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Multicolor FISH Mapping of YAC Clones in 3p14 and Identification of a YAC Spanning both *FRA3B* and the t(3;8) Associated with Hereditary Renal Cell Carcinoma

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Human chromosome band 3p14 contains two tightly linked cytogenetic markers of broad interest, *FRA3B* and the t(3;8) breakpoint associated with hereditary renal cell carcinoma (RCC). The common fragile site at 3p14.2 (*FRA3B*) is the most sensitive site on normal human chromosomes to breakage when DNA replication is perturbed by aphidicolin or folate stress. The t(3;8)(p14.2;q24.1) translocation segregates with RCC in a large family and could mark the location of a tumor suppressor gene involved in renal cancers. In studies aimed at positional cloning of *FRA3B* and the t(3;8) breakpoint, we have used multicolor fluorescence *in situ* hybridization analysis (FISH) on metaphase spreads and interphase nuclei to order 14 yeast artificial chromosomes (YACs) in 3p14. The YACs used in this study were identified by a group of unordered lambda clones that had been previously localized to the 3p14 region and mapped proximal or distal to the t(3;8) breakpoint. FISH analysis was used to order the YACs and to map them in relation both to the t(3;8) translocation breakpoint and to *FRA3B* induced on normal chromosomes by treatment with aphidicolin. YACs that closely flanked both the t(3;8) translocation breakpoint and the fragile site were identified. A YAC walk from the closest distal YAC allowed the identification of a 1.3-Mb YAC derived from the CEPH large insert YAC library that spans both the *FRA3B* and the t(3;8) breakpoint. The order of the YACs and cytogenetic landmarks in 3p14 is cen–(126E1/230B9)–181H6–B15 – D20F4 – 258B7 – 280D2 – 70E12 – 168A8 – 403B2 – 143C5 – 413C6 – 468B10 – [850A6/t(3;8)/*FRA3B*] – 74B2. The location of *FRA3B* and the t(3;8) translocation within the same YAC supports previous cytogenetic studies indicating that these two sites are very closely linked and provides a resource for their molecular analyses. © 1994 Academic Press, Inc.

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INTRODUCTION

Chromosome fragile sites are loci that show gaps or breaks in the metaphase chromosomes of cells that have been grown in the presence of agents that perturb normal DNA replication (Sutherland *et al.*, 1985). The common or constitutive fragile sites represent an interesting and unknown component of chromosome structure. The induction of fragile site expression results in both a visible cytogenetic lesion and a high frequency of recombination events involving fragile sites (Glover and Stein, 1987, 1988), including high-frequency integration of foreign DNA (Rassool *et al.*, 1991; Smith *et al.*, 1992). The fact that common fragile sites are evolutionarily conserved (Miro *et al.*, 1987) and not generally associated with pathogenicity suggests that they play an essential but as yet undefined role in gene expression, chromosome structure, or DNA and cell replication. A model postulating that fragile sites are regions of late replication has been offered to explain their primary expression and recombinogenic characteristics (Laird *et al.*, 1987). Recent experimental evidence has confirmed that the *FMR1* gene from fragile X patients is hypermethylated and replicates later in the cell cycle compared to this same locus in normal controls (Hansen *et al.*, 1992, 1993).

Among the numerous common fragile sites in the human genome, the fragile site at 3p14.2 (*FRA3B*) is the most sensitive to treatment with the DNA polymerase inhibitor aphidicolin and to folate deprivation (Glover *et al.*, 1984). When blood lymphocyte cultures are treated with aphidicolin, up to 80% of metaphase spreads show a break at 3p14 in at least one of the chromosome 3 homologs. In an extensive study charac-

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terizing the specificity of aphidicolin-induced breaks in CHO/human hybrids containing chromosome 3 (Wang *et al.*, 1993), 78% of the breaks induced by 0.4 μM aphidicolin were confined to the 3p14.2 region.

