Mapping of *D4S98/S114/S113* confines the Huntington's defect to a reduced physical region at the telomere of chromosome 4

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ABSTRACT

The dominant gene defect in Huntington's disease (*HD*) is linked to the DNA marker *D4S10*, near the telomere of the chromosome 4 short arm. Two other markers, *D4S43* and *D4S95*, are closer, but still proximal to the *HD* gene in 4p16.3. We have characterized a new locus, *D4S114*, identified by cloning the end of a *NotI* fragment resolved by pulsed-field gel electrophoresis. *D4S114* was localized distal to *D4S43* and *D4S95* by both physical and genetic mapping techniques. The "end"-clone overlaps a previously isolated *NotI* "linking" clone, and is within 150 kb of a second "linking" clone defining *D4S113*. Restriction fragment length polymorphisms for *D4S113* and *D4S114*, one of which is identical to a *SacI* polymorphism detected by the anonymous probe pBS731B-C (*D4S98*), were typed for key crossovers in *HD* and reference pedigrees. The data support the locus order *D4S10*-(*D4S43*,⁺*D4S95*)-*D4S98/S114/S113*-*HD*-telomere. The *D4S98/S114/S113* cluster therefore represents the nearest cloned sequences to *HD*, and provides a valuable new point for launching directional cloning strategies to isolate and characterize this disease gene.

INTRODUCTION

Huntington's disease (*HD*) is a progressive neurological disorder of mid-life onset, characterized by neuronal cell death which is most prominent in the basal ganglia (1). The pre-eminent clinical manifestation of *HD* is a characteristic movement disorder involving progressive chorea, but gene carriers also suffer psychological and intellectual disturbances. The disorder is caused by an autosomal dominant defect that has been mapped to the short arm of chromosome 4 by genetic linkage to the DNA marker, *D4S10* (2,3). Extensive family studies employing multipoint linkage methods combined with physical mapping of the marker loci have established that the *HD* gene is located in 4p16.3, the terminal cytogenetic subband of 4p, about 4 centiMorgans (cM) distal to *D4S10* (4,5). The availability of a linked marker for *HD* has provided the basis for predictive testing (6) and raised the possibility of isolating and characterizing the genetic defect based on its chromosomal location (3). Identification of additional polymorphic DNA markers distal to *D4S10* is of critical importance to these efforts. Recently, we have

reported the characterization of two such markers, *D4S43* and *D4S95*, which map between the telomere and *D4S10* on 4p (7,8). To expand the amount of cloned DNA from the vicinity of one of these markers, *D4S43*, we have attempted to clone the ends of large restriction fragments identified by pulsed-field gel electrophoresis. This effort has serendipitously led to the characterization of an independent locus, *D4S114*. *D4S114* is in close proximity to *D4S113*, defined by a *NotI* "linking" clone (9), and a *SacI* polymorphism within the *D4S114* locus is the same as that revealed by pBS731B-C, which has been assigned the locus symbol *D4S98* (10). The presence of the *D4S98/S114/S113* region on a chromosome 4 in which an interstitial deletion has removed *D4S43* and *D4S95* defines a physical region distal to these other two markers. Genetic analysis of restriction fragment length polymorphisms for *D4S98/S114/S113* demonstrates that *HD* is located between these combined markers and the telomere in this limited portion of 4p16.3.

MATERIALS AND METHODS

Description of Cell Lines

HHW693 is a human/hamster hybrid cell line that contains a compound translocation chromosome composed of the human 4pter-4p15.1 region, the human 5 centromere-5p15.1 region, and a portion of a small hamster chromosome (11). H67 is a human/mouse hybrid cell line with a 4/21 translocation chromosome that is missing 4p16.3 (5). HHW847 is a human/hamster hybrid cell containing the same translocation chromosome as H67 (10). Gus3171, a transformed lymphoblast cell line, is from a patient with a large interstitial deletion that appears to involve 4p16.3-4p15.1 and leaves only the distal portion of 4p16.3 intact. H60 and HHW842 are respective human/mouse and human/hamster hybrid cell lines that each retains only the derivative chromosome 4 from Gus3171 (5,10).

