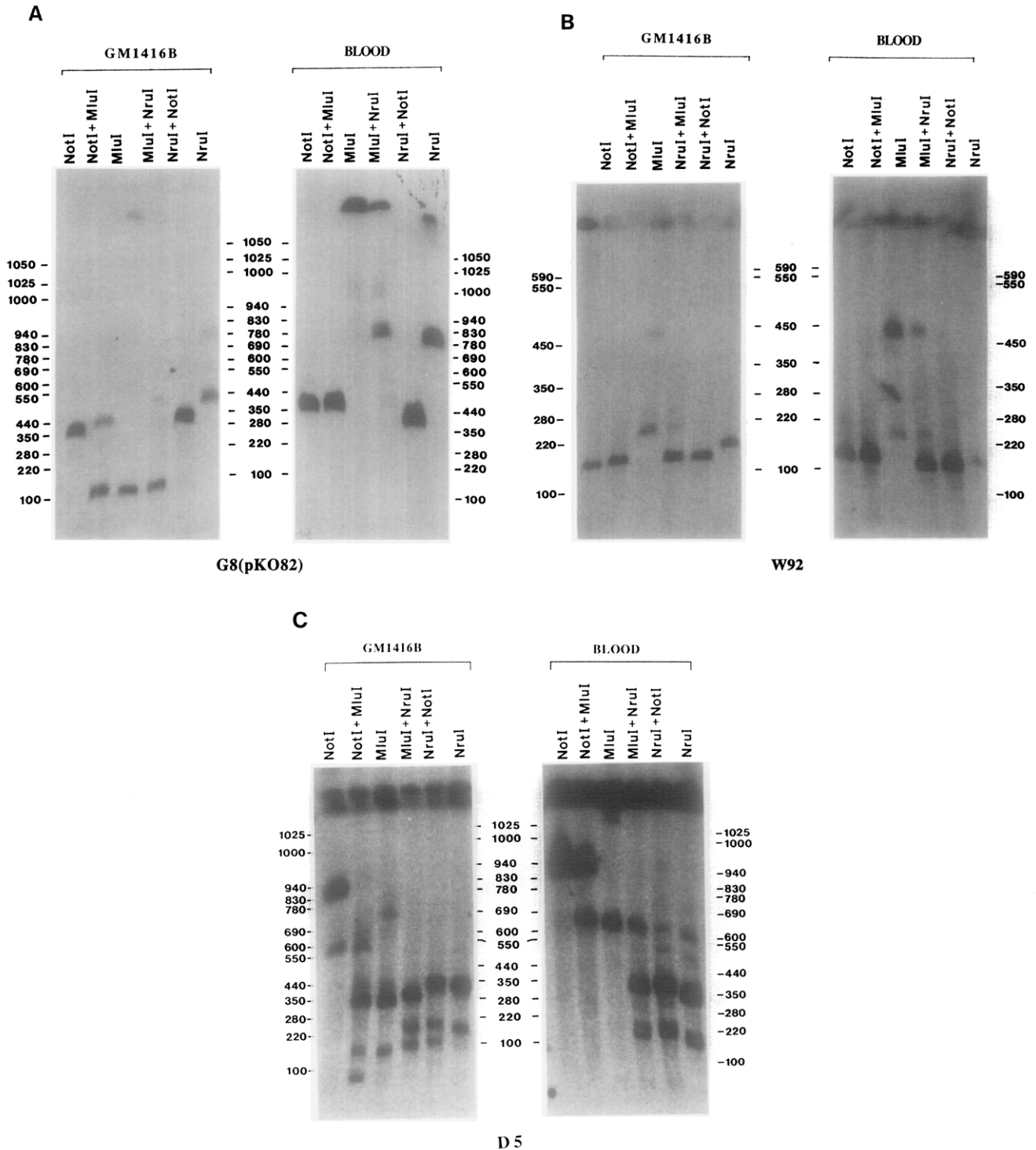
FIG. 1. Pulsed-field gel analysis of DNA isolated from blood and GM1416B cell line. High-molecular-weight DNA was prepared from peripheral blood leukocytes and the cell line GM1416B. DNA samples were digested with *Not*I, *Mlu*I, *Nru*I, and combinations of these enzymes. Electrophoresis was carried out for 40 h in a 0.75% agarose gel at an electric field gradient of 5 V/cm, at 14°C. Separation of DNA fragments up to 600 kb (**B**) was obtained by electrophoresis with a pulse time of 45 s; separation up to 1500 kb (**A, C**) was obtained with a pulse time of 100 s. Filters were analyzed by hybridization with the probes pKO82 (**A**), W92 (**B**), and D5 (**C**). Thirty probes listed in Table 1 were successively hybridized to the filters shown in Fig. 1. Observed fragment sizes are listed in Table 2.