The coincidence of fragile sites with the breakpoints of deletions and translocations observed in solid tumors has led to the suggestion that they may play a role in generating some of these genetic lesions (Yunis and Soreng, 1984). A large number of sporadic solid tumors show loss of heterozygosity, deletions and translocations in the vicinity of 3p14 and 3p21, or deletions of larger portions of 3p that include the 3p14 region (Hibi *et al.*, 1992; Jones and Nakamura, 1992; Kovacs *et al.*, 1987; Yamakawa *et al.*, 1991). Interestingly, a large family with inherited early onset renal cell carcinoma (RCC) and carrying a balanced translocation (t(3;8)(3p14.2;8q24.1)) at a site cytogenetically indistinguishable from *FRA3B* (Glover *et al.*, 1988) has been described (Cohen *et al.*, 1979). We have cloned and initially characterized a putative tumor suppressor gene located at the t(3;8) breakpoint (Boldog *et al.*, 1993). These data taken together suggest the existence of one or several tumor suppressor genes in the 3p14–3p21 region.

In this paper we report the production of a multicolor fluorescence *in situ* hybridization (FISH) map of YAC clones in 3p14 and the identification of a single YAC clone that spans both the common *FRA3B* fragile site and the t(3;8)(3p14.2;8q24.1) translocation associated with heritable RCC. The YAC map was a product of our efforts to clone and characterize the t(3;8) breakpoint and the *FRA3B* fragile site and should aid in further characterization of this region of the human genome.

MATERIALS AND METHODS

Cell culture and slide preparation. Growth of PHA-stimulated whole blood lymphocyte cultures was carried out in RPMI 1640 medium (Irvine Scientific) containing 10% fetal calf serum, 4 mM glutamine, and penicillin/streptomycin (200 Units/ml, 200 μg/ml). Fragile sites were induced with 0.4 μM aphidicolin for 26 h prior to harvesting (Glover *et al.*, 1984).

The immortalized t(3;8)(3p14.2;8q24.1) lymphoblastoid cell line, TL9542, has been previously described (Drabkin *et al.*, 1985) and was derived from a member of the family in which the translocation segregates with a greatly increased risk for RCC (Cohen *et al.*, 1979). Chromosome preparation was modified from Kuwano and Kajii (1991). Briefly, cultures were grown in RPMI 1640 medium as above, synchronized with 0.4 μM aphidicolin for 18 h, washed 2× in RPMI 1640, resuspended in normal growth medium lacking aphidicolin, and permitted to grow for a total of 6–7 h. Ethidium bromide (5 μg/ml) was added for the last 3 h and Colcemid (0.07 μg/ml) for the last 1.5 h of growth prior to harvesting.

B15 is a CHO/human hybrid cell line containing an aphidicolin-induced reciprocal translocation involving human chromosome 3 and a hamster chromosome (Glover and Stein, 1988). The translocation breakpoint was determined to be at or very near *FRA3B*.

A human fibroblast cell line (TC-4728) derived from a male with the fragile X syndrome was used to produce interphase nuclei for multicolor FISH mapping. Cells were cultured in DMEM (Irvine Scientific) supplemented with glutamine and penicillin/streptomycin as above. Cultures were grown to confluency and held for 4 days to enrich for G1 interphase nuclei (Trask *et al.*, 1991) prior to typsinization and harvesting. In all cases, cells were dropped on glass slides after a 20-min treatment in hypotonic medium (0.075 M KCl) and multiple changes of fixative (3:1 methanol:acetic acid).

Description of YACs. YACs were isolated from both the small and large insert CEPH YAC libraries (Albertsen *et al.*, 1990; Bellanne-Chantelot *et al.*, 1992) and from the Washington University YAC library (Brownstein *et al.*, 1989), all of which were prepared in the vector pYAC4 (Burke *et al.*, 1987). Most YACs were isolated at the University of Colorado using a Southern blot hybridization screening method (Mendez *et al.*, 1991) modified by cleavage of yeast DNA with *Eco*RI prior to electrophoretic separation. YAC 850A6 was isolated in the University of Michigan Genome Center from the megabase portion of the CEPH/Genethon YAC library (Bellanne-Chantelot *et al.*, 1992) by PCR screening using primers derived from the right end of YAC 189B12, a member of the YAC contig identified with marker R7K145 (Boldog *et al.*, 1993). The R7K145 marker was known to map in the small region between the t(3;8) translocation and a distal aphidicolin-induced break in the CHO/human hybrid line A5-4 (Boldog *et al.*, 1993; Glover and Stein, 1988).

