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Isolation of DNA Markers in the Direction of the Huntington Disease Gene from the G8 Locus

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Summary

To facilitate identification of additional DNA markers near and on opposite sides of the Huntington disease (HD) gene, we developed a panel of somatic-cell hybrids that allows accurate subregional mapping of DNA fragments in the distal portion of 4p. By means of the hybrid-cell mapping panel and a library of DNA fragments enriched for sequences from the terminal one-third of the short arm of chromosome 4, 105 DNA fragments were mapped to six different physical regions within 4p15-4pter. Four polymorphic DNA fragments of particular interest were identified, at least three of which are distal to the HD-linked D4S10 (G8) locus, a region of 4p previously devoid of DNA markers. Since the HD gene has also recently been shown to be distal to G8, these newly identified DNA markers are in the direction of the HD gene from G8, and one or more of them may be on the opposite side of HD from G8.

Introduction

Huntington disease (HD) is an inherited, autosomal dominant neuropsychiatric disorder. Initially, individuals with an abnormal HD gene are behaviorally and neurologically normal. However, the defective gene is fully penetrant, and eventually carriers begin to show symptoms of the disease, which usually include awkwardness of gait (chorea), impairment of voluntary movements, emotional disorders, and mood changes (Hayden 1981). The onset of the symptoms is usually delayed until the third to fifth decade, on average, although they may either begin as early as age 2 years or not become apparent until as late as the eighth decade of life (Hayden 1981). Regardless of age at onset, the disease progresses relentlessly for 10–20 years until victims are physically and mentally incapacitated; and the disease is invariably fatal. By the time most persons with HD are diagnosed, they are past the reproductive age, and each child of an affected individual is at 50% risk for having inherited the defective gene. In view of the devastating nature of the disease, the delayed onset of symptoms, and the uncertainty faced by at-risk individuals, there is a need to develop an accurate presymptomatic test for the disease not only for genetic-counseling purposes but also so that potentially useful drug therapies might be initiated well before neurologic degeneration has begun.

In addition to heterogeneity in age at onset, there are at least two other features of HD that must ultimately be taken into account in understanding the molecular basis of the disease. The mutation rate at the HD locus, at least with regard to mutations giving the phenotype of HD, is very low. In fact, there are no documented cases of new mutations at the HD locus. Most intriguing is the recent finding that, in terms of both age at onset and severity of symptoms, individuals homozygous for the defective HD gene are indistinguishable from sibs who are heterozygous (Wexler et al. 1987). This finding would seem to indicate that, whatever the nature of the mutation causing HD, it is not one that results in loss of gene function.

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Despite years of intensive study, there are few clues as to the basic biochemical defect in HD, and nothing is known about the nature of the affected gene. Efforts to understand this disease are now directed toward isolating the disease gene on the basis of its location in the genome, a strategy that has recently proved successful in identifying candidates for the genes involved in other genetic disorders, including chronic granulomatous disease (Royer-Pokora et al. 1986), Duchenne muscular dystrophy (Monaco et al. 1986), retinoblastoma (Friend et al. 1986), and cystic fibrosis (Estivill et al. 1987). In 1983, Gusella et al. (1983) identified a random DNA fragment, G8, that defines a polymorphic genetic locus (D4S10) ~4% recombination from the HD gene. Subsequently, G8 (and, by inference, the HD gene) was localized to the terminal portion of the short arm of chromosome 4 (4p), in band 4p16 (Gusella et al. 1985; Landegent et al. 1986; Magenis et al. 1986; Wang et al. 1986). At present, there is a need to identify additional markers closer to and, in particular, on the opposite side of the HD gene from G8. Markers on both sides of the HD gene will help make presymptomatic diagnosis of the disease more accurate and will delineate both endpoints of the genomic region containing the disease gene. The closer that flanking markers are to the disease gene, the more narrowly the region of interest is defined and the less DNA one will have to analyze to identify the gene itself. However, despite several years of searching, no DNA markers on the opposite side of the HD gene from G8 have been identified. The major problem in finding flanking markers for HD has been the inability to isolate DNA fragments from the portion of 4p between G8 and 4pter, the region where the HD gene has recently been localized (Gilliam et al. 1987). No DNA fragments from this region have been reported to date.

In the present report we describe how a recombinant-DNA library enriched for fragments from the distal portion of 4p was used together with a panel of somatic-cell hybrids to provide accurate physical mapping of probes to regions within 4p16—and thereby to identify random DNA probes close and distal to G8. Four DNA probes of particular interest were identified, all of which reveal RFLP. One of these probes, which reveals a highly polymorphic locus, is physically close to G8, but its position relative to G8 and 4pter could not be determined by physical mapping techniques. The remaining three probes are all distal to G8. Since the HD gene is also distal to G8 (Gilliam et al. 1987), these latter three markers at least, and possibly all four, are in the direction of the HD gene from G8, and one or more of them may well be on the opposite side of the HD gene from G8.