TABLE 2

Summary of Fragment Sizes

Probe	Cell line						Blood					
	N	N+M	M	M+R	R+N	R	N	N+M	M	M+R	R+N	R
PK082	420	120	120	120	380	440	420	420	(970) 1300	(800) 1300	380	800
BJ14 HDA29	(160) 300	(160) 300	(160) 420	100 320 420	100 (160) 300	440	420 520	420 (520)	(350) 1300	(350) 800	420 (520)	800
YNZ32	(140) 300	(140) (300)	260 420	360 320 420	(140) 200 300	320	420 520	420 (520)	(350) 1300	(350) 800	420 (520)	800
C4H	200	200	200	230	200	340	200	200	230	200	200	340
39S4.8	80 280	70 (20)	70 (200)	70 (200)	80	340	280		200			340
62.5.BN2.8	30	30	225	85 160	30	150	30 75	30 75	(225) (450)	170 (450)	30 75	170
62.10RB3.5	100	100	225	120	100	120	100	100 (450)	(225)	120	100 290	120 290
J252K0.5	100	100	150	150	100	400	100	100	60 450	60 450	60 450	400
252BN4.0 BS854	250	250	250	225	225	400	250	250	380 450	350 380 450	250	400
157.1BN1.5	250	250	250	30	30	30	250	250 450	380 450	350 450	250	400
BS678	100	100	150	150	20	400	100	100	380 440	380 440	100	400
BS674	500	180 (260) 440	180 (260) 440	180	180	650	500	(180) (260) 440 500	(180) (260) 440 680	180	180	650 (900)
BJ56	500	(80) (260) 440	(80) (260) 440	(80) 120	120	120	500	(260) 440 500	(260) 440 680	120	120	120
W92	150	150	225 260 (450)	150	150	170	150	150	(225) (450)	150 (220) (450)	150	170
BS731 BS385 P102	<20	<20	225 (260) (450)	85 100 160 220	<20	150	>20	>20	(225) 450	80 (120) (170) (220) (300) (450)	<20	170
P133	75	75	225 (450)	70	75	150	75	75	(225) (450)	(170) (220) (450)	75 60	170
42RB1.8	220 280	130 (200)	130 (200)	(80) 130	200	650	280	200	200	80 (200)	200	650 (900)
107BN0.5	50		15			170						

TABLE 2—Continued

Probe	Cell line						Blood					
	N	N+M	M	M+R	R+N	R	N	N+M	M	M+R	R+N	R
107Sal18	150		230			170						
J107	50	50	230	230	50	340						
C107.1	200	200	230	200	200	340						
157.6R8.9	150	150	300 380 700	150 250 350 400	150	150 250 400	150	150	380 (440) 1000	(150) 250 400	150	(150) 250 (400)
417 BN1.8												
D5	(600) 850	(50) (100) 300 600	(100) 300	(100) (180) 300	(100) (180) 350	(180) 300	850	650 850	650 1000	(180) 350 (600)	(180) 350 (600)	(180) 350 (600)

Note. Partially cut bands are indicated in parentheses. All restriction fragment sizes are approximate values (±10%). To confirm the identity of shared bands, several gels run with different pulse times were analyzed. Sizes of partially cut bands larger than 1000 kb are determined as sums of shorter bands due to the larger uncertainty associated with direct size determination of high-molecular-weight bands.

BS674 (*D4S95*) and BJ56 (*D4S127*). *D4S43* maps 4 cM distal to *D4S10* (Gilliam *et al.,* 1987a) but two crossovers place it proximal to the *HD* gene (MacDonald *et al.,* 1989). The locus *D4S43* was extended by chromosome walking from C4H and overlapping cosmids covering 180 kb were isolated (Gilliam *et al.,* 1987a; Zimmer *et al.,* unpublished). A large number of probes isolated from these cosmids were used in genetic linkage analysis and in PFGE mapping studies.

D4S95 defined by probe BS674 was found to be located distal to *D4S10* (G8) by linkage analysis. Initial results did not show recombination with the *HD* locus (Wasmuth *et al.,* 1988), placing it within 0–1.5 cM of the gene. Probe BJ56 was isolated as a human clone from a cosmid library derived from cell line HHW693 and localized to region B by somatic cell genetics.

The map around locus *D4S43* (C4H) is based on the original C4H probe and 42RB1.8, a probe resulting from a chromosome walk from C4H (*D4S43*). 42RB1.8 is adjacent to a partially cleaved *Nru*I site located 150 kb from the C4H and hybridizes to an *Nru*I fragment of approximately 650 kb. An *Nru*I band of the same size is detected by the probe BS674. Coincidental comigration of bands could not be excluded by the use of other enzymes or by the analysis of double-digest patterns, because both probes appear to be located close to the opposite ends of the potentially shared *Nru*I fragment. We therefore analyzed the hybridization patterns of BS674 and 42RB1.8 in DNA isolated from the blood of three different individuals and from nine different lymphoblastoid cell lines digested with the enzyme *Nru*I (Fig. 3). The detection of the same partially cut *Nru*I fragments in the same intensity ratio in DNA from different sources by these probes strongly

suggests that the hybridization of the 650-kb band is due not to random comigration of two different fragments of identical size, but to the location of both probes on the same restriction fragment.

Localization of the probes BS674 and BJ56 is based on their hybridization to identical *Not*I and *Mlu*I fragments. BJ56 does, however, hybridize to a 120-kb *Nru*I fragment different from that detected by BS674. This leads to the following order of markers: BJ56–BS674–42RB1.8–C4H.

The map of the distal subregion of segment II is based on the linking clones P62 (*D4S113*), P133 and P102 (*D4S114*) (Pohl *et al.,* 1988), and P107, probes BS731 (*D4S98*) and BS385 (*D4S99*) isolated from the HHW693 lambda library (Smith *et al.,* 1988), clone W92 (*D4S114*) isolated after preparative pulsed-field gel electrophoresis (Whaley *et al.,* 1988), and clone J107 isolated by chromosome jumping from P107. All clones were localized by somatic cell hybrids to the distal region of 4p16.3 (region A) (Pohl *et al.,* 1988; Smith *et al.,* 1988; Whaley *et al.,* 1988).