Pulsed-Field Gel Electrophoresis

Agarose inserts for pulsed-field gel electrophoresis experiments were made by casting cells (typically 10^6 cells per insert) in 0.5% agarose in molds. The agarose blocks were then digested in sodium dodecyl sulfate/proteinase K solution (12). *NotI* restriction endonuclease was purchased from New England Biolabs. Digests were carried out for an overnight period at 37°C using the recommended buffer. Typically, 50 units per insert were used in a total volume of 250 μ l.

Field-inversion gel electrophoresis (FIGE) was carried out as described (13, 14). For the preparative gel, HHW693 inserts were digested with *NotI* and

resolved by the FIGE technique using 1.0% low melting temperature agarose. The techniques for extracting and cloning the DNA are described in detail elsewhere (14).

Analysis of Cosmid and Phage Clones

Single copy probes were obtained by random subcloning of Sau3AI+HindIII, Sau3AI+EcoRI, or Sau3AI+PstI double-digests, or by competitive hybridization of randomly chosen restriction fragments to blots containing human genomic DNA (15), followed by subcloning and further restriction to produce single copy probes. All subcloning was performed with pGEM3 and pGEM4 vectors (from Promega Biotech Inc.).

Linkage Analysis

Screening of probes for restriction fragment length polymorphism (RFLP) and analysis of linkage using the extended Venezuela reference pedigree with the LINKAGE programs were performed as previously described (16-18).

RESULTS

Attempted expansion of the D4S43 region

C4H, the anonymous probe used to define the D4S43 locus, detects an approximately 200 kb Not I fragment in human DNA. One end of this fragment maps within a region already cloned in overlapping cosmids (7). The goal of our strategy was to isolate the alternate end as a NotI-Sau-3AI fragment after fractionation of NotI-digested DNA by preparative pulsed-field gel electrophoresis of NotI-digested DNA followed by partial Sau3AI digestion and cloning of selected DNA fractions. The hybrid cell line HHW693, which contains 4pter-4p15.1 and 5p15.1-5cen as the only human material on a hamster background (11), was used as the source of starting DNA to minimize contamination of the fractions by NotI fragments from other human chromosomal regions. Putative "end"-clones from the experiment were screened to identify those of human origin (19) and these were mapped on either chromosome 4 or 5, using a mapping panel of somatic cell hybrids constructed to facilitate the regional assignment of DNA probes to the 4p16.3 region containing the HD gene (5,10). We anticipated identifying a clone with the same pattern of hybridization to the mapping panel as C4H.

Identification and physical mapping of D4S114

One clone, W92, containing 17 kb of human DNA was identified repeatedly. When a single-copy subclone, p311, was hybridized to the somatic cell hybrid panel, however, the pattern obtained differed from that observed for the D4S10, D4S43 and D4S95 probes, and clearly placed W92 distal to these other

Figure 1--Regional Localization of *D4S114* Distal to *D4S10*, *D4S43* and *D4S95*

Hybridization of DNA probes pKO82 (*D4S10*), C4H (*D4S43*), p674 (*D4S95*) and p311 (*D4S114*) to DNA from cell lines HHW693, H67, H60, HHW847, and HHW842 was used to regionally assign the corresponding loci (5,10). The only physical region common to H60, HHW842 and HHW693 that is absent from H67 and HHW847 is the terminal-most portion of 4p16.3. Since the entire 4p16.3 subband represents about 3% of the cytogenetic length of chromosome 4, the region of 4p16.3 remaining in the H60 and HHW842 hybrids is unlikely to contain more than 4×10^6 bp of DNA.

loci (Figure 1). Like G8 (*D4S10*), C4H (*D4S43*) and pBS674 (*D4S95*), p311 failed to hybridize to DNA from hybrids H67 and HHW847 which lack 4p16.3. By contrast, however, p311 did detect the expected fragment in hybrids H60 and HHW842 which each contain a chromosome 4 with an interstitial deletion in which the most terminal portion of 4p16.3 remains intact. G8, pBS674 and C4H showed no signal in H60 or HHW842 DNA and therefore map proximal to W92.

