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Radiation Hybrid Map Spanning the Huntington Disease Gene Region of Chromosome 4

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Radiation hybrid (RH) mapping was used to construct a map of 11 markers in the distal 4 Mb of the short arm of chromosome 4, the region containing the Huntington disease gene. Two different methods for deriving the order of the markers were compared and both arrived at the same order as being the most likely. This order is also consistent with both the physical map constructed using pulsed-field gel electrophoresis (PFGE) and the meiotic linkage map. Comparing the RH map to the map determined by PFGE provided the means to equate RH map units (centirays) with actual physical distance in kilobases of DNA. In addition, a simple procedure for reducing the complexity of human DNA in radiation hybrids is described. One cell line isolated using this procedure contains, as its only human DNA, ~2 Mb surrounding the Huntington disease gene. © 1992 Academic Press, Inc.

INTRODUCTION

Radiation hybrid (RH) mapping is a somatic cell genetic technique for constructing physical maps over tens of millions of basepairs of DNA at approximately 50 Kb resolution (Cox *et al.*, 1990). In many respects, RH mapping is the physical mapping equivalent of meiotic mapping; the frequency of breakage or recombination between loci is directly related to the distance between them. In contrast to meiotic mapping, however, loci do not have to be polymorphic for RH mapping. In this report, we describe the construction of an RH map of 11 loci in the distal 4 Mb of the short (p) arm of chromosome 4, the region containing the Huntington disease (HD) gene. An RH map of this region is particularly useful in several respects:

1. It provided a way to correlate or equate RH map units (centirays or cR) with actual physical distances separating loci in kilobases of DNA as a long-range restriction map of this region has been compiled by using PFGE (Bucan *et al.*, 1990; Bates *et al.*, 1991).
2. Since one locus included in the RH map of distal 4p is within 300 kb of the telomere, the map provided data concerning the effect of the telomere on the retention of loci very near the ends of chromosomes.
3. The RH map could be used to predict the size of a gap that currently exists in the PFGE map of this region.
4. A region between two markers placed on the RH map, D4S10 and D5S125, has been shown to be a "hot spot" for meiotic recombination (Allitto *et al.*, 1991). An RH map of this same region showed that the much higher than normal frequency of meiotic recombination was not correlated with a higher than average frequency of radiation-induced breakage.

In addition, two different methods for analyzing the data and constructing the RH map were compared. Interestingly, both methods arrived at the same order of loci as being the most likely, which is in agreement with the physical map determined by PFGE studies (Bucan *et al.*, 1990; Bates *et al.*, 1991). In addition to being mapping reagents, individual radiation hybrids with small, defined segments of the human genome provide a means to selectively clone DNA from specific regions. For example, Doucette-Stamm *et al.* (1991) described a microcell, irradiation, refusion procedure for deriving radiation hybrids that retain, under selective pressure, just the distal portion of 4p. This cell line was used to isolate 14 new DNA clones from the Huntington disease gene candidate region. In this report we describe a simple procedure for reducing the amount of human DNA in radiation hybrids that does not involve any direct selection. This technique is useful for any set of radiation hybrids for any chromosome and allows the isolation of cell lines that retain single human DNA fragments from a region of interest.

MATERIALS AND METHODS

Isolation of radiation hybrids. The isolation of the 109 radiation hybrids used in these studies has been described (Warrington *et al.*, 1991). The radiation hybrids were generated from the human–Chinese hamster cell hybrid HHW 661, which retains a derivative chromosome 5 [4pter–4p15.1::5p14–5qter] as its only human DNA (Was-

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muth *et al.*, 1986). The region of chromosome 4 (p15.1-pter) present on this derivative chromosome includes the HD gene and the linked genetic markers (Smith *et al.*, 1988; Lin *et al.*, 1991).