Material and Methods

Lymphoblastoid and Hybrid Cell Lines

The isolation and characterization of three interspecific (CHO-human) somatic-cell hybrids—HHW105, which retains a human chromosome 5 exclusively (Dana and Wasmuth 1982*a*); HHW416, which retains an intact human chromosome 4 exclusively (Carlock et al. 1986); and HHW693—have been described elsewhere. HHW693 retains, as its only human DNA, the short (p) arm of a translocated human chromosome 5 in which the terminal one-third of 5p has been replaced with the terminal one-third of 4p (5 cen-5p15::4p15-4pter) (Wasmuth et al. 1986).

Human lymphoblastoid cultures established from patients with various rearrangements of the short arm of chromosome 4 (4p) were obtained from Dr. James Gusella (Massachusetts General Hospital, Boston) and from Dr. David Ledbetter (Baylor College of Medicine, Houston). Information concerning these cell lines, including the rearrangements of 4p and references to the original description of the patients and their karyotypes, are included in table 1. Gus1548 was established from a patient with a terminal deletion that removed all of 4p16 (Gusella et al. 1985). Gus2275 was established from a patient whose terminal deletion breakpoint is in the midregion of 4p16.1 (Magenis et al. 1986). LZ was established from a patient who also has a terminal deletion in the midregion of 4p16.1 (D. Ledbetter, personal communication). Gus3171 was established from a patient with a large interstitial deletion, with the proximal breakpoint within 4p14 and the terminal breakpoint very near the 4p16.1-4p16.2 border (Magenis et al. 1986). As discussed in detail in the Results section, this deletion is indeed interstitial, as determined on the basis of molecular techniques. Gus1654 was established from a patient reported to have a small interstitial deletion that removes the distal portion of 4p16.1, all of 4p16.2, and the proximal portion of 4p16.3 (Wang et al. 1986). Gus4066 was established from a patient with an unbalanced 4;21 translocation with the breakpoint on 4p originally reported to be within 4p16.3 (Carpenter et al., in press). However, a

Table 1

Lymphoblastoid Cell Lines Used to Isolate Cell Hybrids

Lymphoblastoid Culture Designation	Patient Designation in Appropriate Reference	Rearrangement of 4p[a]	Reference
1. Gus1548	–	del(4)(4qter-4p15.3:)	Gusella et al. 1985
2. Gus2275	DQ	del(4)(4qter-4p16.1:)	Magenis et al. 1986
3. LZ	–	del(4)(4qter-4p16.1:)	Unpublished data
4. Gus3171	TA	del(4)(4qter-4p14::4p16.1-4pter)	Magenis et al. 1986
5. Gus1654	1	del(4)(4qter-4p16.1::4p16.3-4pter)	Wang et al. 1986
6. Gus4066	–	t(4;21)(4qter-4p16.1::21q21.1-21qter)	Carpenter et al., in press

[a] Locations of some chromosome breakpoints within 4p16 are based on a combination of cytogenetic and DNA probe data, as described in the text.

reexamination of the karyotype of this patient indicated that the breakpoint could be interpreted as being in the very distal portion of 4p16.1 (N. Carpenter, unpublished results), which would be most compatible with the DNA probe data described in the Results section. Ideograms depicting the portions of 4p deleted from each of these abnormal chromosomes 4 are shown in figure 3.

Lymphoblastoid cells from each of these six patients were fused to the CHO cell line UCW56, which has a thermolabile leucyl-tRNA synthetase, rendering the cell line nonviable at 39 C, as described elsewhere (Dana and Wasmuth 1982*b*). Hybrids that retained the human LARS gene on chromosome 5, a situation that complements the temperature-sensitive phenotype of the CHO-cell parent, were selected at 39 C. A large number of independent hybrids from each fusion were isolated, and metaphase chromosome preparations were analyzed by means of trypsin-Giemsa banding and alkaline-Giemsa (G-11) staining to identify hybrids that, by chance, retained the abnormal chromosome 4 of interest. Hybrids determined by cytogenetic analysis to retain the abnormal chromosome 4 apart from the normal homologue were then analyzed by using appropriate DNA probes from chromosome 4, as described in the Results section. From each patient's cells, at least two independent hybrids were identified that retained the abnormal chromosome 4 in $\geq$80% of the cells examined and that did not contain a normal chromosome 4. It should be noted that initial attempts were made to isolate cell hybrids that retained chromosomes 4 under selective pressure, by using a CHO Ade A^- mutant to select for retention of the human PPAT gene (Stanley and Chu 1978). However, virtually every hybrid isolated by means of this selective system had extensive rearrangements of human chromosomes, making these hybrids unsuitable for our purposes. In contrast, hybrids isolated by means of the selective system described above for chromosome 5 rarely contain spontaneous rearrangements of human chromosomes (Carlock et al. 1986).