In situ hybridization. Details of inter-*Alu* PCR with the CL1 and CL2 primers, probe labeling with biotin–14-dATP or digoxigenin–11-dUTP, probe hybridization, immunologic detection, fluorescence microscopy, photography, and digital image acquisition for single-color and multicolor FISH analysis have recently been published (Flejter *et al.*, 1993; Mercer *et al.*, 1993). These procedures were modified from Lengauer *et al.* (1992), Pinkel *et al.* (1986), Lemieux *et al.* (1992), Trask *et al.* (1991), and Ried *et al.* (1992).

Two-color FISH mapping on metaphase chromosomes was accomplished by simultaneously hybridizing the PCR-amplified products from two YACs, one labeled with biotin and the other with digoxigenin, to early metaphase spreads. Digoxigenin-labeled probes were detected with FITC-conjugated antibody and biotin-labeled probes with avidin–Texas red. After detection the resulting green and red signals were scored relative to each other and to the chromosome 3 centromere as proximal, distal, or even on individual chromosomes. In the three-color experiments, the PCR products of three YACs were simultaneously hybridized to G1 interphase nuclei. Three colors were produced by labeling one probe with biotin and the second with digoxigenin and mixing biotin and digoxigenin labeled products for the third (Flejter *et al.*, 1993). The detection protocol gave rise to easily distinguishable red, green, and yellow/orange signals, respectively, which were viewed through a triple-band pass filter (Omega). The order of the signals in a given nucleus was scored by direct visualization, and the results were tabulated. Only the published photographs were digitally captured and imaged (Flejter *et al.*, 1993). For both the two-color and three-color FISH experiments, the chromosomes or nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole). In the single-color FISH experiments, biotin-labeled probes were detected with avidin–FITC, and the chromosomes were counterstained with propidium iodide.

Statistical analysis of the mapping data follows that of Flejter *et al.* (1993) and Guo *et al.* (1994) and was performed on a Sun SPARC II workstation using the FISHMAP program written by S.-W.G. The program evaluates the Bayesian posterior probability that the most frequently observed locus order in two-color metaphase or three-color interphase experiments is the true locus order. In our analysis we demanded a minimum of 10 informative observations and a posterior probability of 0.90 or greater for the data to be incorporated into the map. The posterior probability of the map was calculated using the method of Guo *et al.* (1994), assuming unequal error probabilities.

RESULTS

Prior to the initiation of this study, a number of YACs had been isolated using lambda clones mapped by somatic cell hybrids to the immediate vicinity of the t(3;8)

breakpoint and *FRA3B*. It was concluded that FISH analysis would be the most efficient and rapid approach to order these YACs with respect to each other and to the two chromosomal landmarks and to identify YACs within this collection that either crossed the breakpoints or were in close proximity to it. Preliminary single-color FISH experiments were carried out using metaphases from three different cell lines, B15, TL9542, and normal female lymphocytes from blood cultures in which *FRA3B* was induced by aphidicolin treatment. The translocation breakpoints and fragile site in these cells subdivide 3p14 and allowed us to establish gross groupings of the YACs in this region (data not shown). These data confirmed that 74B2, a YAC corresponding to the lambda probe R7K145, which is distal to the t(3;8) break (Boldog *et al.,* 1993), is distal to both the B15 and the t(3;8) breaks. When 74B2 was used as a probe against normal aphidicolin-treated metaphase spreads, it most frequently hybridized to the distal edge, but sometimes fell within the *FRA3B* fragile site. The distal end of 74B2 was known to cross the aphidicolin-induced break in the CHO/human hybrid strain A5-4 (Boldog *et al.,* 1993). The other YACs were grouped as follows: 468B10, 403B2, 143C5, D20F4, 258B7, 168A8, 181H6, 230B9, and 126E1 were all proximal to the t(3;8) break. A subset of these clones tested against B15 showed that 143C5, D20F4, and 168A8 were distal to the B15 break, while members of the YAC contig associated with lambda 214, 181H6, 230B9, and 126E1 were proximal to the B15 break, thus showing that the B15 breakpoint is proximal to *FRA3B*.