Polymerase chain reaction. The presence or absence of each of 11 different markers from distal 4p in the 109 radiation hybrids was determined by PCR. Table 1 lists the loci that were tested, including clone names, HGMW-approved symbols, and the retention frequency of each in the radiation hybrids. The sequences of oligonucleotide primer sets for each marker has been reported, as have the annealing temperatures (Gusella *et al.*, 1992). PCR reactions were carried out exactly as previously described (Warrington *et al.*, 1991). The PCR primer sets for each locus were designed from genomic DNA sequence, and none were conserved in Chinese hamster, making the scoring of the radiation hybrids very straightforward.

Two-point and four-point analysis of RH data. The relative distances between and the order of the 11 loci were determined using both two-point and four-point analysis programs as described by Cox *et al.* (1990). The distances between markers is reported in cR_{6500}, where 6500 represents the radiation dose (in rads) the irradiated parent was exposed to in constructing the set of radiation hybrids. cR_{6500} is abbreviated as cR throughout the text.

Maximum likelihood multipoint analysis of RH data. The 4p RH mapping data were also analyzed using a maximum likelihood multipoint approach (Boehnke *et al.*, 1991). This approach makes use of data on all loci simultaneously, including information on partially typed hybrids. Like the two-locus method of Cox *et al.* (1990), the multipoint method assumes that breakage occurs at random along the chromosome and that fragments are retained independently. In the N-locus case, the likelihood of the RH data is a function of $N - 1$ breakage probabilities θ_i between adjacent loci i and $i + 1$ and one or more retention probabilities for the various fragments that result. In the current analysis, we assume that all fragments have the same probability r of being taken up in a radiation hybrid.

For a given order, breakage and retention probabilities are estimated by those values that maximize the likelihood of the RH mapping data. Orders can be compared by their maximum likelihoods, the order with the largest maximum likelihood being best supported by the data. Since it is not practical to consider all $N!/2$ locus orders explicitly, we used a branch-and-bound algorithm to identify the best locus order. Branch and bound guarantees that the best locus order is found, but requires explicit consideration of only a small subset of the possible orders.

Refusion of radiation hybrids to reduce the amount of human DNA. The radiation hybrid XZH 147 was fused with the CHO cell line UCW 932 in monolayer culture using polyethylene glycol as described previously (Wasmuth *et al.*, 1981). UCW 932 has a mutation in the LARS gene, which renders leucyl-tRNA synthetase thermolobile and the cell line nonviable at 39°C (Thompson *et al.*, 1973; Wasmuth and Chu, 1980). The temperature-sensitive phenotype is recessive. The cell line was also selected as being resistant to 1 m*M* ouabain, which behaves as a dominant selectable marker (Baker *et al.*, 1974). The radiation hybrids are temperature resistant and sensitive to concentrations of ouabain above 0.1 m*M*. One-day postfusion, the cells were transferred to 39°C (to select against the UCW 932 parent) in medium with 1 m*M* ouabain (to select against the XZH 147 parent). Only hybrids between the two cell lines are viable. Independent hybrid clones were isolated and maintained at 39°C.

Fluorescence in situ hybridization. In situ hybridization was performed as described previously, using either total human DNA or a chromosome 4-specific plasmid library as the biotinylated probe (Altherr *et al.*, 1991; Collins *et al.*, 1991).

RESULTS

Ordering of Markers Using Two-Point and Multipoint Methods

The presence or absence of the 11 loci from distal 4p in 109 radiation hybrids was determined by PCR as described under Materials and Methods. The various markers were nonselectively retained in 22–29% of the radiation hybrids, as shown in Table 1. The data were analyzed using two-point and four-point analysis programs as described by Cox *et al.* (1990). Table 2 summarizes the two-point linkage data, including the distances between markers in centirays (cR), the estimates for θ, and the lod scores (for marker pairs with lod scores over 3.0).

