

Generation and Characterization of Irradiation Hybrids of Human Chromosome 4

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Abstract—*In recent years investigators have attempted to develop more rapid and precise methods to isolate specific chromosomal DNA regions. In this paper we demonstrate a modification of the method first developed by Goss and Harris for generation of irradiation hybrids. The gene encoding the dominant selectable marker for resistance to neomycin was introduced into human chromosome 4 using retroviral insertion into human fibroblasts. Transfer of these chromosomes via microcells into the mouse cell line NIH3T6 produced a somatic cell line containing chromosome 4 as the only human chromosome. Irradiation of this cell line followed by fusion with the hamster cell line CHTG49 generated hybrids containing only small portions of chromosome 4p on a hamster background. The use of selection produced stable hybrids that retained chromosome 4 fragments over long periods of tissue culture passage. To obtain new polymorphic markers for Huntington's disease, one of these hybrids was used to isolate new genomic fragments. We identified 41 single-copy fragments, of which 27 have been mapped to specific regions of chromosome 4; 52% of these fragments map to the region of chromosome 4 containing the HD gene.*

INTRODUCTION

The study of genetic diseases necessitates isolation of DNA fragments from a specific chromosomal region. Creation of somatic cell hybrids containing one human chromosome on a rodent genomic background has been the method most often used. If the gene responsible for the disorder has been localized to a specific chromosomal subregion, development of methods that allow for efficient cloning of DNA from these subregions would greatly facilitate gene identification by reducing the complexity of

DNA to be sorted through. Hybrids containing translocated human chromosomes can be useful as initial starting reagents, but these still contain large amounts of human DNA from other regions. To obtain small human chromosomal fragments on a rodent background, the method first developed by Goss and Harris has been used to generate irradiation hybrids (1–3) and recently has been used to create a map of human chromosomes 21 and 11 (4, 5) and mouse chromosome 1 (6).

Huntington's disease (HD) is a progressive neurological genetic disorder character-

ized by involuntary movements (chorea), intellectual decline, and severe personality and mood disturbances (7). HD usually presents in the third or fourth decade of life, although it can occur as early as the first decade or as late as the eighth. The average duration of HD from age of onset is 17 years, but it may be as long as 30 years (8). HD is inherited as an autosomal dominant gene with complete penetrance but a delayed onset of symptoms. In terms of age of onset and severity of symptoms, individuals homozygous for the *HD* gene are indistinguishable from siblings who are heterozygous for *HD* (9). It therefore appears that *HD* is a true dominant gene suggesting that the *HD* mutation is not one resulting in a loss of gene function. By the time the majority of persons with HD are diagnosed, they are past the reproductive age and have passed the *HD* gene on to their offspring. The mutation rate at the *HD* locus appears to be low, as only a few possible sporadic cases have been reported (10, 11).

In 1983 genetic linkage was first shown between *HD* and the DNA marker D4S10 located on chromosome 4 (12). D4S10 was localized to the short arm of chromosome 4 in band 4p16 and more recently to the most terminal cytogenetic subband 4p16.3 (13–15) (Fig. 1). Multipoint linkage analysis indicated that the *HD* gene was located between D4S10 and the telomere (16). However, the use of these markers for predictive testing was limited due to the distance of D4S10 (4–6 cM) from the *HD* gene. Two additional polymorphic markers, D4S43 and D4S95, recently have been located distal to D4S10 and show tighter linkage with the *HD* gene (17, 18). The locus cluster D4S98/S113/S114 suggested that *HD* was located more distal to all these markers (19). Additional methods recently have generated new markers within this region of chromosome 4 (20–22). The markers that have been generated to date have been assembled into a comprehensive physical map (23). Therefore, at present

there are extensive genetic and physical maps of the *HD* region. Recombination events in HD families, however, have not identified a specific position for the *HD* gene in the 4p16.3 region and in fact give contradictory placements for the *HD* gene (24, 25). Linkage disequilibrium data seem to support the presence of the *HD* gene within 4p16.3, proximal to D4S115 (26, 27). To date no DNA markers have been found that clearly flank the *HD* gene.