High resolution pulsed-field gel electrophoresis revealed that W92 derived from a 150 kb Not I fragment, distinct from that containing C4H, though the two were apparently present in the same preparative gel fraction (Figure 2). The size of this fragment remains unaltered in a cell line, GUS3171, from the patient whose interstitial deletion chromosome is present in the H60 and HHW842 hybrids, indicating that the 150 kb Not I fragment probably does not cross the distal breakpoint of this deletion. Thus, W92

Figure 2-- Hybridization of p311 and C4H to a blot of a gel prepared by the OFAGE technique.

The gel was prepared with 1.0% agarose in 0.5X TBE. Electrophoresis was carried out in the double-inhomogeneous mode on an LKB 2015 "Pulsaphor" unit for 44 hours at 330V using a 25 second pulse time. Lanes 1 and 2 contain undigested and NotI digested DNA, respectively, from a normal human lymphoblastoid cell line. Lanes 3 and 4 are agarose inserts of Gus3171 and HHW693, respectively, that have been completely digested with NotI. Lambda ladders for use as size markers were prepared from λ ci857 with a monomer size of 48.5 kb. Two different preparations of these were loaded in lanes flanking the sample lanes.

defines a new locus, *D4S114*, which maps between *D4S43* and the telomere, in the most terminal portion of 4p16.3 containing the *HD* gene.

RFLP for the *D4S114* region

A cosmid, C101, with an insert of 40 kb was isolated by hybridization of p311 to a library of DNA from the hybrid cell line HHW693 in pCOS2 (20). Subclone p312, containing a 3.0 kb XbaI fragment from C101, detects a three-allele SacI RFLP with allelic fragments of 7.5 kb, 6.7 kb and 12 kb present on 70%, 27% and 3% of chromosomes, respectively (N=78). The polymorphism information content (PIC) (21) of this locus is 0.36, making it moderately informative for linkage studies. The locations of p311 and p312 with respect to W92 and C101 are indicated in Figure 3.

The SacI RFLP was typed in a number of known cases of recombination

Figure 3--Relative locations of *D4S113* and *D4S114*

Positions relative to NotI sites (N) are shown for NotI "end"-clone W92, NotI "linking" clones 102 and 62, cosmids C101 and A62.5, anonymous clone pBS731B-C, and subcloned probes 311, p312 and p337.

between *D4S10* and *HD* to determine whether *D4S114* was proximal to the defect, or alternately might represent a flanking marker. One recombination event previously detected by *D4S10* was also detected by *D4S114* (Figure 4), indicating that *HD* is located between this new locus and the telomere, in the the physical region defined by the H60 and HHW842 hybrids. To obtain an estimate of the genetic distance between *D4S114* and the other DNA markers, the SacI RFLP was typed in the Venezuela reference pedigree (17), a resource for estimating recombination between DNA markers, but few informative meioses were observed.

Relationship of *D4S114* with *D4S98* and *D4S113*

Comparison of C101 with other cloned segments from 4p16.3 by both hybridization and restriction mapping revealed that it overlapped with 102, a member of a previously reported cluster of phage NotI "linking" clones (9). The relationship between C101 and W92 with phage linking clones (102 and 62) and a second cosmid clone (A62.5, locus *D4S113*) from the cluster are

B.

Figure 4--Crossovers between *D4S114*, *D4S113* and *HD*

Symbols: circle, female; square, male; slashed symbol, deceased; darkened symbol, affected by HD. Each individual is designated by a sequential number assigned within each generation. Genotypes in brackets are inferred from progeny. Alleles enclosed within rectangles are inferred to be on the HD chromosome.