Two-color metaphase mapping. Two-color metaphase FISH mapping was employed to determine YAC order within the larger groupings established by the initial FISH analysis described above. The results of two-color metaphase mapping are shown in Table 1 and summarized in Fig. 2. Representative FISH results are shown in Figs. 1a–1d. The data in Table 1 resolve the 12 probes tested into 6 groups (Fig. 2a). Three of the YAC groups contain clones whose relative positions could not be resolved by two-color metaphase mapping. For example, the position of the hybridization signals from YACs 403B2 and 143C5 could not be distinguished in the great majority of the metaphase spreads and therefore provide no ordering information. Similar results were obtained for the probe combinations 70E12 and 258B7, and 181H6 and 126E1 or 230B9. The other 16 probe combinations in Table 1, such as 70E12 and 230B9, gave clear ordering information with probabilities greater than 0.90. The order of the YACs most consistent with the data is centromere–126E1/230B9/181H6/D20F4/280D2 – 70E12/258B7 – 168A8 – 403B2/143C5–468B10–74B2–telomere, with the slashes indicating those YACs that are not resolved on metaphase spreads and the dashes separating resolved groups.

Three-color interphase mapping. Three-color mapping on interphase chromatin was employed to resolve YAC order within the groups identified by two-color analysis. The results are shown in Table 2 and summarized in Fig. 2. In addition to those YACs examined by metaphase analysis, clone 413C6, which is known to overlap with 468B10 (Boldog *et al.,* pers. commun.), was included in the three-color experiments. Representative results are shown in Figs. 1e–1f.

The data in Table 2 resolved several markers that were unresolved by the metaphase mapping experiments. Experiment 1 places 181H6 between 70E12 and 230B9, ordering the YACs of the lambda 214 contig. Since the positions of the YACs within this contig are known, experiment 1 also allows us to place 126E1 on the map in Figs. 2 and 3 as shown. Experiments 2 and 7 place D20F4 proximal to 258B7. Although D20F4 was not resolved from 181H6 in a mapping experiment, it was found to lie distal to the B15 break, whereas 181H6 is proximal, effectively resolving these two clones. Experiments 4 and 5, in conjunction with the 70E12 and 280D2 two-color result, place 70E12 distal to 258B7, with 280D2 in between them. Experiments 11, 12, and 13 all resolve 403B2 from 143C5 and taken together with experiments 15 and 16 confirm the order of 403B2, 143C5, 413C6, and 468B10. Combining the data from the metaphase and interphase mapping and including the positions of the B15 and t(3;8) translocation breaks and the newly isolated YAC clone 850A6 (see below) allows the construction of the consensus map shown in Fig. 2c. The posterior probability of the consensus map

TABLE 1

Two-Color FISH Metaphase Mapping in 3p14

Position of	Relative to	Number of Chromatids			Probability of correct order[a]
		Distal	Proximal	Even	
1 70E12[b]	181H6	31	0	32	1.000000
2 70E12	230B9	23	1	14	0.999999
3 70E12	D20F4	21	0	25	1.000000
4 181H6	126E1	2	0	41	0.875000
5 181H6	230B9	0	0	34	
6 70E12	280D2	21	12	35	0.939275
7 168A8	70E12	28	1	71	1.000000
8 70E12	258B7[c]	12	10	70	0.661180
9 168A8	258B7	30	1	36	1.000000
10 168A8	D20F4	40	1	32	1.000000
11 143C5	230B9	14	0	24	0.999969
12 143C5	70E12	15	1	25	0.999863
13 143C5	258B7	9	2	25	0.980713
14 143C5	168A8	16	1	23	0.999928
15 403B2	143C5	5	4	88	0.623047
16 403B2	258B7	55	0	70	1.000000
17 403B2	168A8	21	2	46	0.999982
18 468B10	143C5	14	2	40	0.998825
19 468B10	403B2	13	1	53	0.999512
20 74B2	468B10	14	1	30	0.999741