To obtain new DNA markers from chromosome 4p16.3, we employed a technique that would enrich for obtaining DNA fragments from human chromosome 4. Introduction of a dominant selectable marker into chromosome 4 produced stable hybrids that retained the human chromosome 4 indefinitely in tissue culture under selection. These hybrids were then used to generate Goss-Harris irradiation hybrids that retained only small portions of human chromosome 4 flanking the introduced neomycin-resistance gene. This method generated new mapping reagents and provide a new source for isolation of genomic DNA fragments from a specific chromosomal subregion. We have characterized these hybrids extensively and used one of the hybrids (HD8000-3) to isolate new DNA markers.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Primary diploid fibroblasts were obtained from a sample of foreskin propagated in F/DV medium (a 1:1 mixture of DME and Ham's F12) supplemented with 15% fetal calf serum and 2 mM glutamine. NIH3T6 cells are an established line of mouse fibroblasts; these were maintained in F/DV medium plus 10% fetal calf serum. CHTG49, an established hamster cell line that is resistant to 6-thioguanine, was grown in DME with 10% fetal calf serum and 5 μ g/ml thioguanine. HD113.2B is a human mouse cell hybrid maintained in DME with 15%

fetal calf serum and 500 $\mu\text{g}/\text{ml}$ neomycin. Goss-Harris hybrids were grown in DME with 10% fetal calf serum, 500 $\mu\text{g}/\text{ml}$ neomycin, and 5 $\mu\text{g}/\text{ml}$ thioguanine.

Generation and Characterization of Cell Line HD113.2B. Culture medium containing virus was harvested from Ψ -AM producer cells filtered through a 0.45- μm filter assembly and incubated with human fibroblasts for 2 h at 37°C in the presence of 8 $\mu\text{g}/\text{ml}$ polybrene (28). Infected cell clones were selected by growth in 2 mg G418. The infected human fibroblasts were plated onto plastic bullets in the presence of 10 $\mu\text{g}/\text{ml}$ colcemid for 48 h (29, 30). After micronuclei had formed, the cells were centrifuged in the presence of 5 μg of cytochalasin B per milliliter of serum-free growth media to enucleate the cells. The NIH3T3 cells and microcells were fused in the presence of 44% polyethylene glycol. Individual hybrid colonies were picked using glass cloning rings and passaged through media containing 500 $\mu\text{g}/\text{ml}$ G418 and 3×10^{-6} M ouabain to eliminate any contaminating human donor cells.

Twenty microcell hybrids were screened by Southern blotting with human chromosome 4 markers to identify hybrids containing human chromosome 4. HD113.2B was further identified as containing human chromosome 4 by karyotypic analysis (31). HD113.2B was characterized by fluorescence in situ hybridization with nick-translated interspersed repetitive sequence-PCR products from HD113.2B DNA onto normal human metaphases and nick-translated total human DNA onto HD113.2B metaphases (32–34). The chromosomal locations of integrated SVX(neo) retrovirus were mapped by in situ hybridization as previously described (35).

Preparation of Goss-Harris Hybrids. Using the somatic cell genetics approach originally developed by Goss and Harris, large chromosome fragments surrounding the neo^R marker in cell line HD113.2B were

transferred to hamster cells (1–3). The cell line HD113.2B was gamma-irradiated with 500, 1000, 1500, 2000, 4000, and 8000 rads. Then 1×10^7 irradiated HD113.2B cells were fused to 2×10^7 CHTG49 hamster cells using Koch Light PEG 1000 for 3 min. The fused cells were washed three times in DME with 15% fetal calf serum and plated onto 100-mm petri dishes. The hybrids were allowed to recover for 48 h in selection-free DME prior to selection in 500 $\mu\text{g}/\text{ml}$ G418 and 5 $\mu\text{g}/\text{ml}$ thioguanine. After 10–14 days under selection, single clones were picked, replated, and grown for isolation of DNA.

DNA Probes and PCR Analysis. Initially, Southern blotting was performed using standard conditions (36) on a small sample of the hybrids with the DNA probes D4S10 and D4S90 to detect those hybrids that had retained the terminal portion of chromosome 4 (12, 37) (Fig. 1). PCR analysis of 100 ng of DNA from each hybrid was done under cycling conditions previously reported (38, Gusella et al., manuscript in Preparation).