DNA Probes and Southern Blot Hybridization

The preparation of a complete genomic DNA library from hybrid HHW693 in the λ vector EMBL-4, the isolation of recombinant phage with human DNA inserts, the preparation of phage DNA, and the isolation of single-copy DNA fragments from phage inserts all have been described in detail elsewhere (Overhauser et al. 1986*a*, 1986*b*; Wasmuth et al. 1986).

High-molecular-weight DNA extracted from lymphoblastoid cells or from human–hamster cell hybrids was digested to completion with different restriction enzymes and subjected to electrophoresis through 0.8% agarose gels. Gels were soaked in 0.25 N HCl for 7 min, rinsed with H_2O, then placed on top of Whatman paper that was saturated with 0.5 N NaOH. Nylon membranes that had been prewetted in water were put on top of the gels and Whatman paper, and paper towels and a weight were placed on top. Two to 12 h later the membranes were removed and rinsed in 3 × SSC before use.

DNA fragments were labeled by means of the random-primer method according to Feinberg and Vogelstein (1983). Normally, 30–90 ng of each DNA fragment was labeled to a specific activity of $\sim 10^8$ cpm/μg for each Southern blot hybridization. In some cases whole recombinant phage DNAs were used as hybridization probes without first isolating low-copy fragments. For these experiments, 50 ng of

sonicated phage DNA was labeled with ^{32}P and prehybridized with 500 μg of sonicated human DNA, according to a method described by Sealey et al. (1985), before adding the labeled DNA to hybridization mixtures.

Membranes were prehybridized for 2–4 h at 65 C in hybridization solution consisting of 10% PEG, 7% SDS, 1% BSA, 0.25 m sodium phosphate pH 7.2, 0.25 M NaCl, 1 mM EDTA. The membranes were then placed in plastic bags with 10 ml hybridization solution, 250 μg sonicated denatured carrier DNA, and 10^7 cpm radiolabeled probe. The membranes were incubated at 65 C for 16–24 h and then washed in 2 × SSC for 0.5 h at 65 C and in 0.5 × SSC for 0.5 h at 65 C before autoradiography.

Results

Isolation of DNA Fragments from Distal 4p

The isolation of an interspecific (Chinese hamster ovary–human) somatic-cell hybrid that retains, as its only human DNA, the short arm of a translocated human chromosome 5 in which the terminal one-third of 5p has been replaced with the terminal one-third of 4p (5cen-5p15::4p15-4pter) has been described elsewhere (Wasmuth et al. 1986). In this cell hybrid, HHW693, only 3%–4% of the DNA is human, approximately one-third to one-half of which is the region 4p15-4pter. A complete genomic DNA library was prepared from this cell line (Wasmuth et al. 1986) in the λ vector EMBL-4, and recombinant phage with human DNA inserts were identified on the basis of their hybridization to ^{32}P-labeled total human DNA. Three hundred recombinants with human DNA inserts were plaque-purified, and DNA was prepared from each. Determining which of the 300 phage had inserts from the 4p as opposed to the 5p portion of the translocated chromosome was accomplished by using the phage DNAs as probes in Southern blots of an appropriate set of somatic-cell hybrids as follows: Whole phage DNAs were labeled with ^{32}P and then prehybridized with unlabeled total human DNA to prevent hybridization signals from repeat sequences. Each labeled phage DNA was then hybridized to filters containing *Eco*RI-digested DNA from (1) CHO cells, (2) a cell hybrid with chromosome 4 exclusively, (3) cell hybrids with the 4;5 translocation chromosome described above (to serve as positive controls), (4) a cell hybrid with an intact chromosome 5 exclusively, and (5) human lymphoblasts. Figure 1 shows examples of the autoradiograms obtained when DNA from three different recombinant phage from the HHW693 library were hybridized to filters containing this series of genomic DNAs. In each panel, the order of digested genomic DNAs is as indicated above. The important lanes for distinguishing recombinant phage with inserts from 4p15-4pter from those phage with inserts from proximal 5p are lanes 2 (DNA from a hybrid with chromosome 4 only) and 5 (DNA from a hybrid with chromosome 5 only). Thus, the recombinant phage used as probes in figure 1*A* and figure 1*C* have inserts from the 4p portion of the translocated chromosome (hybridization to human-derived restriction fragments in lane 2 and no hybridization in lane 5). The recombinant phage used as a hybridization probe in figure 1*B* has an insert derived from the 5p portion of the translocated chromosome (hybridization to human-derived restriction fragments in lane 5 and no hybridization in lane 2). By means of this type of analysis, the inserts from 163 phage from the HHW693 library were assigned to the 4p region of the translocated chromosome, i.e., to 4p15-4pter.