[a] The probability that the most frequently observed order is the true observed order.
[b] Chimeric with chromosome 1.
[c] Chimeric with both a C group and a D group chromosome.

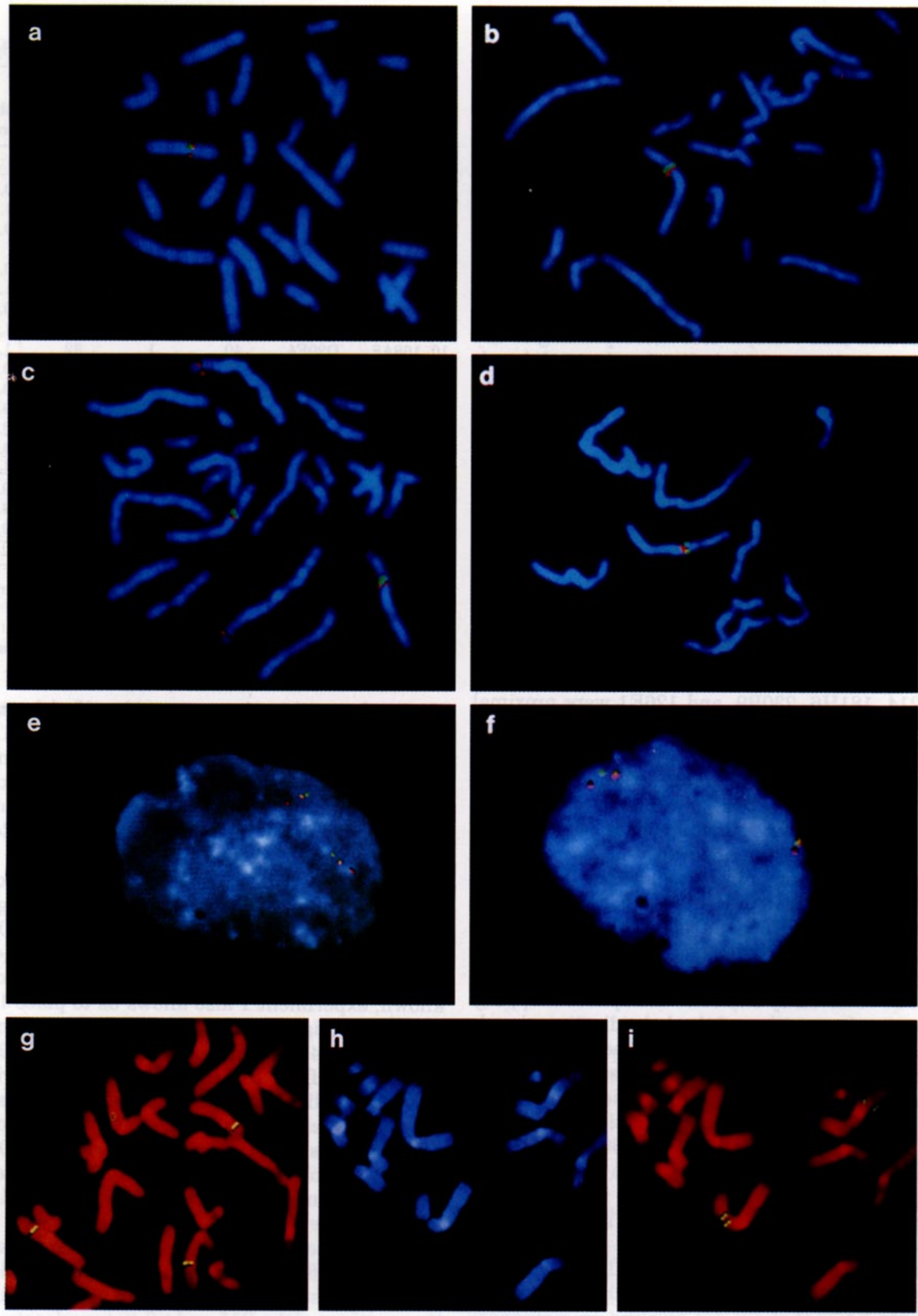

FIG. 1. FISH of YACs in 3p14. (**a–d**) Two-color metaphase mapping results. (**a**) 74B2 (red) is distal to 468B10 (green); (**b**) 468B10 (green) is distal to 403B2 (red); (**c**) 143C5 (green) is distal to 70E12 (red), and 70E12 is chimeric with chromosome 1; (**d**) 143C5 (green) is distal to 230B9 (red). (**e** and **f**) Three-color interphase mapping results. (**e**) 468B10 (orange) maps between 74B2 (red) and 403B2 (green); (**f**) 258B7 (green) maps between D20F4 (red) and 168A8 (orange). (**g–i**) Single-color FISH experiments demonstrating that YAC 850A6 spans both the t(3;8) breakpoint and *FRA3B*. (**g**) Metaphase with the t(3;8). Normal chromosome 3, lower left; der(3), upper right; der(8), lower middle. (**h** and **i**) metaphase expressing *FRA3B* (see text for details).