Lad-4 Phage Library Construction and Analysis. Goss-Harris hybrid HD8000-3 was partially digested with *Sau3A* to give aver-

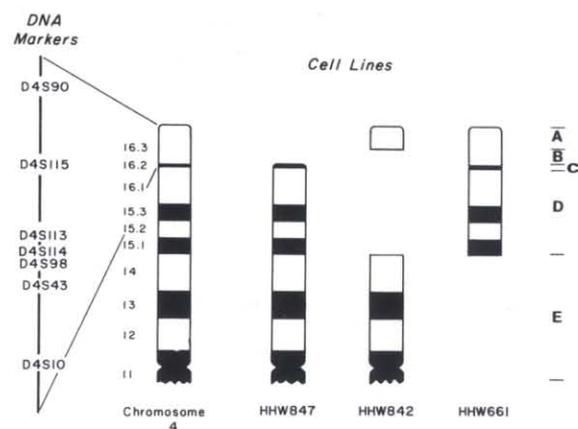


Fig. 1. Chromosome 4p16.3 DNA markers and HHW cell lines. Depicted are the previously published DNA markers for chromosome band 4p16.3 from the most proximal marker D4S10 to the most distal marker D4S90. The cell lines used for mapping the single-copy DNA fragments identified in this paper are depicted on the right. The individual fragments identified using these hybrids are shown by the letters A–E on the far right.

age DNA fragment sizes of 18–23 kb and ligated into the phage vector EMBL-4 as previously described (39). Titration of the library showed the genomic representation to be three haploid genomes per microgram of DNA. Then 500,000 recombinant phage were screened with CotI human repeat-enriched DNA (40). Positive plaques were picked and purified. Five hundred milliliters of phage preparations of each phage were digested with Sall to cut out the inserts and determine the size of the fragment DNA.

Isolation and Localization of Single-Copy Fragments. In order to identify single copy DNA fragments, the phage were digested to completion with Sau3A followed by cloning into BamHI-digested pUC19. White recombinant colonies were toothpicked onto triplicate gridded nitrocellulose filters and probed with EMBL-4 and CotI. Colonies that did not hybridize to either of these probes were presumed to contain single-copy fragments and were picked and used to hybridize to human genomic DNA blots. Single-copy fragments were localized by hybridization to mapping panel blots. Cell line HHW847 contains 4p16.3-4qter, HHW842 contains 4pter-1/2 4p16.3 and 4p14.1-4qter, and HHW661 contains 4pter-4p15.1 (13, 20, 41) (Fig. 1).

RESULTS

Generation of Neo-Tagged Chromosome 4 Hybrid. A population of human diploid fibroblasts was infected with amphotropic retrovirus SVX(neo) and selected with G418. Microcell-mediated gene transfer to NIH3T6 cells produced human–mouse hybrids with the neo marker integrated into several human chromosomes (33). Cytogenetic analysis and Southern blotting with chromosome 4 probes confirmed the presence of chromosome 4 as the only human chromosome retained in one of the hybrid cell lines, HD113.2B. Fluorescence in situ hybridiza-

tion using total human DNA nick translated with ¹¹dUTP to metaphase spreads of cell line HD113.2B detected three human chromosomes on a mouse background (Fig. 2A). Furthermore, fluorescence in situ hybridization using interspersed repetitive sequence-PCR products from the cell line HD113.2B onto normal human metaphases labeled specifically chromosome 4 (Fig. 2B). Southern analysis demonstrated a single integrated proviral genome was present in this cell line. In situ hybridization indicated that the integration occurred within chromosome bands 4p14-p16 (data not shown).

Goss-Harris Hybrid Analysis. Large fragments of human chromosome 4 from hybrid HD113.2B were transferred to CHTG49 hamster cells by the method of Goss and Harris (1–3). At least two hybrids from each irradiation dose were characterized with chromosome 4 probes (Fig. 1). Southern blotting showed that all but one of the hybrids retained the marker D4S10 while only the lower irradiation dose hybrids (500–3000 rads) and one 8000-rad hybrid (HD8000-3) retained the most distal chromosome 4 marker D4S90. Efficient retention of DNA markers from band 4p16.3 is consistent with previous in situ hybridization on the location of the site of retroviral integration and suggests that the site of integration is close to band 4p16.3.