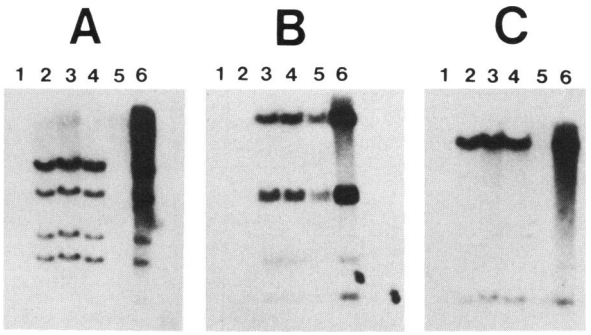

Figure 1 Blot hybridization of DNA from recombinant phage to identify inserts derived from 4p15-4pter. Phage DNAs were labeled, prehybridized to total human DNA as described in Material and Methods, then hybridized to filters containing *Eco*RI-digested DNA from CHO (lanes 1), HHW416 (chromosome 4 only) (lanes 2), HHW693 (lanes 3), HHW661 (the parent of HHW693; see Wasmuth et al. 1986) (lanes 4), HHW105 (chromosome 5 only)(lanes 5), and human lymphoblasts (lanes 6). Panels *A*–*C* represent autoradiograms that use DNA from three independent recombinant phage as probes.

Regional Localization of DNA Fragments in Distal 4p by Means of Deletion Mapping

To facilitate identification of those DNA fragments that are likely to be closest to the HD gene, it was necessary to develop a means to physically map many probes with reasonable accuracy. Therefore, somatic-cell hybrids that retained chromosomes 4

from six different patients with either terminal deletions, interstitial deletions, or translocations involving 4p16 were isolated. The isolation of cell hybrids, as well as karyotype analysis of the patients from whom they were derived, are described in Material and Methods. For cells from each patient, at least two cell hybrids were isolated that appeared, on the basis of karyotype analysis, to retain the abnormal chromosome 4 apart from the normal homologue. To verify the cytogenetic data, each of these hybrids was examined by Southern blot analysis with the following two DNA probes: 622, which is derived from the long arm of chromosome 4 (B. Smith, unpublished results), and pKO82, a plasmid subclone of G8. The 622 probe served to verify that a chromosome 4 was present in the majority of the cells from each hybrid. The pKO82 probe enabled us to verify that the chromosome 4 retained was the abnormal one, since previous studies, using either in situ hybridization to metaphase chromosomes (Magenis et al. 1986; Wang et al. 1986) or quantitative blot hybridization (Gusella et al. 1985; J. Gusella, personal communication; B. Smith and J. J. Wasmuth, unpublished results) had shown it to be missing from each of the abnormal chromosomes 4 in question. Figure 2 shows autoradiograms of blot hybridizations with 622 (fig. 2*A*) and pKO82 (fig. 2*B*) when one cell hybrid with the rearranged chromosome 4 from each patient was used. As can be seen in figure 2*A* (lanes 4–10), the intensity of hybridization signal from the control probe, 622, is similar in DNAs from all the hybrids derived from the patients. It is clear from the results shown in figure 2*B* (lanes 4–10) that G8 is deleted from the abnormal chromosome 4 of each of the six patients. This result confirms that the deletions in the six different patients have a common region of overlap, which appears to include the very distal portion of 4p16.1 and possibly the proximal portion of 4p16.2. That these results with pKO82 are not due to undetected, spontaneous rearrangements of some of the chromosomes 4 in cell hybrids is evidenced by the fact that at least two independent cell hybrids with the abnormal chromosome 4 of each patient gave identical results. In addition, analysis of a similar panel of cell hybrids isolated in another laboratory gave the same result (M. MacDonald and J. Gusella, personal communication). Ideograms depicting the rearranged chromosomes 4 retained in cell hybrids are shown in figure 3 along with the G8 subregional localization based on these results.