This region of the map of segment II is based on the closely spaced *Not*I linking clones P62, P133, P102, and P107 sharing a 450-kb *Mlu*I partial digestion product, and could be extended on one side by a cosmid isolated using P62 as probe. To extend the map on the other side, we used P107 to screen a *Not*I chromosome jumping library (Poustka and Lehrach, 1986, 1988; Poustka *et al.,* 1987), containing sequences from both ends of single *Not*I fragments brought together by circularization and cloning of the junction fragments. The resulting jumping clone J107, a 50-kb jump from P107, hybridized to the same *Mlu*I and *Nru*I fragments as C4H. In addition, probes on the other side of the *Not*I
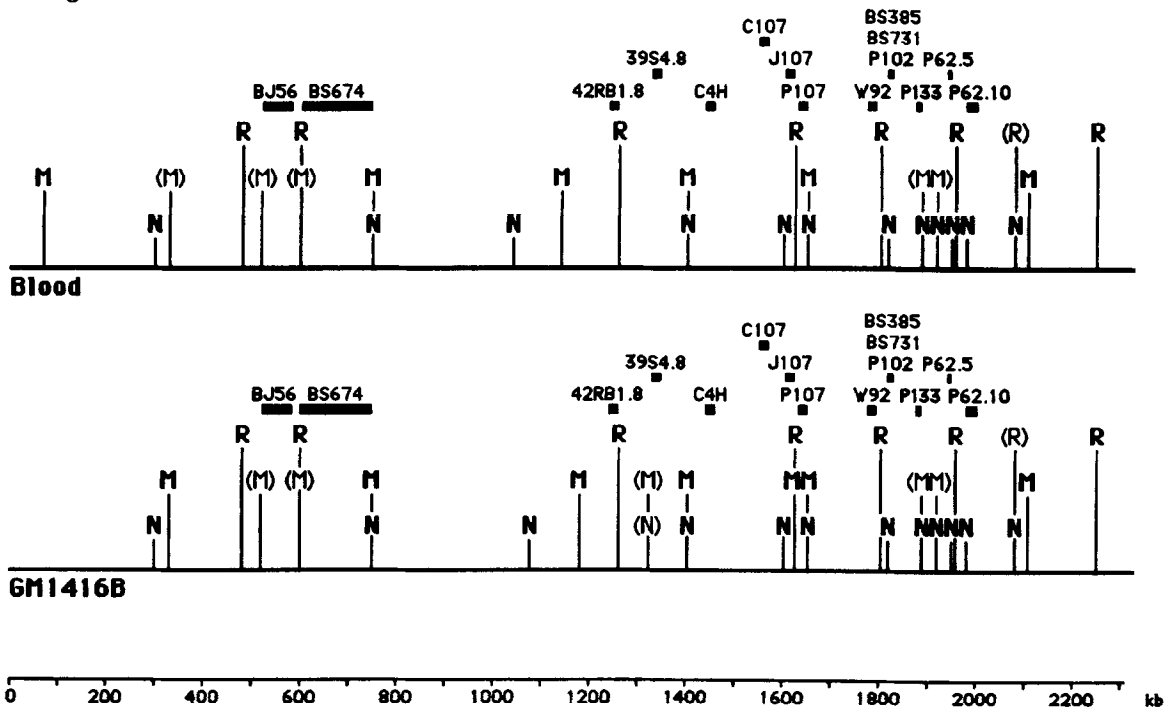
Segment I
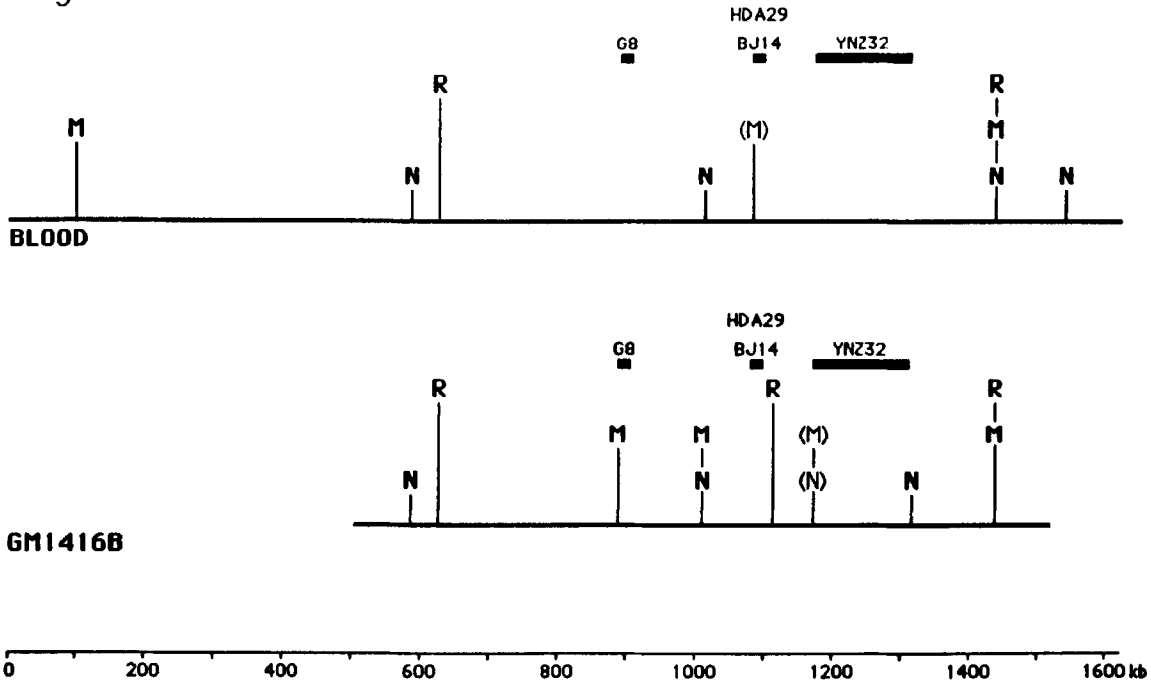
Segment II

FIG. 2. Physical maps of band 4p16.3. Long-range maps of the DNA in the blood of one individual or in the cell line GM1416B were constructed by analysis of the data in Table 2. The maps indicate sites for the enzymes *Not*I (N), *Nru*I (R), and *Mlu*I (M). Partially cut sites are shown in parentheses. The scale (in kb) is shown beneath the map determined with GM1416B DNA. The positions of probes are indicated by black squares above the map. Longer black boxes indicate the limits of uncertainty of probe locations.