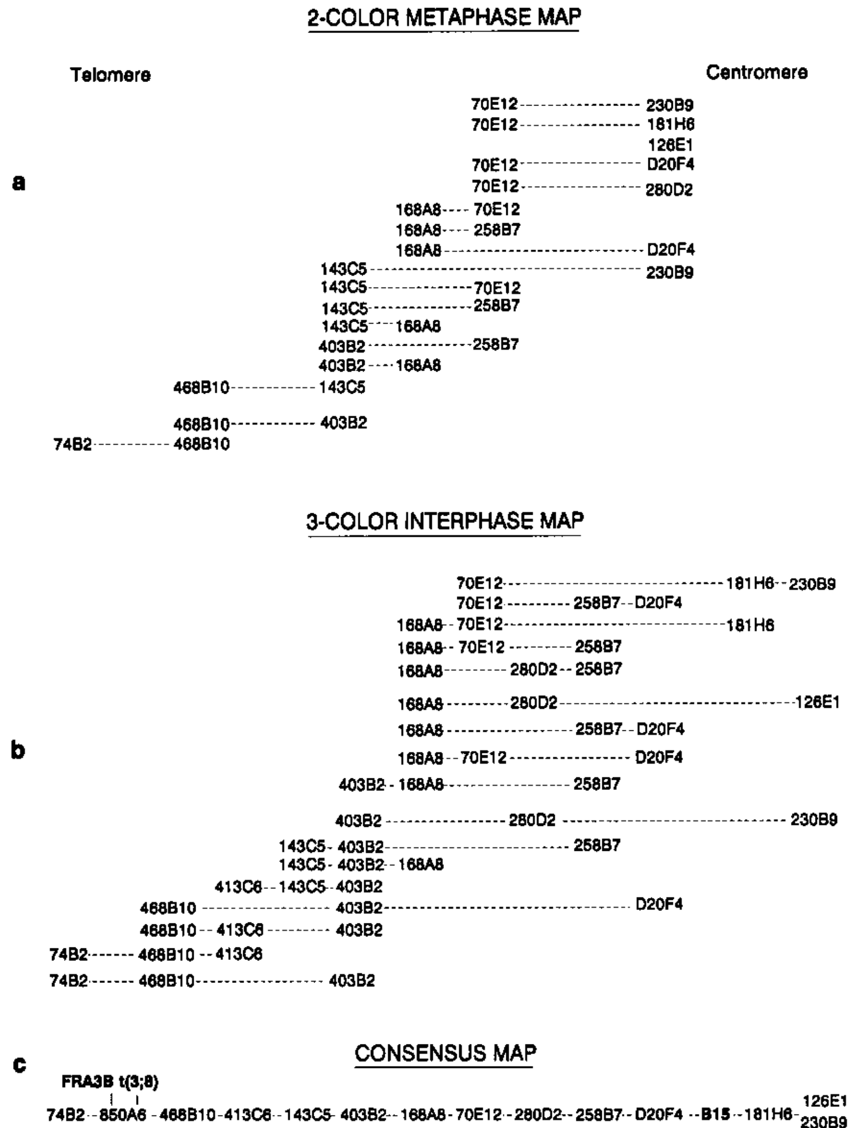

FIG. 2. Metaphase and interphase FISH mapping results and consensus map of YACs in 3p14. (**a**) A preliminary ordering of the YACs generated by the two-color FISH experiments. YACs that could not be resolved are positioned in six vertical columns. (**b**) The increased resolution of the closely linked YACs provided by the three-color interphase mapping experiments. (**c**) The composite map of the 3p14 YACs from the combined data of the metaphase and interphase FISH mapping, as well as FISH localizations relative to the B15 and t(3;8) translocations.

is 0.939 and was calculated by combining the data from metaphase (but excluding experiments 4, 5, 8, and 15) and interphase experiments and taking into account the fact that D20F4 is distal to 181H6. This map is superimposed on the 3p14 region in Fig. 3.

Identification of a YAC spanning both FRA3B and the t(3;8) translocation. Preliminary single-color FISH results involving metaphase chromosomes in which *FRA3B* had been induced suggested that 74B2 was very close, distally, to *FRA3B* and the t(3;8) break. End-clone PCR primers from YAC 189B12, which is partially collinear with 74B2, were used to screen the Genethon Megabase YAC library. The initial clone isolated was nonchimeric and 1.3 Mb in length. Figures 1g–1i show the results of single-color FISH analysis with 850A6 to metaphase spreads of TL9542 and normal male lymphocytes treated with aphidicolin to induce *FRA3B*. In Fig. 1g, 850A6 is seen to hybridize to the normal chromosome 3, the der(3) and der(8) chromosomes of the t(3;8) translocation. Figure 1h shows a DAPI-stained metaphase spread of aphidicolin-treated lymphocytes expressing *FRA3B,* and Fig. 1i shows the same spread with hybridization signals of 850A6 spanning the fragile site. An initial characterization of this YAC in the t(3;8) region has previously been described (Boldog *et al.,* 1993).