TABLE 1

Markers Placed on the RH Map

Locus	Probe from which primers were designed	Retention frequency in hybrids
D4S10	G8	0.26
D4S125	YNZ32	0.24
D4S180	L19	0.22
D4S95	BS674	0.26
D4S43	C9A	0.25
D4S166	L6	0.27
FGFR3	BS385 HA	0.23
D4S168	E4	0.24
D4S97	BS854	0.28
D4S115	252	0.28
D4S90	CD2	0.23

Note. The sequences of the oligonucleotide primer sets for each locus have been described, as have the PCR conditions (Gusella *et al.*, 1992).

Initially, the order of the 11 loci was resolved by arranging them so that the sum of the distances between adjacent markers was minimized. The likelihood of one order over another for sets of four markers also aided in ordering the loci, as described by Cox *et al.* (1990). Figure 1 shows the predicted order of and distances between the 11 loci as determined by the two-point and four-point analyses. The loci D4S97 and D4S115 were not separated in any radiation hybrids, thus the order of these is arbitrary.

Recently, Boehnke *et al.* (1991) described a method for multipoint analysis of RH mapping that was also used to analyze the data for the 11 loci described above. Table 3 presents the maximum likelihood locus order and distance estimates for that order, together with all other locus orders with maximum likelihood no more than 1000 times less than the best order. The best locus order was the same as that arrived at using the approach described above. However, several other locus orders had maximum likelihoods only marginally smaller. The fact that some of the nearly best locus orders are not simple two-locus inversions stresses the importance of providing a list of the best locus orders, rather than simply listing comparisons of likelihoods for locus inversions, which was done for the map shown in Fig. 1. This latter practice can substantially overstate the evidence in favor of the best locus order. The most likely order arrived at by using both RH mapping methods is the same as the order determined by PFGE mapping (Bucan *et al.*, 1990; Bates *et al.*, 1991).

Correlation between RH Map Units and Kilobases of DNA

To equate RH map units (cR) with actual physical distances in kilobases of DNA, the RH map distances between adjacent loci were compared to the kilobases of

TABLE 2

RH Map Analysis and Two-Point Distances

Marker A	Marker B	No. of clones observed ++	+−	−+	−−	TOT	θ	cR_{6500}	LOD
S97	S115	28	0	0	69	97	0	0	0
S166	S43	25	2	0	72	99	0.0521	5	20.64
S115	S168	22	2	0	69	93	0.0557	6	18.67
S97	S168	22	2	0	68	92	0.0559	6	18.57
S168	FGFR	18	2	1	70	91	0.0916	10	15.18
S95	S125	23	3	1	72	99	0.1069	11	17.3
S97	S166	25	3	1	68	97	0.1020	11	17.89
S125	S10	23	1	3	72	99	0.1069	11	17.3
S95	S180	22	4	0	73	99	0.1097	12	16.9
FGFR	S43	20	2	2	70	94	0.1155	12	15.37
S97	FGFR	21	4	0	68	93	0.1109	12	15.87
FGFR	S166	21	1	3	69	94	0.1122	12	15.89
S180	S125	21	1	3	74	99	0.1132	12	16.36
S115	S166	25	3	2	69	99	0.1258	13	17.06
S43	S95	23	2	3	71	99	0.1320	14	16.24
S115	FGFR	21	4	1	68	94	0.1383	15	14.93
S97	S43	23	5	1	68	97	0.1564	17	15.11
S95	S10	23	3	3	70	99	0.1564	17	15.28
S180	S10	21	1	5	72	99	0.1646	18	14.5
S168	S166	20	2	4	67	93	0.1696	19	13.41
S168	S43	19	3	3	68	93	0.1745	19	12.92
S166	S95	23	4	3	69	99	0.1803	20	14.4
S115	S43	23	5	2	69	99	0.1801	20	14.44
S43	S180	20	5	2	72	99	0.1950	22	13.16
FGFR	S95	18	4	4	68	94	0.2277	26	11.05
S43	S125	20	5	4	70	99	0.2440	28	11.61
S166	S180	20	7	2	70	99	0.2432	28	11.72
S166	S90	20	7	3	69	99	0.2669	31	10.98
S43	S90	19	6	4	70	99	0.2748	32	10.52
S97	S95	21	7	4	65	97	0.2837	33	10.47
S43	S10	20	5	6	68	99	0.2904	34	10.27
S115	S90	20	8	3	68	99	0.2895	34	10.37
S168	S95	17	5	5	66	93	0.2867	34	9.18
S166	S125	20	7	4	68	99	0.2901	34	10.3
S97	S90	20	8	3	66	97	0.2931	35	10.2
FGFR	S180	16	6	4	68	94	0.3020	36	8.77
S115	S95	21	7	5	66	99	0.3053	36	10.03
FGFR	S10	16	6	5	67	94	0.3131	38	8.3
FGFR	S90	16	6	5	67	94	0.3272	40	8.19
S166	S10	20	7	6	66	99	0.3348	41	9.07
FGFR	S125	15	7	5	67	94	0.3516	43	7.27
S97	S180	18	10	3	66	97	0.3502	43	8.31
S168	S90	15	7	5	66	93	0.3594	45	7.17
S97	S10	19	9	5	64	97	0.3611	45	8.02
S115	S180	18	10	4	67	99	0.3727	47	7.93
S168	S10	15	7	6	65	93	0.3728	47	6.75
S168	S180	14	8	5	66	93	0.3952	50	6.27
S97	S125	18	10	5	64	97	0.3953	50	7.2
S95	S90	17	9	6	67	99	0.4062	52	6.97
S115	S10	19	9	7	64	99	0.4071	52	7.17
S168	S125	14	8	6	65	93	0.4132	53	5.84
S115	S125	18	10	6	65	99	0.4164	54	6.88
S180	S90	15	7	8	69	99	0.4313	56	6.17
S10	S90	16	10	7	66	99	0.4604	62	5.73
S125	S90	15	9	8	67	99	0.4742	64	5.35