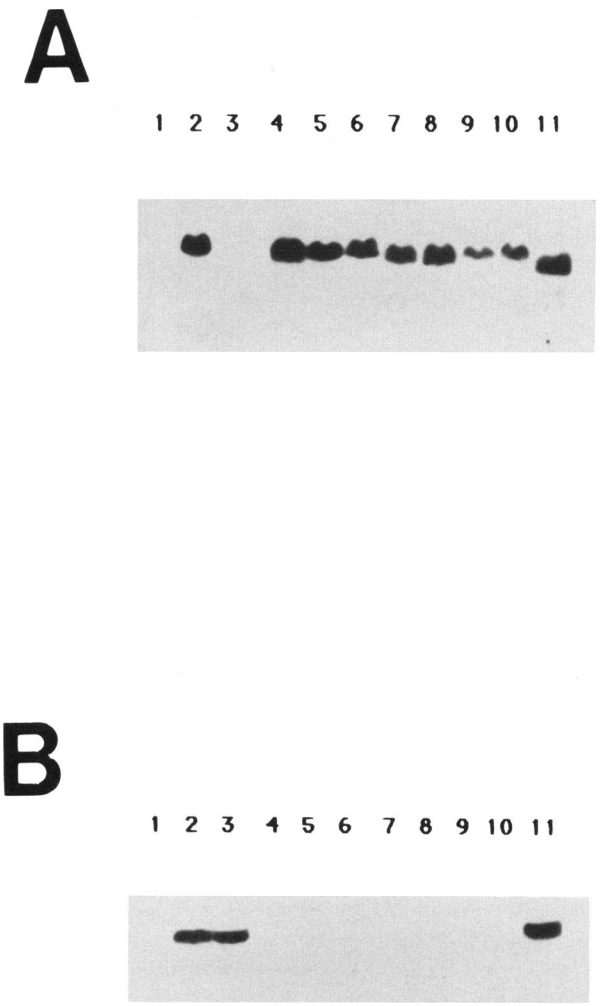

Figure 2 Blot hybridization of pKO82 and control probe 622 to the hybrid-cell deletion mapping panel. The lanes contain *Eco*RI-digested DNA from CHO (lanes 1)l HHW416 (chromosome 4 only)(lanes 2); HHW693 (lanes 3); HHW847, subclone 1 (lanes 4); HHW832 (lanes 5); HHW842 (lanes 6); HHW878 (lanes 7); HHW882 (lanes 8); HHW886 (lanes 9); HHW847, subclone 2 (lanes 10); and human lymphoblasts (lanes 11). *A*, 622 Probe; *B*, pKO82 probe. There is no hybridization to DNA from HHW693 with the 622 probe, since it is derived from the long arm of chromosome 4, which is not present in this cell line.

Identification of DNA Probes Distal to G8

The inserts from 105 phage determined as described above to be derived from 4p15-4pter were screened by means of Southern blot hybridization with this same panel of cell hybrids. On the basis of these probes' patterns of hybridization to the deletion-mapping panel, they were assigned to the six distinct physical regions of 4p shown in figure 4.

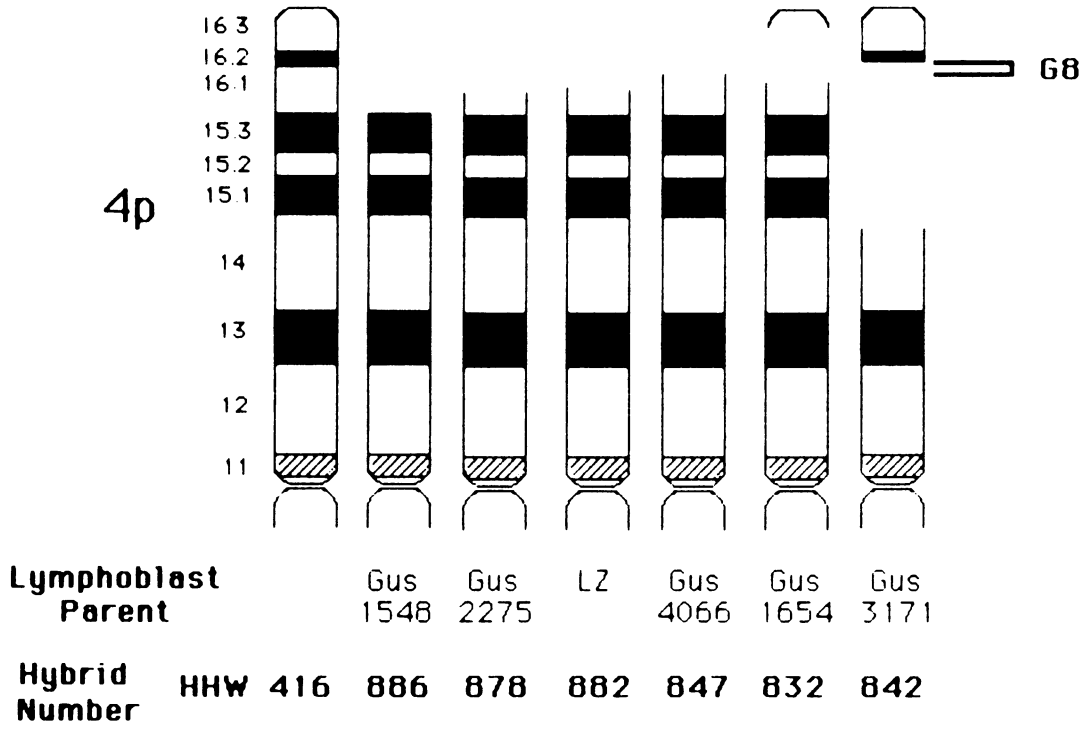

Figure 3 Ideograms depicting the regions of 4p deleted from the abnormal chromosomes 4 retained in cell hybrids.