A. Typing results are presented for an *EcoRI* RFLP at the *D4S10* locus (detected by probe pK081) and for the *D4S114* *SacI* RFLP in a nuclear family displaying a crossover for each of these loci with HD. Affected individual II-3 clearly received different alleles at both loci from the affected mother (I-1) relative to the affected sibs (II-2 and II-4).

B. Typing results are presented for a haplotype of *D4S10* constructed by combining data from *HindIII* (probe pK082) and *EcoRI* (probe pK083) RFLPs and for the *StuI* RFLP of *D4S113*. The alleles present in individuals III-1 and IV-1 indicate that the HD gene is cosegregating in this part of the family with the C2 haplotype at the *D4S10* locus and the 1 allele for the *D4S113* RFLP. However, affected individual III-3 apparently possesses a C1 haplotype for *D4S10* based on the alleles segregated to his progeny. It cannot be distinguished whether the HD gene in this individual is on the chromosome with the C1 haplotype or with the A2 haplotype, since his parents are deceased and neither of his children have yet shown symptoms of HD. The apparent crossover in this pedigree is also detected by the *D4S113* RFLP since individual III-3 has a 2 allele on each of his chromosome 4s. It cannot be determined whether the apparent crossover which distinguishes the two branches of this pedigree occurred during meiosis in I-1, II-1 or II-3. Because the individuals whose genotypes reveal the presence of a crossover are separated by more than one generation, the possibility exists of two separate recombination events affecting both loci independently. Although this cannot be discounted, the genetic distances involved make the likelihood of two separate crossovers extremely low.

Table 1--Pairwise Lod scores for linkage of D4S98/S114/S113 to D4S10, D4S43 and D4S95

Locus A	Locus B	RECOMBINATION FRACTION (θ)								Peak Score ($\hat{\theta}$)	Confidence Interval (1-lod unit)
		0.00	0.01	0.05	0.10	0.20	0.30	0.40			
D4S98/S114/S113	D4S10	∞	43.02	44.81	41.69	32.05	20.45	8.19	45.12, $\hat{\theta}=0.035$	$\theta = 0.015$ to 0.065	
D4S98/S114/S113	D4S43	∞	44.53	41.79	37.63	28.40	18.22	7.47	44.65, $\hat{\theta}=0.005$	$\theta = 0.001$ to 0.026	
D4S98/S114/S113	D4S95	34.43	33.80	31.21	27.88	20.74	13.17	5.41	34.43, $\hat{\theta}=0.000$	$\theta = 0.000$ to 0.016	

illustrated in Figure 3. Surprisingly, the probe pBS731B-C (designated locus D4S98) (10), isolated independently from a phage library of HHW693 also hybridizes to C101, and detects the same SacI (=SstI) RFLP as p311. Like D4S114, D4S98 has been physically mapped to the very distal portion of 4p using somatic cell hybrid analysis. It is not clear whether the cloning of segments of D4S98/S114 by three different approaches in independent experiments reflects the relatively small size of the 4p16.3 region defined by the somatic cell hybrid panels, or a growth advantage on the part of recombinants containing these sequences.

RFLP for D4S113

Since the D4S98/S114 locus is only 150 kb from the 62 "linking" clone which has been given the locus name D4S113, we isolated single copy probes from the cosmid A62.5 in order to identify additional RFLPs more informative in the Venezuela reference pedigree. A 300 bp EcoRI-Sau3AI fragment subcloned as p337 (Figure 4) detected both StuI and BglII RFLPs in strong linkage disequilibrium with each other. The StuI polymorphism was most easily visualized, giving allele frequencies of 0.18 and 0.82 corresponding to fragments of 6.3 and 5.6 kb, respectively (N= 78; PIC=0.25). When the known crossovers between D4S10 and HD were typed, the p337 StuI RFLP detected one additional recombination event that had been uninformative for the p311 SacI RFLP, providing further evidence that the D4S98/S114/S113 cluster is proximal to HD. The p337 StuI RFLP was also informative in the Venezuela reference pedigree, allowing an assessment of the genetic position of this cluster of clones.