Note. For each marker pair, + + indicates the number of hybrids containing markers A and B, + − indicates that marker A is present and marker B is absent, − + indicates that marker A is absent and marker B is present, and − − indicates that neither marker is present. θ denotes the estimated frequency of breakage, cR_{6500} is the estimated map distance, and the lod score is a measure of the likelihood that two markers are linked (Cox *et al.*, 1990).

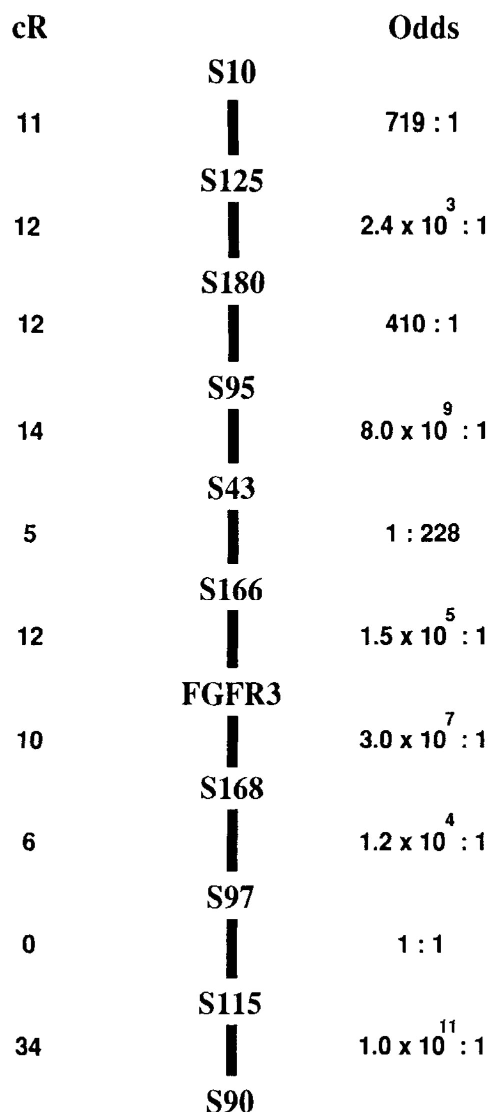

FIG. 1. Radiation hybrid map as determined by two-point and four-point methods. The distances between markers (**left**) are expressed in cR_{6500}. The order was determined as described in the text. The numbers on the right are the odds of the given order versus the likelihood of the order in which a given pair of markers is inverted. Four-point analysis was performed on each set of four markers, and the odds for any two middle markers was compared to the odds where the middle markers were inverted. The odds for the two most distal markers and the two most proximal markers were determined by using the four most distal and proximal markers, respectively, and inverting the position of the two markers at each end of the map.

DNA separating them based upon extensive long-range restriction maps of this region (Bucan *et al.*, 1990; Bates *et al.*, 1991). Figure 2 shows the distances between markers in kilobases and centirays using data from both the two-point and multipoint methods. Taking the average for each pair of adjacent loci, 1 cR corresponds to 32 kb of DNA using the two-point data or 27 kb of DNA using the multipoint data. In addition, as also shown in Fig. 2, the relative distances between markers, whether determined by PFGE, two-point analysis, or multipoint analysis, are within twofold of one another for most pairs of adjacent markers.

Two other points concerning the RH map of this region are worth noting. The retention frequency for the 11 loci in the RH hybrids varies only from 22 to 29% with no significant gradation from the most telomeric locus (D4S90) to the most centromeric (D4S10). Since D4S90 is only ~300 kb from the telomere of 4p, this result suggests that DNA fragments very near telomeres are no more or less likely to be retained in RH hybrids than the more proximal loci. In addition, the retention frequencies in this set of radiation hybrids for 13 loci on the long arm of chromosome 5 (most of which are tens of megabases from the telomere) are comparable (16–28%) to those observed for the distal 4p markers, further suggesting the lack of a "telomere" effect on retention of loci in radiation hybrids (Warrington *et al.