DISCUSSION

In this paper we report the use of FISH to order 14 YAC clones in 3p14 and to aid in the direct cloning

of the two tightly linked loci, the t(3;8) translocation breakpoint associated with hereditary RCC and *FRA3B*. This approach was successful because of the high concentration of DNA probes in 3p14 and the availability of complete human YAC libraries. The identification of a single YAC containing both the t(3;8) hereditary RCC translocation and the 3p14.2 fragile site provides the essential materials for determining the relationship between these two sites, as well as the relationship of *FRA3B* to the many aphidicolin-induced breaks that have been isolated in the 3p14 region (Glover and Stein, 1988; Wang *et al.*, 1993). Furthermore, it provides a valuable resource for isolation of genes and DNA sequences at the translocation breakpoint (Boldog *et al.*, 1993) and the fragile site region. The clustering of aphidicolin-induced breaks in hybrids and the chromosome abnormalities found in cancers involving the 3p14 region suggest that *FRA3B* may play a role in generating some of these recombination events. The fact that FRA3B and the t(3;8) breakpoint are contained within a single YAC confirms earlier predictions that these two cytogenetic entities are closely linked (Glover *et al.*, 1988). Preliminary FISH data using lambda subclones from 850A6 on aphidicolin-treated metaphase spreads show that FRA3B and the t(3;8) breakpoint are separated by less than 180 kb (Wilke *et al.*, 1993).