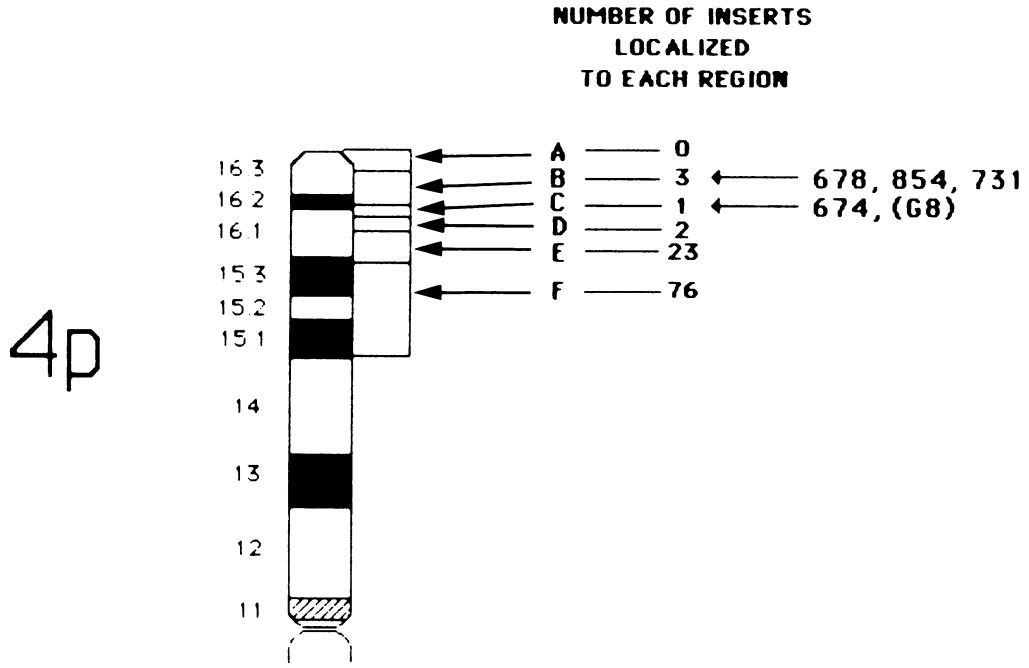

Figure 4 Regional location of DNA fragments from the HHW693 library. The 105 DNA fragments were assigned to the six different physical regions A–F, defined by hybridization patterns to the deletion-mapping panel, as described in the text. The numbers to the far right represent the designations of the recombinant phage whose inserts map to the indicated regions of particular interest.

Only those DNA fragments in the regions (B–D) closest to G8 will be discussed further. The two probes localized to region D are missing from all of the rearranged chromosomes 4 except the one with the 4;21 translocation (HHW847). This demonstrates that the translocation breakpoint in 4p16 in this chromosome is distal to all of the terminal deletion breakpoints. These probes, therefore, are just proximal to G8 in 4p16. The one DNA fragment localized to region C (probe 674) is, like G8, missing from every abnormal chromosome 4, as shown in figure 5*A* (no hybridization in lanes 4–10). This probe is, therefore, very close to G8, but its order relative to G8 and 4pter cannot be determined by means of this physical mapping approach. However, as mentioned below, preliminary linkage studies indicate that 674 is distal to G8. Of particular interest are the three probes (678, 731, and 854) localized to region B, which are missing from all of the abnormal chromosomes 4 except the one with the large interstitial deletion (hybrid HHW842). An example of the pattern of hybridization of one of these probes, 854, to the deletion-mapping panel is shown in figure 5*B*. This probe does not show hybridization signals in those lanes (4–10) containing DNA from cell hybrids having the deleted chromosomes 4 from the different patients—except in lane 6 (hybrid HHW842), where a strong hybridization signal is apparent. Probes 678 and 731 gave analogous results when tested in the same manner.

These latter three probes, in region B, are of significance for three reasons. They confirm, by molecular techniques, the cytogenetic interpretation that the deletion of the chromosome 4 retained in HHW842 is interstitial and that this chromosome retains some DNA above 4p16.2. These results demonstrate that G8 is not at the very terminus of 4p and indicate that there may be a considerable amount of DNA between G8 and 4pter. In this regard, it should be noted that none of the inserts from the three independent phage with DNA derived from this region have any regions of overlap. Most important, these probes represent the first DNA fragments that have been assigned to the region of 4p distal to G8 and thus are in the direction of the HD gene from G8. It is likely, therefore, that one or more of these DNA fragments is between HD and 4pter.