*, 1991). Second, the region of greatly increased meiotic recombination, which is between D4S10 and D4S125, does not correlate with an increased likelihood of radiation-induced breakage. The genetic distance between these loci, which are less than 500 kb apart, is ~3.5 cM or close to 10 times the overall average for the human genome (Allitto *et al.*, 1991). However, the relationship between kilobases of DNA and the centiray distance between these loci using two-point data (43 kb/cR) is slightly higher than the overall average for the entire region (32 kb/cR), indicating a lower than average frequency of breakage. Using the multipoint data, the kilobase to centiray relationship between D4S10 and D4S125 is slightly lower (17 kb/cR) than the average for the region (27 kb/cR). However, this difference is nowhere near the 10-fold increased frequency of meiotic recombination.

Estimating the Size of a Gap in the PFGE Map of Distal 4p

There is currently one gap, of unknown size, in the long-range restriction map from D4S10 to the 4p telomere. The closest markers flanking this gap are D4S168 on the proximal side and D4S115 on the distal side (Bates *et al.*, 1991). The two-point centiray distance between these loci is 6 cR, which would correspond to 192 kb based upon the average of 32 kb/cR. Thus, the RH map would predict that the gap in the PFGE map is small. In fact, if there is no gap in the PFGE map, existing data indicate the distance between these loci to be 175 kb (G. Bates, personal communication). It is likely that a high density of very well cut restriction sites within this region have precluded closure of the PFGE map. Until very recently there was a second, more proximal gap in the long-range restriction map (between D4S95 and D4S180), which recently was closed and shown to be ~480 kb (Bates *et al.*, 1991). Before this distance was established, the centiray distance between these loci (12 cR) predicted the gap to be ~385 kb. In regions of the genome that have a very high frequency of sites for rare-cutting enzymes, like distal 4p, the compilation of a restriction map over several megabases can be very difficult. In regions such as this, an RH map can provide reasonable estimates of kilobase distances separating loci, assuming one has enough markers and PFGE mapping data to be able to establish a good correlation between RH map units and actual physical distance.