Twelve hybrid cell lines were selected for more extensive characterization using PCR analysis of eight different DNA sequences in the 4p16.3 subband, QDPR at 4p16.1-4p15.1, and AFP at 4q11-4q13 (Table 1) (41). Three hybrids did not retain D4S10, the most proximal 4p16.3 marker, while five hybrids did not retain the most distal marker, D4S90. Only one hybrid, HD1500-4, retained the *AFP* locus at 4q11-4q13 and seven of the lower-dose irradiation hybrids retained the *QDPR* locus at 4p11-4p13. The markers that were present appear to be retained in a contiguous segment of DNA in all of the hybrids except HD500-4, HD1000-4, and

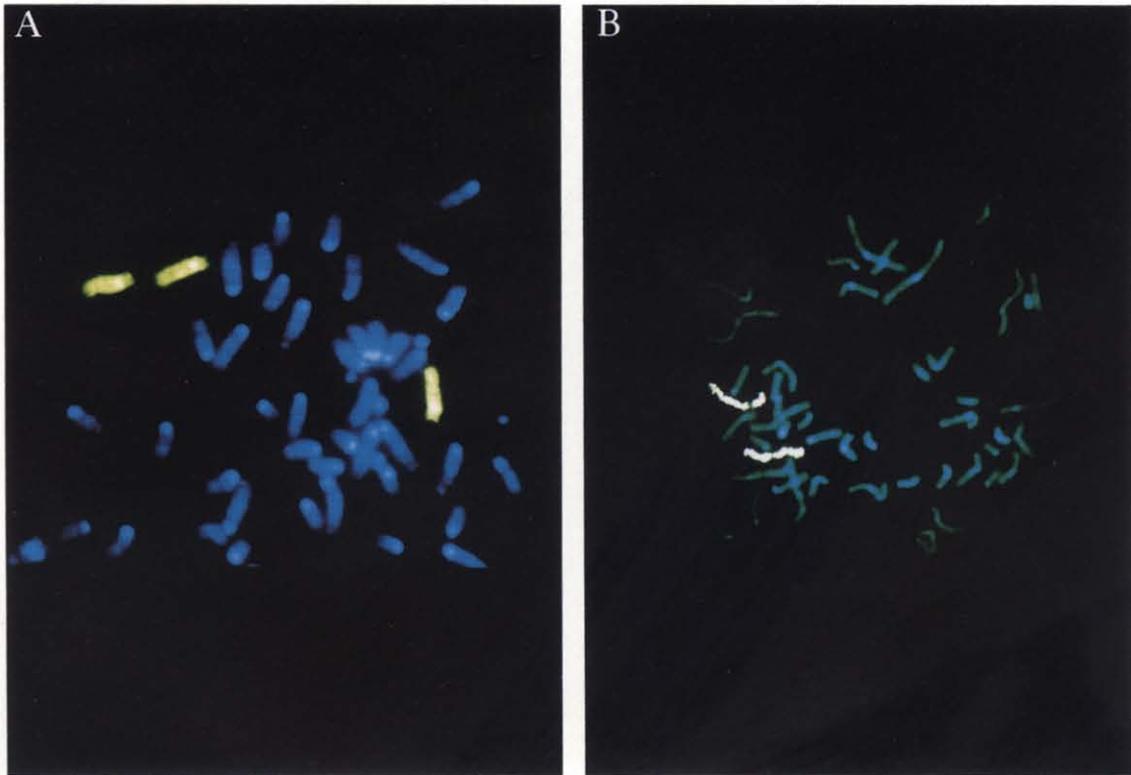


Fig. 2. Fluorescence in situ hybridization of chromosomal content of cell line HD113.2B. All probes were labeled with biotinylated-dUTP, detected with avidin-FITC, and counterstained with DAPI. (A) Total human DNA was nick translated and hybridized to metaphase spreads of cell line HD113.2B. (B) The PCR reaction was carried out in a total volume of 100 μ l, primer at 100 μ M in 1.5 μ M $MgCl_2$ reaction buffer (Perkin-Elmer/Cetus), 1.2 mM dCTP, 1.2 mM dGTP, 1.2 mM dATP, 1.2 mM dTTP, and 2.5 units Taq polymerase. An automated thermal cycler (Perkin-Elmer/Cetus) was used following the procedure of Nelson et al. (33) and primer 559 is referred to as TC-65 (31). The PCR products were nick-translated and hybridized to normal human metaphases. Negative controls were run using the primer 559 on total mouse (RAC) and hamster (E36) DNA.