One other point that should be noted concerning these physical mapping studies is that no probes have been localized to the most telomeric region (A). This region is defined as being above the distal breakpoint

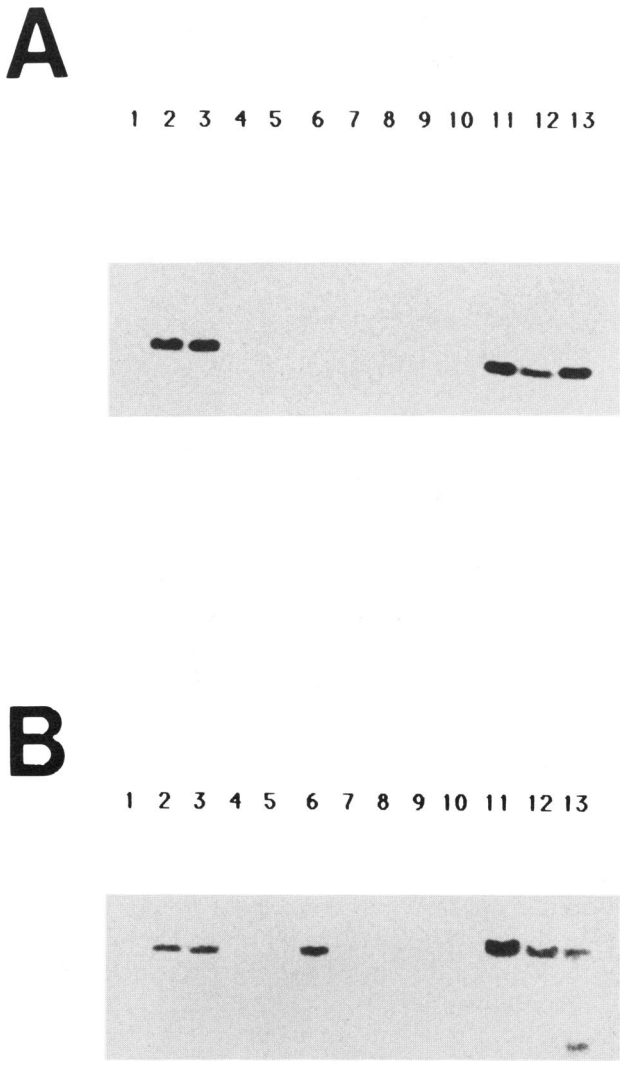

Figure 5 Blot hybridization of single-copy DNA fragments from regions B and C to the hybrid-cell deletion-mapping panel. Subcloned fragments pBS674E-D (*A*), derived from phage 674, and fragment pBS854P-B (*B*), derived from phage 854, were hybridized to filters containing *Eco*RI-digested DNA from: CHO (lanes 1); HHW416 (lanes 2); HHW693 (lanes 3); HHW847, subclone 1 (lanes 4); HHW832 (lanes 5); HHW842 (lanes 6); HHW878 (lanes 7); HHW882 (lanes 8); HHW886 (lanes 9); HHW847, subclone 2 (lanes 10); and human lymphoblasts (lanes 11–13). Note that pBS674E-D is missing from each hybrid with a deletion involving 4p16 (lanes 4–10), as is G8. pBS854P-B shows the same pattern of deletion as pBS674E-D, except that it is present in hybrid HHW842 (lane 6), which retains the chromosome 4 with a large interstitial deletion (see fig. 3).

Table 2

Polymorphisms Revealed by DNA Fragments in Distal 4p16

Probe (HGM Symbol)	Regional Location[a]	Enzymes Revealing RFLP	No. of Alleles	Frequency of Major Allele[b]
pBS674E-D (D4S95)	C	*Hin*dIII	2	.69
		*Pst*I	2	.84
		*Mbo*I	2	.58
		*Taq*I	2	.67
pBS678E-D (D4S96)	B	*Msp*I	2	.50
pBS854P-B (4S97)	B	*Rsa*I[c]	2	.75
		*Mbo*I[c]	2	.75
pBS731B-C (D4S98)	B	*Sst*I	3	.67

[a] Physical regions of 4p16 shown in fig. 4.
[b] Based on an examination of 6–12 unrelated individuals.
[c] These polymorphisms are in linkage disequilibrium.

of the chromosome 4 in hybrid HHW832, which is thought to have a small interstitial deletion. This may indicate either that there is an underrepresentation of fragments from this region in the library or that the deletion is actually terminal as opposed to interstitial. This question remains to be resolved.