TABLE 3

Maximum Likelihood Locus Orders for the Multipoint Analysis

Locus order											Relative likelihood
	35	5	8	11	5	14	12	12	11	29	
S90	S115	S97	S168	FGFR3	S166	S43	S95	S180	S125	S10	1:1
S90	S115	S97	S168	FGFR3	S166	S43	S95	**S125**	**S10**	**S180**	30:1
S90	S115	S97	S168	FGFR3	S166	S43	S95	**S10**	**S125**	**S180**	32:1
S90	**FGFR3**	**S168**	**S115**	**S97**	S166	S43	S95	S180	S125	S10	75:1
S90	S115	S97	S168	FGFR3	S166	S43	S95	S180	**S10**	**S125**	88:1
S90	S115	S97	S168	FGFR3	S166	S43	**180**	**S95**	S125	S10	162:1
S90	S115	S97	S168	FGFR3	**S43**	**S166**	S95	S180	S125	S10	186:1
S90	S115	S97	S168	FGFR3	S166	S43	S95	**S125**	**S180**	S10	253:1

Note. Relative likelihood compares the maximum likelihood for the given locus order to that for the overall maximum likelihood order. Boldface numbers indicate inversions of two or more loci relative to the best locus order; boxed boldface numbers indicate more complex modifications. The order of S115 and S97 is arbitrary. Numbers listed above the best locus order are estimates of the distances between the loci (in centirays).

Reducing the Complexity of Human DNA in Radiation Hybrids by Refusion

In addition to providing physical mapping information, selected RH hybrids provide a simple means to cloning human DNA from small, specific regions of particular interest. In this case, the region of interest is between the two closest markers flanking the HD gene, D4S126 and D4S168. For example, one RH hybrid we characterized (XZH 147) is positive for loci from D4S168 to D4S95, but negative for other markers from 4p tested. If this were the only human DNA in the cell line, it would represent a very enriched source of most of the DNA in the HD region, which could be recovered either by interspersed-repeat sequence PCR (IRS–PCR) or construction of a genomic library. However, like the majority of RH hybrids we have characterized, XZH 147 was found to retain multiple (six to seven) fragments of human DNA, as determined by fluorescent *in situ* hybridization with total human DNA (Fig. 3A). Only one of the fragments is derived from the chromosome 4 portion of the derivative human chromosome in the parent (HHW 661) of the radiation hybrids, as determined by chromosome "painting" with a chromosome 4-specific plasmid library (Fig. 3B).

In an attempt to derive a cell line containing the fragment of distal 4p exclusively, the RH hybrid XZH 147 was refused (without irradiation) to a Chinese hamster ovary (CHO) cell line with two selectable genetic markers. One of the markers, which is recessive, results from a mutation in the gene encoding leucyl-tRNA synthetase, which renders the cell line nonviable at 39°C (Thompson *et al.*, 1973). The second marker, resistant to 1 m*M* ouabain, is a dominant selectable marker (Baker *et al.*, 1974). Secondary hybrids between the XZH 147 and the CHO cell lines were selected as described under Materials and Methods.

The rationale for this approach is as follows: The hybrids between the two cell lines are, for all practical purposes, intraspecific Chinese hamster × Chinese hamster cell hybrids, which are usually pseudotetraploid. That is, they retain most of the chromosomes from each parent but usually have a chromosome number 10–20% less than a true tetraploid karyotype, apparently because a few chromosomes from one or both parents segregate after fusion. If the chromosomes from the XZH parent with the human fragments are as likely to segregate after refusion as any other chromosomes, it should be possible to identify "secondary" hybrids in which, by chance, the chromosome with the human fragment of interest is present without the chromosomes containing the unwanted human fragments.