Segment I; HDA29, pK082 (G8), BJ14, and YNZ32 all recognize two large DNA fragments in DNA isolated from peripheral blood leukocytes: an *Nru*I fragment of approximately 800 kb and an *Mlu*I fragment of about 1300 kb. All four probes detect a *Not*I band of 420 kb; however, a partial *Not*I fragment of 520 kb hybridizes only to HDA29, BJ14, and YNZ32, indicating that the probe pK082 hybridizes to a different comigrating *Not*I fragment. In the cell line GM1416B probes pK082, BJ14, and HDA29 all detect an *Nru*I fragment of approximately 440 kb, while YNZ32 detects an *Nru*I fragment of about 320 kb in length. These results suggest that the probes BJ14 and HDA29 map between pK082 and YNZ32. Probes BJ14 and HDA29 show the same hybridization pattern using *Mlu*I, *Not*I, and *Nru*I single and double digests. Hybridization

Segment III
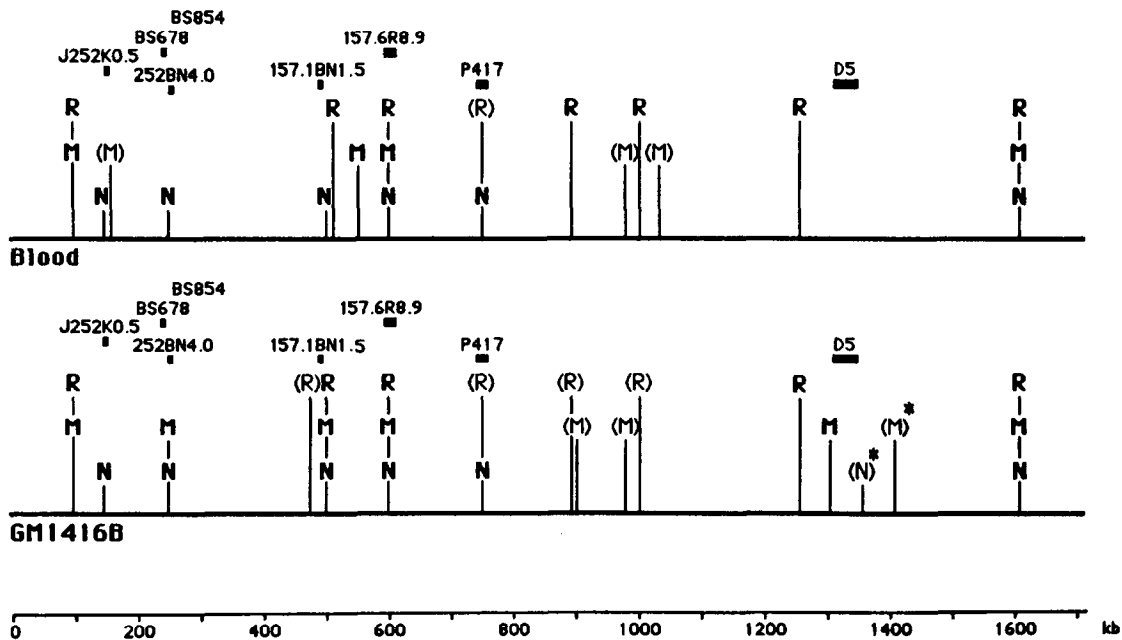
Fig. 2—*Continued*

site isolated from a cosmid clone identified by J107 also hybridized to the same *Not*I and *Nru*I fragments as C4H, verifying the connection between the proximal and the distal parts of segment II.

This establishes the final order of markers as BJ56–BS674–42RB1.8–C4H–J107–P107–W92–P102–P62 embedded in a map covering approximately 2.2 Mb. Using four enzyme digests (*Not*I, *Mlu*I, *Nru*I, and *Sal*I) no shared restriction fragments between YNZ32, the most distal probe of segment I, and BJ56, the most proximal probe of segment II, could be detected.

Segment III

Map segment III (Fig. 2C) was established using the linking clones P157 and P417, the jumping clones J157

(linking clone P252) and J252, probes from a chromosomal walk from P157, and the probe D5 (*D4S90*) isolated from a chromosome 4-specific library and mapped to the distal portion of 4p16.3 (region A) (Youngman *et al.*, 1988; Youngman *et al.*, 1989). *D4S90* (D5) was mapped by linkage analysis to a position 6 cM distal of *D4S10* (Youngman *et al.*, 1989; MacDonald *et al.*, 1989), distal to all other described markers.