Table 1. PCR Analysis of Chromosome 4 Goss-Harris Hybrids^a

	AFP	QDPR	D4S10	D4S95	D4S43	D4S99	D4S97	D4S115	I14	D4S90
HD500-3	-	+	+	+	+	+	+	+	+	+
HD500-4	-	+	+	+	+	+	-	+	+	+
HD1000-2	-	-	-	-	-	-	+	+	+	+
HD1000-3	-	+	+	+	+	+	+	+	+	+
HD1000-4	-	+	+	+	+	+	-	+	+	+
HD1500-4	+	+	+	+	+	+	+	+	+	-
HD2000-1	-	+	-	+	+	+	+	+	+	-
HD2000-2	-	+	+	+	+	+	+	+	+	+
HD2000-4	-	-	+	+	-	-	-	-	-	-
HD4000-2	-	-	+	+	+	+	+	+	-	-
HD4000-3	-	-	-	-	+	+	+	+	+	-
HD8000-3	-	-	+	+	+	+	+	+	+	+

^aA "+" indicates the presence of the marker and "-" indicates the absence of the marker in the PCR assay. All of the markers are in linear map order with AFP mapping to the 4q arm and D4S90 being the most telomeric marker on arm 4p.

HD2000-1. In these three hybrids it appears that small deletions have occurred during formation of the hybrids.

Characterization of *Lad-4* Library. The hybrid cell line HD8000-3 retained all of the *HD* region and was therefore chosen for isolation of additional markers from the chromosome 4p16.3 region. Additional hybrids retained these markers but since HD8000-3 was generated with the highest dose of irradiation, this hybrid was likely to contain the least amount of extraneous chromosome 4 material (i.e., below 4p16.3). It is also possible that there are small deletions undetected in HD8000-3 as a very high dose of irradiation was used.

An EMBL-4 lambda phage library was constructed from HD8000-3 hybrid DNA in order to isolate new DNA fragments from the 4p16.3 region. Phage (5×10^5) were screened with *CotI* repetitive human DNA and 73 positive phage were isolated. Therefore approximately one in every 7000 phage recombinants was a human clone. However, additional human clones probably went undetected due to isolation of only strong positives. The remainder of the recombinant clones contained either mouse or hamster DNA sequences. Of the phage analyzed, only two of 50 were duplicates, indicating that we had isolated approximately 1.4 Mb of human DNA from cell line HD8000-3.

Isolation of Single-Copy Sequences. To isolate new DNA markers from chromosome 4p in the *HD* region, we analyzed the phage for single-copy sequences. Subcloned fragments of the phage inserts that did not hybridize with repetitive human DNA were tested further by Southern hybridization to digested human DNA blots. Single-copy fragments were identified in 84% (41/49) of the phage characterized. Figure 3 shows four of the single-copy fragments hybridized to blots of digested human DNA. The single-copy fragments ranged in size from 200 bp to 5 kb.

Mapping of Single Copy Sequences. Using three established somatic cell hybrids containing only portions of chromosome 4,

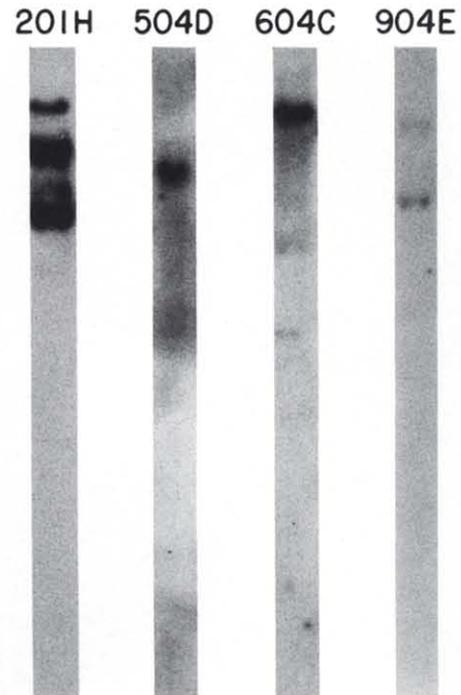


Fig. 3. Southern analysis of four of the single-copy DNA fragments. Genomic human DNA (10 μ g) was digested with *EcoRI*. Filters were hybridized with the indicated DNA markers. DNA marker 201H detects three *EcoRI* fragments, 504D one *EcoRI* fragment, and 604C and 904E three *EcoRI* fragments. Filters were washed to a stringency of $0.5 \times$ SSC (0.15 M NaCl, 0.015 M Na citrate).

several of the single-copy DNA fragments were localized (13, 20, 42). Figure 4 shows four of these fragments mapped to four different regions of chromosome 4p. We have definitively mapped 27 of the isolated fragments to specific regions of chromosome 4p (Table 2). Most important, 52% (14/27) of the single-copy fragments map to 4p16.2-4p16.3, which is the region known to contain the *HD* gene. The remainder of the fragments map as follows: 4% (1/27) to 4p16.2-4p15.1, 7% (2/27) to 4p15.1-4p14.1, and 37% (10/27) to 4p14.1-4p14.2.