Linkage mapping of the combined marker D4S98/S114/S113

Pairwise lod scores for D4S98/S114/S113 with D4S10, D4S43, and D4S95 are presented in Table 1. The results of multipoint analysis of the loci are

Figure 5--Graphic presentation of results from multipoint linkage analysis.

The curve was generated using the program LINKMAP (18) by holding *D4S10* and a combined point for the very tightly linked *D4S43/D4S95* markers (MacDonald et al., in preparation) at a fixed distance of 4 cM from each other and by iterating the position of *D4S98/S114/S113*. The difference in log₁₀ likelihoods between the selected point and a very distant point (-10, simulating the "unlinked state") is plotted. The maximum log₁₀ likelihood difference occurs at 0.5 cM distal to *D4S43/S95*, representing the most probable position of *D4S98/S114/S113*. This exceeds by 30 fold the best score for a location between *D4S10* and *D4S43/S95*. The downward plunges (to $-\infty$) in the curve result from crossovers between this marker and both *D4S10* and *D4S43/S95*.

displayed graphically in Figure 5, with the relative heights of the peaks in the log₁₀ likelihood difference curve providing a measure of the most likely position of *D4S98/S114/S113*. The data are entirely consistent with the somatic cell hybrid analysis, with the most likely order of the loci, *D4S10*-(*D4S43*, *D4S95*)- *D4S98/S114/S113* favored by about 30:1 over the next most likely order, *D4S10*-*D4S98/S114/S113*-(*D4S43*, *D4S95*).

DISCUSSION

To date the probes, p312, p337 and pBS731B-C, represent the closest cloned segments mapped relative to the HD gene. They are distal to *D4S10*, *D4S43* and *D4S95* by both somatic hybrid cell mapping and genetic linkage

analysis. Although the genetic distance between the combined marker *D4S98/S114/S113* and the closely linked pair *D4S43* and *D4S95* is only about 0.5 cM, the two locus clusters are separable by somatic cell hybrid analysis, and long-range restriction mapping using pulsed-field gel electrophoresis has thus far failed to connect them (Bucan *et al.*, in preparation; Whaley *et al.*, unpublished results). The genetic distance between *D4S43* and *HD* has been estimated to have an upper limit of 1.5 cM (7). Consequently, *D4S98/S113/S114* should be considerably less than 1 cM from the *HD* gene, but such small distances are difficult to estimate accurately without analyzing very large numbers of meiotic events. The two individual crossovers between *D4S98/S114/S113* and *HD* suggest strongly that the genetic defect is located distal to this cluster of cloned DNA. The search for the disease gene can now be concentrated in a considerably reduced physical region between *D4S98/S114/S113* and the telomere which is distal to previously reported markers and is likely to contain an upper limit of 4×10^6 base pairs of DNA. Most importantly, this terminal portion of 4p16.3 containing the *HD* gene is physically delineated by the H60 and HHW842 hybrid lines which thereby provide a ready means to saturate the region with DNA markers. Several additional loci have recently been assigned to this segment of the chromosome (9,10,22) by use of the somatic cell hybrid panels. Development of RFLPs and typing of these loci in known recombinants from both HD and reference pedigrees should define the minimum genetic region that must contain the *HD* gene. *D4S98/S114/S113* itself represents a valuable addition to the markers available for predictive testing in HD. Foremost, however, *D4S98/S114/S113* provides the closest defined point to launch directional cloning strategies such as chromosome walking, cloning of pulsed-field gel fragments (14), and screening of "jumping" clone libraries (12,23), aimed at isolating the *HD* gene.

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