The clone P157 was isolated from a *Not*I linking library and mapped by somatic cell hybrids to the distal portion of 4p16.3 (Pohl *et al.*, 1988). To expand the PFGE map and to isolate genetic and physical markers from the vicinity of P157 we used the fragment 157.1BN1.5, which flanks the *Not*I site, to screen a *Not*I chromosome jumping library. Two clones hybridizing to the start probe were identified. The endpoints of both jumps were mapped to the subregion A by so-
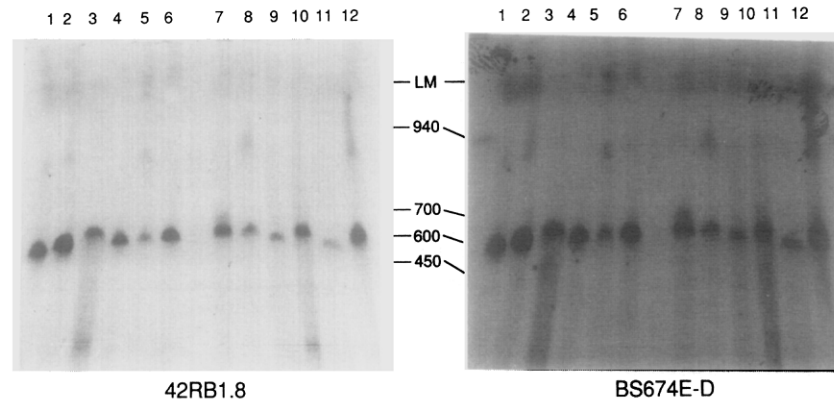
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FIG. 3. Physical linkage of probes 42RB1.8 and BS674. High-molecular-weight DNA from the blood of three individuals (**1, 2, 3**), sperm (**4**), and lymphoblastoid cell lines GM1416B (**5**), AH148 (**6**), GusHM1 (**7**), GusHM2 (**8**), GusHM3 (**9**), Gus641 (**10**), Gus115 (**11**), and EJ167T12 (**12**) was digested with the enzyme *Nru*I. Electrophoresis was carried out for 40 h in a 0.75% agarose gel at an electric field gradient of 5 V/cm, with a pulse time of 100 s, at 14°C. The filter was analyzed by hybridization with the probes 42RB1.8 and BS674. Probes 42RB1.8 and BS674 detect only one strong fragment of 650 kb in DNA from 12 different sources. In addition, both probes detect the same weak partially digested fragment of 900 kb in DNA isolated from cell lines GM1416B and GusHM2, and a partially digested fragment of 700 kb in DNA from cell line GusHM1.

matic cell hybrids and localized relative to the startpoint by PFGE analysis (Fig. 4). Startpoint clone 157.1BN1.5 and endpoint 252BN4.0 are located on opposite ends of the 250-kb *Not*I fragment. In contrast, a probe from the endpoint of the other jump (J252K0.5) hybridizes to a *Not*I fragment of 100 kb, suggesting, that this clone represents a jump over a *Not*I site only partially cleaved during library construction, positioning the endpoint of this jump 350 (250 + 100) kb from the start. This interpretation was confirmed by isolating a linking clone (P252) which detects two adjacent *Not*I fragments of 100 and 250 kb.

Two clones isolated from the HHW693 lambda library, BS854 and BS678 (Smith *et al.,* 1988), map in the vicinity of P252. The probe BS854 shows the same hybridization pattern as 252BN4.0, localizing BS854 between 252BN4.0 and 157.1BN1.5. The probe BS678 was shown to hybridize to a cosmid isolated using 252BN4.0 as a probe, and is located between 252BN4.0 and J252.

Since fragments in the direction opposite to the jumps 252 and J252 were small, the cloned region was extended in this direction by cosmid walking through a region containing a number of well-cleaved *Not*I, *Nru*I, and *Mlu*I sites (Zimmer *et al.,* unpublished). The map was then further extended with the probe 157.6R8.9 located 100 kb away from the linking clone P157, flanking well-cleaved *Nru*I, *Not*I, and *Mlu*I sites. D5, the only remaining region A probe, could initially not be unequivocally connected to the other probes. There was, however, hybridization of both 157.6R8.9 and D5 to a 1000-kb *Mlu*I partial digestion product. Using the strategy applied before in verifying linkage between the markers BS674 and 42RB1.8, we took advantage of methylation (or sequence) polymorphism to verify the location of 157.6R8.9 and D5 on the iden-

tical *Mlu*I fragment. Both probes were successively hybridized to the same filter containing 15 DNA samples isolated from the blood of different individuals and from different lymphoblastoid cell lines (Fig. 5). The same hybridization pattern and identical intensity ratios between *Mlu*I partial bands detected by D5 and 157.6R8.9 confirmed that the two probes hybridize to the same 1000-kb partial *Mlu*I fragment. The physical linkage of 157.6R8.9 and D5 was proven by linking clone P417 that detects the same *Mlu*I partially cut band and shares one *Not*I fragment with 157.6R8.9 and the other with D5. The analysis of the probe D5 in DNA isolated from GM1416B allowed the construction of the map around D5, placing this probe 700–750 kb away from 157.6R8.9.

In constructing the map of this region in GM1416B, the observed hybridization pattern can be explained by postulating different methylation profiles on the two homologous chromosomes (Fig. 2c). In this context, it is interesting to note that imprinting by differential methylation of paternal and maternal chromosomes has been suggested as a possible source of the difference in the age of onset of paternally and maternally transmitted chromosomes in HD (Erickson, 1985; Reik, 1988).