DISCUSSION

We have modified the method of Goss and Harris for production of irradiation hybrids that contain specific regions of human chromosomes. Retroviral insertion of

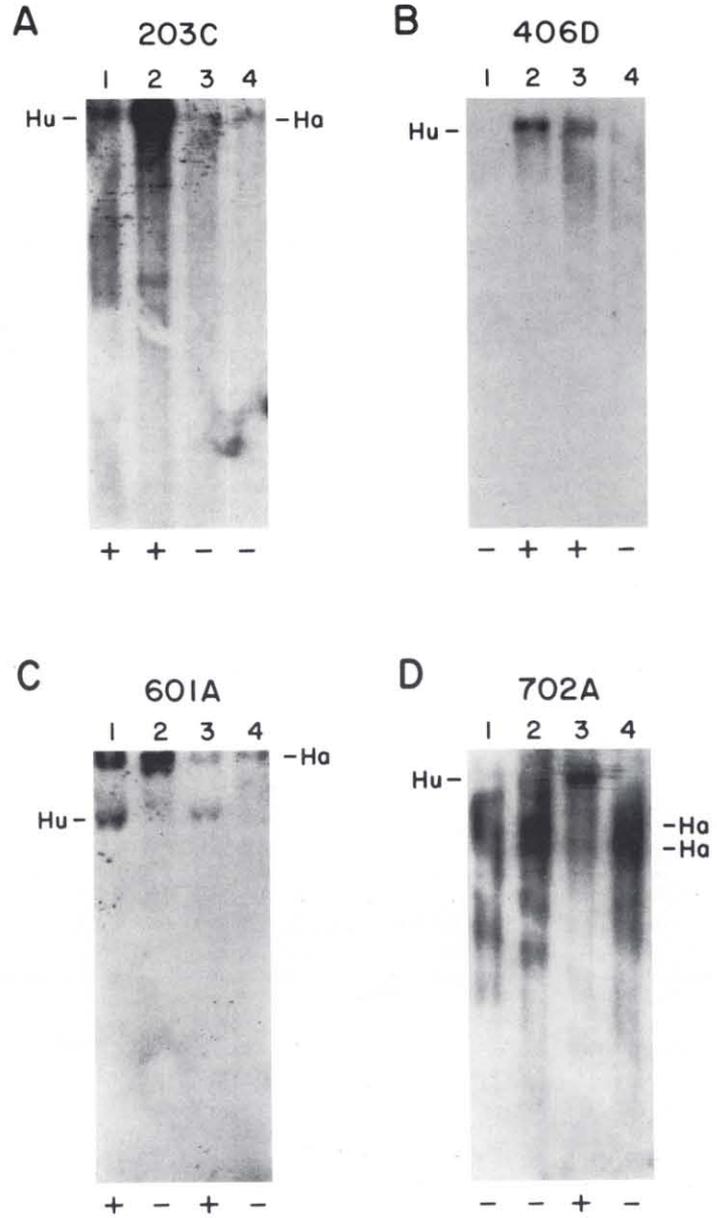


Fig. 4. Chromosome 4 mapping of single-copy DNA fragments. Genomic DNA (10 μ g) of cell lines HHW847 (lane 1), HHW842 (lane 2), HHW661 (lane 3), and CHTG49 hamster cells (lane 4) were digested with EcoRI and analyzed by Southern blotting. Refer to Fig. 1 for DNA content. (A) DNA fragment 203C detects an EcoRI human fragment in lanes 1 and 2, demonstrating a map position of 4p14-4qter. 203C also detects a cross-hybridizing hamster band. (B) DNA fragment 406D detects an EcoRI fragment in lanes 2 and 3, demonstrating a map position of 4pter-1/2 4p16.3. (C) DNA fragment 601A detects an EcoRI fragment in lanes 1 and 3, demonstrating a map position 4p16.2-4p15.1. 601A also detects a cross-hybridizing hamster band. (D) DNA fragment 702A detects an EcoRI fragment only in lane 3, demonstrating a map position of 1/2 4p16.3-4p15.1. 702A also detects two cross hybridizing hamster bands.

a dominant selectable marker into human chromosome 4 has enabled the production of a stable human-mouse hybrid that retains only this human chromosome (HD113.2B). This hybrid has been maintained in culture for more than two years and retains the human chromosome 4 in all cells due to the presence of the selectable marker encoding neomycin resistance at chromosome band 4p16.3. Using this hybrid as a donor, we have now created somatic cell hybrids that contain fragments of human chromosome 4 that

flank the integration site on a rodent background.