RFLPs Revealed by Probes Distal to G8

At least one unique-sequence DNA fragment from each of the four phage with inserts that map to regions B and C was subcloned into pUC8. These subcloned fragments were hybridized to six unrelated individuals' DNAs, each of which had been digested with 12–24 different restriction endonucleases, to identify those probes that reveal RFLP. The results of these studies are summarized in table 2. Each of the probes tested revealed at least one polymorphism. The probe closest to G8, pBS674 E-D (D4S95), is the most polymorphic, revealing RFLP with four different restriction endonucleases, none of which are in complete linkage disequilibrium. For each polymorphism listed in table 2, Mendelian inheritance was confirmed by following the segregation of alleles in one or more nuclear families in which at least one parent was heterozygous. We are continuing to isolate additional unique-sequence fragments from each phage and are expanding each locus by means of cosmid walking in an attempt to identify additional polymorphisms and thereby make each locus as informative as possible for linkage studies in families in which HD is segregating. It is hoped that this will enable us to determine which, if any, of these DNA probes represent flanking markers for HD.

Discussion

The joint use of DNA libraries enriched for specific portions of the human genome and patient material or somatic-cell hybrids that contain chromosomes with deletions of the regions under study has proved to be an effective method for high-resolution physical mapping of human chromosomes. This combination of techniques was important in isolating DNA markers near the genes responsible for Duchenne muscular dystrophy, chronic granulomatous disease, retinoblastoma, and cystic fibrosis, which were first steps in identifying candidates for these disease genes (Friend et al. 1986; Monaco et al. 1986; Royer-Pokora et al. 1986; Estivill et al. 1987).

As described herein, this same strategy applied to identification of DNA fragments in the vicinity of the HD gene has already resulted in the isolation of four probes of interest, three of which (678, 731, and 854) were shown by physical mapping to be distal to G8, i.e., in the direction of the HD gene from this marker. In addition, although the position of the remaining probe of interest (674) relative to G8 and 4pter could not be determined by means of physical mapping, preliminary linkage studies on reference pedigrees indicate that it is also distal to G8. Since the HD gene is also toward the telomere from G8, any one or all of these new markers may be on the opposite side of the HD gene from G8. All of the new DNA markers described herein are being used for linkage studies in large pedigrees in which HD is segregating, to define their order relative to one another and to the HD gene. The identification of DNA markers close to and

on both sides of the HD gene will have two important ramifications. First, if the two closest flanking markers can be made highly polymorphic, presymptomatic diagnosis of HD could be made virtually 100% accurate. Currently, G8 is the only DNA marker close enough to HD to be used for preclinical diagnosis of the disease. Since G8 and HD are ~4 cm apart, there is a 4% chance of an incorrect preclinical diagnosis if G8 is used alone in such a test. Second, the two closest flanking DNA probes will delineate both endpoints of the region of DNA that must contain the HD gene, a demarcation that will help considerably in attempts to isolate the disease gene, as discussed below.

It is not obvious why it has previously been so difficult to identify DNA fragments distal to G8. The region from G8 to the terminus of 4p represents, in terms of cytogenetic distance, ~3%–5% of the total length of chromosome 4. Thus, even in screening DNA fragments from a library specific for all of chromosome 4, one would theoretically expect three to five of every 100 fragments to be in this region. The 105 DNA fragments whose physical locations we have determined in the present report were all derived from 4p15-4pter, which represents, at most, 20% of the cytogenetic length of chromosome 4. However, only four of these 105 DNA fragments are located within or above 4p16.2; this is approximately one-fifth the number that would be expected on the basis of cytogenetic distances. The paucity of DNA fragments identified as being in this region could be due to any one of the following factors: (1) There may be an underrepresentation of DNA fragments from this region in the library, possibly owing to its having a high proportion of sequences that are unstable in or do not replicate well in the cloning vector or *E. coli* host strain used to prepare the library. (2) The method used to identify recombinant phage having human DNA inserts (hybridization to repetitive human DNA sequences) results in a bias against isolating recombinants having inserts from regions that contain very little repetitive DNA. This could be the case for the terminal portion of 4p16. (3) The distribution of DNA over the cytogenetic region 4p15-4pter may not be uniform, and 4p16.2-4pter may contain much less DNA than would be expected on the basis of its cytogenetic length. This latter possibility is being examined using DNA fragments from this region in conjunction with pulsed-field gel electrophoresis in an attempt to compile a long-range restriction map of the region. If a contiguous long-range restriction map that physically links DNA probes found to be on opposite sides of the HD gene can be compiled, it will be possible to determine exactly how much DNA is in the region that must contain the disease gene. It will then be obvious in which direction from each of the endpoints one should move to get closer to the HD gene.

Note added in proof.—The RFLP revealed with pBS674E-D in *Hin*dIII digested DNA was recently found to be a multiallele rather than a two-allele system. *Acc*I reveals this same multiallele polymorphism and produces allelic fragments that are more easily resolved than the corresponding fragments produced by *Hin*dIII.

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