No evidence for physical linkage between segments I and II was detected. The other end of the map is defined by a site observed with all enzymes tested (*Not*I, *Mlu*I, *Nru*I, *Sal*I, *Bss*HII). No fragments resulting from either partial or complete restriction digest extending beyond this site were observed indicating either a cluster of very well cut rare cutter restriction sites or possibly the end of the chromosome. Since repeated attempts to reach the position next to the presumptive island by either chromosome jumping in a *Bss*HII chromosome jumping library or by the selective cloning
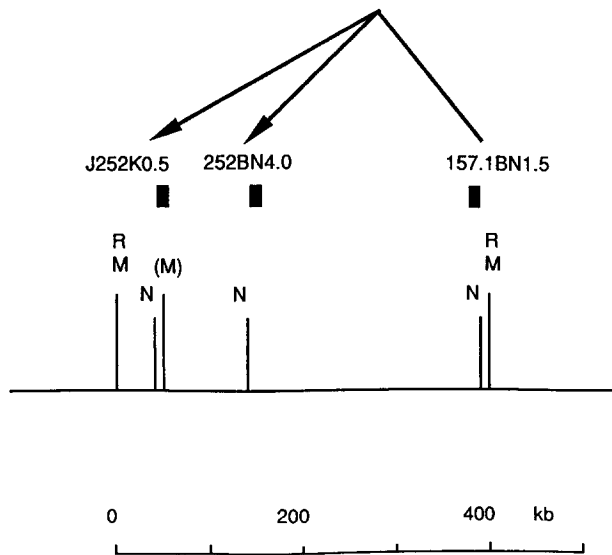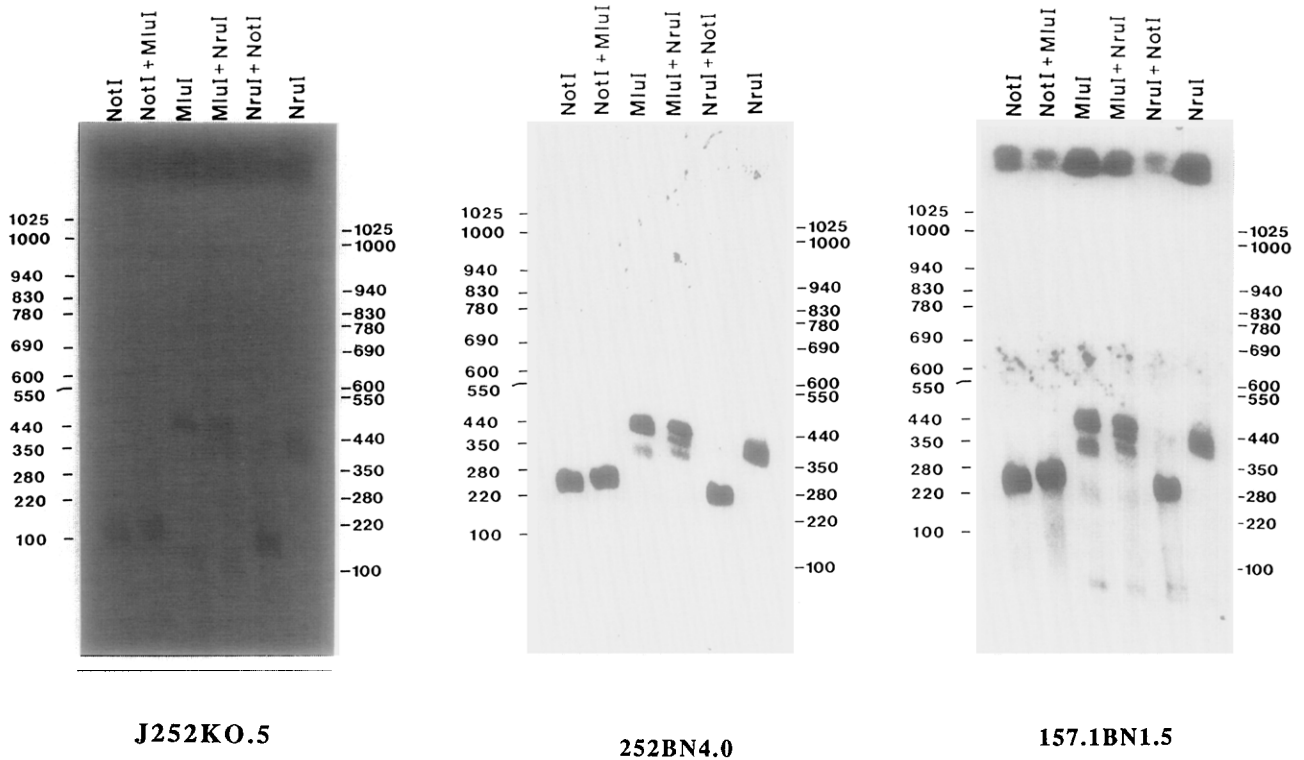
J252K0.5   **252BN4.0**   **157.1BN1.5**