Irradiation of cell line HD113.2B followed by fusion to hamster cell line CHTG49 generated hybrids that, under neomycin selection, retained portions of the telomeric region of chromosome 4. Three of the hybrids did not retain the most proximal 4p16.3 marker D4S10 while five of the hybrids did not retain the most distal marker D4S90. When the hybrids were tested for markers outside of the 4p16.3 region, we

Table 2. Sublocalization of Single-Copy DNA Fragments on Chromosome 4

Subclone	Cell line ^a			Chromosome 4 region
	HHW847	HHW842	HHW661	
102E (D4S149)	-	+	+	A
205E	-	+	+	A
302C (D4S154)	-	+	+	A
304E (D4S147)	-	+	+	A
401B	-	+	+	A
405E	-	+	+	A
406D (D4S148)	-	+	+	A
502C	-	+	+	A
604C	-	+	+	A
609B (D4S153)	-	+	+	A
802C	-	+	+	A
905C	-	+	+	A
1003C (D4S152)	-	+	+	A
702A	-	-	+	B
601A	+	-	+	C
303D	+	-	-	D
409A	+	-	-	D
101B (D4S149)	+	+	-	E
201H (D4S153)	+	+	-	E
203C (D4S159)	+	+	-	E
403B (D4S158)	+	+	-	E
503D	+	+	-	E
505G (D4S150)	+	+	-	E
703A	+	+	-	E
803B	+	+	-	E
901D	+	+	-	E
906D (D4S156)	+	+	-	E

^aA "+" indicates presence of marker in the hybrid and "-" indicates absence of the marker in the hybrid. The chromosomal regions The subclones map to are depicted in Fig. 1.

found that the 4q arm was only retained in one hybrid while the lower-dose hybrids tended to also retain DNA from the 4p15.3 region. As expected, irradiation dose correlates inversely with the fragment size transferred, i.e., low doses of irradiation generated large DNA fragments. The markers appear to be retained in a contiguous DNA segment in the majority of the hybrids. However, it should be noted that this conclusion is based on the use of markers that are generally a large distance apart. It is therefore possible that small deletions could have occurred that are not detected in this analysis. In hybrids HD500-4 and HD1000-4 there are small deletions of chromosome 4 DNA detectable around the D4S97 region, and in the hybrid HD2000-1 around the

D4S10 region. When deletions occur, it is likely that the surrounding DNA fragments are fused together so that the DNA is retained under neomycin selection or perhaps retained as separate fragments.

DNA from the hybrid cell line HD8000-3 was used to produce an EMBL-4 library, which has been used successfully for the isolation of new sequences from the chromosome 4 region. This hybrid was selected for two reasons. Southern blotting showed the presence of the most distal (D4S90) and proximal 4p16.3 markers (D4S10), which delineate the *HD* region. Secondly, the hybrid was produced using the highest irradiation dose (8000 rads), which increases the likelihood that it would contain a limited amount of other chromosome 4 material

(i.e., DNA segments distal to the neomycin viral integration site).

Screening of the HD8000-3 phage library yielded 73 human clones when probed with CotI DNA. We successfully identified single-copy fragments in 84% (41/49) of the phage characterized by hybridization to human genomic DNA. These DNA fragments ranged in size from 200 bp to 5 kb. Since the goal of this study was to obtain new probes from the chromosome 4 region containing the *HD* gene, we mapped 27 of these DNA fragments using established cell lines (Table 2). We report here the isolation of 14 new single-copy DNA fragments mapping to the chromosome 4pter-1/2 4p16.3 region. Therefore this method has substantially enriched for DNA sequences from the region of chromosome 4 containing the *HD* gene with 52% of isolated clones falling within this region.

Continuing characterization of these hybrids will better define small deletions that occur. These hybrids now provide new reagents for mapping other DNA sequences in the region. The newly isolated DNA sequences can be used further to identify conserved sequences and to search for new polymorphic markers in the search for the *HD* gene. These conserved sequences possibly represent genes of interest from the 4p16.3 region and are being studied further.

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