A detailed molecular characterization of *FRA3B* is an important first step leading to an understanding of the basis of its fragility. An understanding of *FRA3B*, the most sensitive common fragile site in the genome, is likely to lead to a more general understanding of common fragile sites. It will be interesting to compare

TABLE 2

Three-Color Interphase Mapping

Experiment and probe combinations (red/orange/green)	Observed color scheme			Probability of correct order[a]
	RGO	ROG	GRO	
1 70E12/181H6/230B9	7	50	12	1.000000
2 D20F4/258B7/70E12	8	36	3	0.999992
3 181H6/168A8/70E12	28	8	2	0.999623
4 70E12/258B7/168A8	13	15	90	1.000000
5 258B7/280D2/168A8	17	60	41	0.970300
6 168A8/280D2/126E1	2	30	3	1.000000
7 D20F4/168A8/258B7	30	2	2	1.000000
8 D20F4/168A8/70E12	40	13	3	0.999912
9 403B2/258B7/168A8	40	6	9	0.999997
10 403B2/280D2/230B9	13	40	2	0.999912
11 403B2/258B7/143C5	16	2	40	0.999368
12 403B2/143C5/168A8	10	14	80	1.000000
13 413C6/403B2/143C5	28	9	6	0.999115
14 468B10/403B2/D20F4	3	22	6	0.998801
15 468B10/413C6/403B2	2	35	9	0.999967
16 74B2/468B10/413C6	5	39	8	0.999998
17 74B2/468B10/403B2	3	33	1	1.000000

[a] The probability that the most frequently observed order is the true observed order.

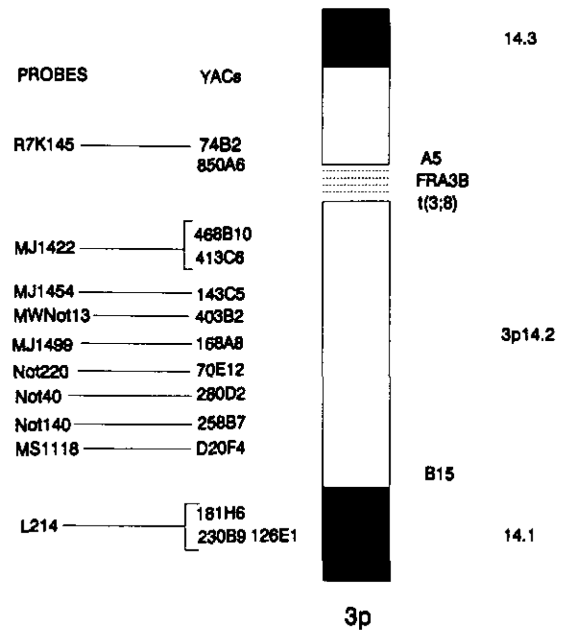

FIG. 3. A schematic representation of the YAC order in 3p14. The lambda probes used to isolate the YACs are shown on the left. Brackets indicate overlapping YAC clones.

the mechanisms responsible for *FRA3B* expression with those recently delineated for the *FRAXA* and *FRAXE* loci (Knight *et al.*, 1993; Verkerk *et al.*, 1991). Unlike these rare fragile sites, which involve the unstable expansion of trinucleotide repeats, the expression of *FRA3B* and other common fragile sites is constitutive and shows little variability between individuals (Tedeschi *et al.*, 1992). This suggests that the molecular basis for the expression of common fragile sites is equally constant and conserved and may therefore mark chromosome positions that encode essential functions for the cell.

The order of the YACs derived from this multicolor FISH analysis provides a detailed outline of 3p14 and is consistent with a detailed physical map of the region (Boldog *et al.*, pers. commun.). This information will be integrated with the extensive mapping data that have accumulated for chromosome 3 (e.g., Smith *et al.*, 1989; Drabkin *et al.*, 1990; Haas *et al.*, 1993; LaForgia *et al.*, 1993). It will also be useful in developing informative genetic markers and in determining the extent of involvement of 3p14 in tumorigenesis.

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