FIG. 4. Chromosome jumping from linking clone 157. (**Top**) PFGE analysis of the startpoint clone (157.1BN1.5) and the two endpoints (252BN4.0, J252K0.5). High-molecular-weight DNA was prepared from peripheral blood leukocytes and digested with *Not*I, *Mlu*I, *Nru*I, and combinations thereof. Electrophoresis was carried out for 40 h in a 0.75% agarose gel at an electric field gradient of 5 V/cm, at 14°C, with a pulse time of 100 s. The filters were analyzed by hybridization with the probes J252K0.5, 252BN4.0, and 157.1BN1.5. (**Bottom**) Long-range map of the region surrounding the startpoint probe 157.1BN1.5 and the endpoints probes 252BN4.0 and J252K0.5. The map indicates sites for the enzymes *Not*I (N), *Nru*I (R), and *Mlu*I (M). Partially cut sites are shown in parentheses. The scale (in kb) is shown beneath the map. The positions of probes are indicated by black squares above the map. The arrows indicate the direction of jumps relative to the startpoint. Both the startpoint probe 157.1BN1.5 and a probe from one of the endpoints (252BN4.0) hybridize to a *Not*I fragment of about 250 kb, two *Mlu*I fragments of about 380 and 440 kb, and an *Nru*I fragment of 400 kb in blood DNA (**top, right,** and **middle**). Using additional enzymes which cut between these two clones (*Bss*HII, *Sfi* I, and *Sal*I) we were able to show that the jumping clone 252 maps, as expected, to the opposite end of the *Not*I fragment of 250 kb (data not shown). A probe from the second endpoint (J252K5.0) hybridizes to a *Not*I fragment of 100 kg, two *Mlu*I fragments of 60 and 450 kb, and an *Nru*I fragment of 400 kb, suggesting that this clone represents a jump over an uncut *Not*I site (**top left**). Clone J252 therefore maps 350 kb (250 + 100) away from the probe 157.1BN1.5 used as a starting point.
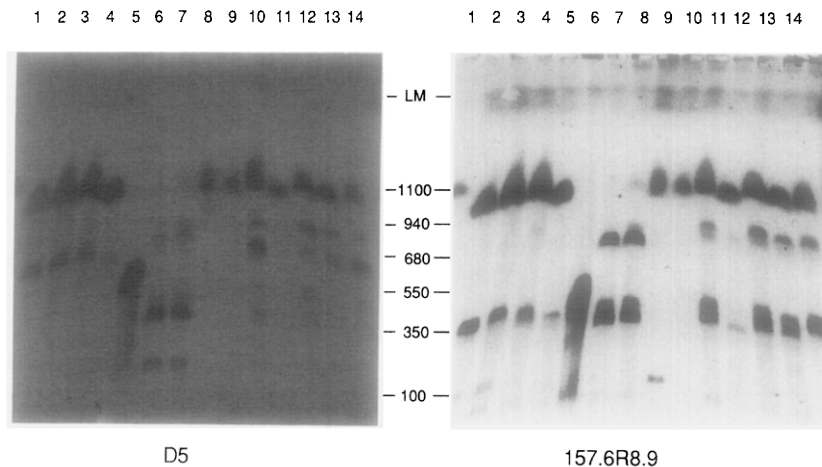
FIG. 5. Physical linkage of probes 157.6R8.9 and D5. High-molecular-weight DNA from the blood of four individuals (**1, 2, 3, 4**), sperm (**5**), and DNA from two different passages of the lymphoblastoid cell lines GM1416B (**6, 7**), HHW842 (**8**), HHW693 (**9**), Gus641 (**10**), Gus115 (**11**), two different passages of GusHM3 (**12,13**), and GusHM1 was digested with the enzyme *MluI*. Electrophoresis was carried out for 40 h in a 0.75% agarose gel at an electric field gradient of 5 V/cm, with a pulse time of 100 s, at 14°C. The filter was analyzed by hybridization with the probes D5 and 157.6R8.9.

of fragments ending in *Not*I sites were unsuccessful, the location of an CpG island at this position appears unlikely, strengthening the second interpretation. This interpretation has been verified recently by the isolation of a telomere clone in a yeast artificial chromosome vector (Bates *et al.,* submitted) covering the last 100 kb of the map. The endpoint of the map, drawn as a cluster of rare cutter sites, is therefore the end of the chromosome.

The map segment around *D4S112* (J252), *D4S115* (252), *D4S97* (BS854), *D4S111* (P157), *D4S96* (BS678), and *D4S90* (D5) covers 1.5 Mb. Genetic linkage data (Youngman *et al.,* 1989; MacDonald *et al.,* 1989) allowed the orientation of segment III on the chromosome, positioning *D4S90* (D5) distal to the *D4S111* (157) region, with three-point crosses showing the order cen-*D4S10-D4S111-D4S90*-tel.

Order and Orientation of Segments along the Chromosome

The combination of physical, genetic, and somatic cell hybrid mapping data allowed the establishment of a map of the region 4p16.3 covering at least 5 Mb. This map consists of three segments, which have been ordered and oriented by genetic linkage analysis and somatic cell hybrid data.

Segment I   G8–(BJ14–HDA29)–YNZ32
Segment II   BJ56–BS674–C4H–J107–P107–W92–
               (P102–BS731–BS385)–P133–P62
Segment III   J252–P252–P157–P417–D5

The relative order of the maps along the chromosome, with segment I being the most centromeric and segment

III the most telomeric (Fig. 6), was determined using somatic cell hybrids and genetic linkage analysis with polymorphic markers from each segment. The probes from the region covered by segment I and a part of segment II are located within the proximal part of 4p16.3, and probes from the region covered by the rest of segment II and segment III lie within the distal portion of 4p16.3. To further define the order of the maps, we used data from genetic linkage analysis in the CEPH and Venezuela reference family, and in HD pedigrees. Genetic linkage data using G8 and C4H (Gilliam *et al.,* 1987a; MacDonald *et al.,* 1989) and G8 and BS674 (Wasmuth *et al.,* 1988) placed segment I proximal to segment II. Genetic linkage data described by Youngman *et al.* (1989) and MacDonald *et al.* (1989) suggest the following order of markers: G8–(BS674–C4H)–(BS731, W92, P62)–(P252, BS678, P157)–D5. This order of probes indicates that segment II maps proximal to segment III.