Sixteen independent hybrids between the two cell lines were isolated and tested immediately after cloning for the presence of two loci in the 4p segment retained in

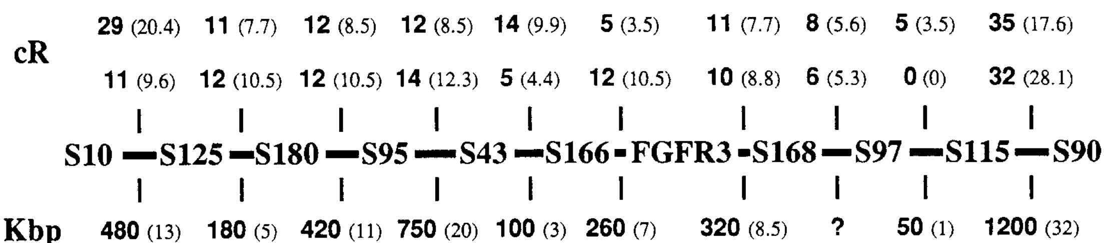

FIG. 2. Distances between markers as determined by PFGE, two-point analysis and multipoint analysis. The physical distance between adjacent markers in kb of DNA is shown below each pair of markers. The cR distance between markers, as determined by two-point analysis, is shown just above each pair of markers. The cR distances determined by multipoint analysis are the uppermost numbers. The number in parentheses represent the relative distances between adjacent markers as determined by the different methods. The distance between D4S10 and D4S90 was set at 100; the fraction of this that each interval represented was then calculated.

XZH 147 (D4S43 and D4S95). Ten of the 16 hybrids retained the 4p markers, and these were then tested by IRS–PCR to determine which contained the least amount of human DNA. Two of the 10 hybrids that had the least complex patterns of human fragments amplified by IRS–PCR were then examined by fluorescent *in situ* hybridization using total human DNA as a probe. One of these secondary hybrids contained only a single fragment of human DNA, as shown in Fig. 3C. Thus, the simple refusion technique, together with the PCR assays, represents an easy way to produce and characterize RH hybrids with single, defined segments of the human genome. DNA from one of the secondary hybrids has been used along with IRS–PCR to isolate several human DNA fragments, all of which map to the expected region of 4p. Although most of the DNA in the HD gene region has already been cloned, the technique of reducing the complexity of human DNA in RH hybrids via refusion provides a convenient means to developing cell hybrid reagents to allow selective cloning of human DNA from other regions of particular interest where very little cloned DNA is available.

DISCUSSION

The two different methods used to construct the RH map of distal 4p both arrived at the same order as being most likely. However, several other orders had likelihoods not much lower than the best. In constructing maps using RH hybrids, the two-point/four-point method becomes cumbersome with more than 10 or so markers, while the multipoint method can easily handle at least twice this number. However, it would seem important to analyze the data using both methods, since the two-point approach requires a more careful examination of the raw data.

One particular advantage to compiling an RH map of distal 4p is that it provided an easy and accurate means of equating centirays to real physical distances in kilobases of DNA, since an extensive PFGE map of this region exists (Bucan *et al.*, 1990; Bates *et al.*, 1991). This information was useful for estimating the size of an apparently small gap in the PFGE map. In addition, this same set of radiation hybrids is being used to compile a physical map of the long arm of chromosome 5 (5q) (Warrington *et al.*, 1991). Even though an extensive