High Density of Unmethylated CpG Sequences

We observed a general high density of unmethylated CpG islands in 4p16.3, reflected by the exceptionally small restriction fragments generated by rare cutting enzymes in many parts of the map. While the average spacing of unmethylated *Not*I sites in the human genome was estimated as 750 kb (Lindsay and Bird, 1987), the average size of fragments generated by *Not*I in this region is only 170 kb. This phenomenon is especially apparent in two regions, P102–P133–P62 and 157.1–157.6, where a very high density of unmethylated CpG sequences in regions of 100 kb has been observed. The analysis of some DNA fragments flanking unmethylated CpG islands showed their cross-hybridization with other species (Zimmer *et al.,* unpublished).
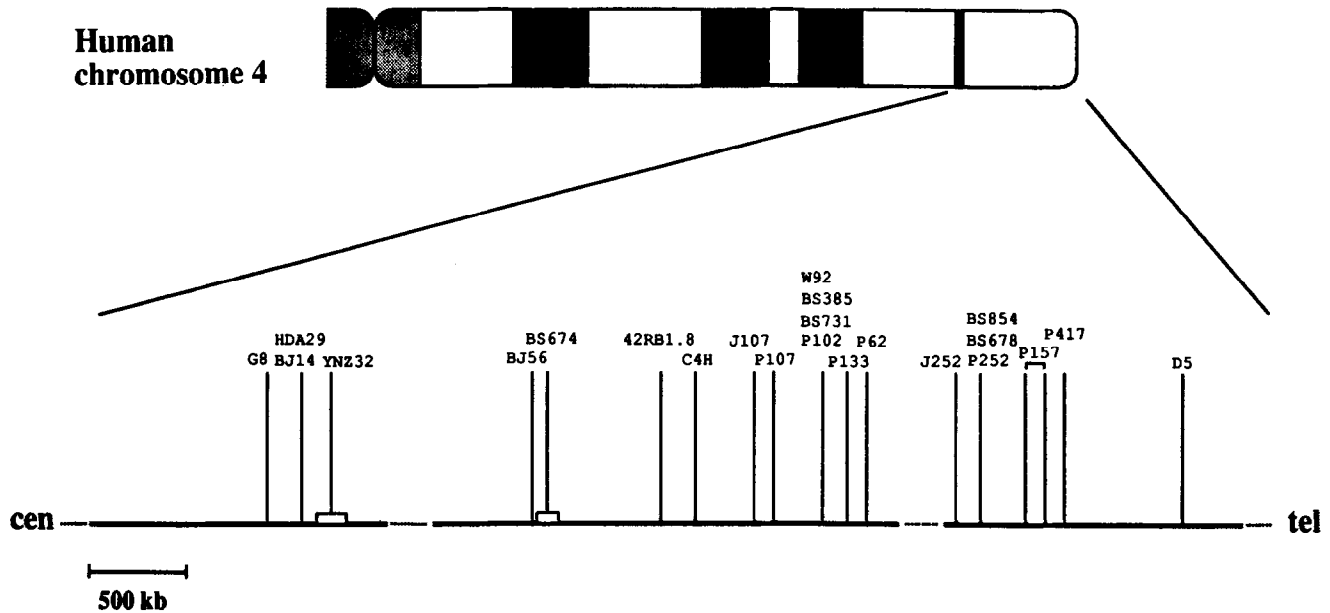
FIG. 6. Summary map of 4p16.3 region linking map segments I, II, and III together. The map shows the position of 23 molecular markers from the 4p16.3 region grouped into three clusters (I, II, III). The telomere–centromere orientation is shown (tel; cen). The gaps between segments are indicated by dotted lines.

These conserved sequences may represent genes often found near unmethylated CpG islands (Brown and Bird, 1986) or may simply reflect an unusual structural feature of regions near telomeres (Brown, 1988). Since the region 4p16.3–4pter contains G-light-staining DNA our observation of a high density of unmethylated CpG isands is in agreement with recent reports of CpG-rich sequences in the G-light bands, and A/T-rich sequences in G-dark bands (Bernardi *et al.*, 1985; Holmquist, 1987; Korenberg and Rykowski, 1988; Burmeister *et al.*, 1988).

Position of the HD Gene

The positions of the probes with respect to the *HD* gene can be deduced from genetic linkage data obtained by linkage analysis in HD pedigrees. Two individual recombination events between *D4S114/D4S113* and *HD* strongly suggest that the defect is located distal to segment II, placing the *HD* gene in the region bordered by the *D4S114/D4S113* loci and the telomere (Whaley *et al.*, 1988). However, genetic linkage analysis using polymorphic markers from segment III, located distal to *D4S114*, provides equivocal information as to whether the disease gene maps between segments II and III or within the distal part of segment III (MacDonald *et al.*, 1989).

D4S10 was mapped by somatic cell hybrids (Gusella *et al.*, 1983) and *in situ* hybridization (Magenis *et al.*, 1986; Landegent *et al.*, 1986) to 4p16.3, the terminal subband of the short arm. If DNA were evenly distributed along the cytogenetic length of the chromosome, the upper limit for the region between *D4S10* and the

telomere would correspond to approximately 6×10^6 bp, 0.2% of the human genome (Gilliam *et al.*, 1987b). The physical map of 4p16.3 presented in this paper is distributed over at least 5 Mb, suggesting that the distance between *D4S10* and the telomere is minimally 4.3 Mb. This implies that the majority of the region 4p16.3 is covered by the map and that the gaps between the three segments should not be large. Since it has been shown that the *HD* gene maps either between segments II and III or at the distal end of segment III, future effort will be devoted to bridge the gap between these segments and to obtain probes located distal to D5.

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