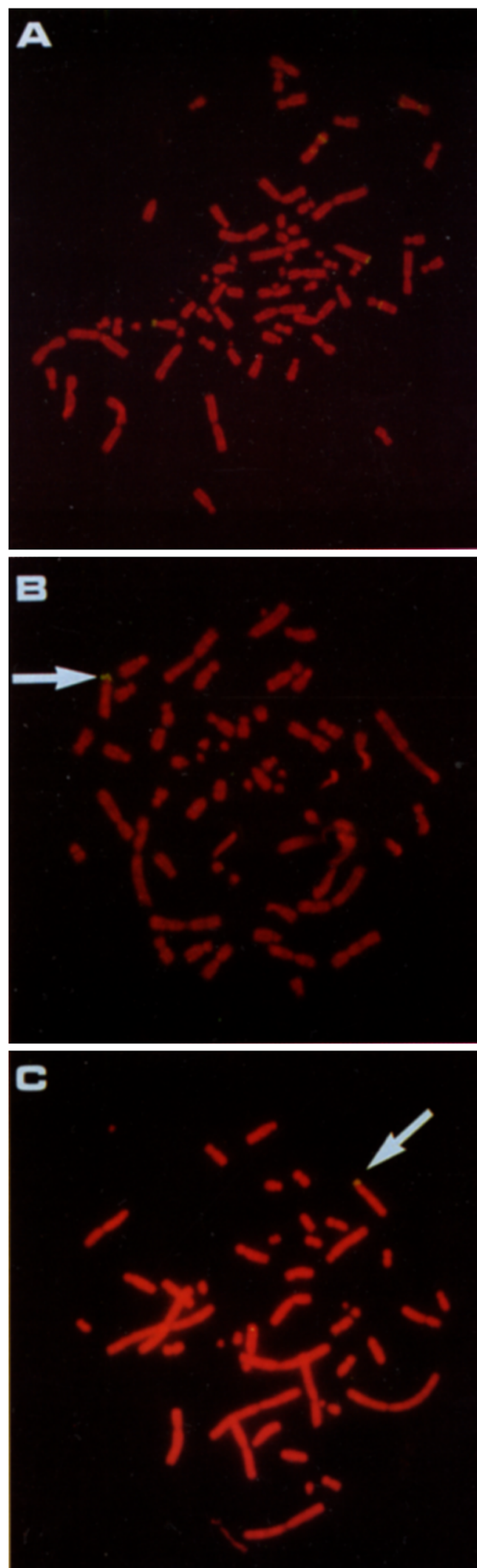

FIG. 3. *In situ* hybridization of biotinylated total human or chromosome 4-specific DNA to a radiation hybrid and a secondary refusion hybrid. (**A**) Metaphase chromosome preparations from radiation hybrid XZH 147 were hybridized with labeled total human DNA. Six-seven different fragments of human DNA are visible. (**B**) Metaphase chromosome preparations from XZH 147 were hybridized with labeled DNA prepared from a human chromosome 4-specific plasmid library (Collins *et al.*, 1991). Only one of the fragments in **A** shows hybridization to the chromosome 4-specific probe, and it appears to be telomeric. (**C**) Metaphase chromosome preparations from a secondary refusion hybrid (derived from XZH 147) hybridized with labeled total human DNA. Only one human DNA fragment is detected, and it appears to be the same telomeric fragment detected in XZH 147 with the chromosome 4-specific label, as in **B**.

PFGE map does not exist for any region of 5q, we can estimate the real physical distances between markers using the centiray to kilobase relationship determined for 4p. As the map of 5q becomes more refined, it will be interesting to determine whether the relationship between a centiray and a kilobase of DNA is consistent over most of the derivative chromosome 5 retained by the irradiated parent of these hybrids.

The refusion procedure described here for reducing the complexity of human DNA in radiation hybrids is applicable to any set of radiation hybrids for any chromosome. A large number of refusion, secondary hybrids can be screened with ease using PCR and just a few cells. Only those clones that are the best candidates for having the region of interest without any "extraneous" human DNA need to be analyzed by *in situ* hybridization. We have used the same technique for isolating refusion hybrids that contain single, small fragments of 5q, including one with just the region surrounding the gene for spinal muscular atrophy (Brzustowicz *et al.*, 1990; Melki *et al.*, 1990) and one with just the region surrounding the gene for Treacher Collins syndrome (Dixon *et al.*, 1991; Jabs *et al.*, 1991).

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