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A 1.5-megabase yeast artificial chromosome contig from human chromosome 10q11.2 connecting three genetic loci (*RET*, *D10S94*, and *D10S102*) closely linked to the *MEN2A* locus

(multiple endocrine neoplasia type 2/genomic walking/yeast artificial chromosome clones/microsatellite polymorphisms)

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ABSTRACT **The genetic loci *RET*, *D10S94*, and *D10S102* from human chromosome 10q11.2 are very closely linked to a locus responsible for the multiple endocrine neoplasia type 2 (MEN2A and MEN2B) and medullary thyroid carcinoma (MTC1) familial cancer syndromes. We have constructed a 1.5-megabase contig consisting of six genomic yeast artificial chromosome clones which include these loci and define their physical order. A critical crossover event has been identified within the map interval; this event places the *MEN2A* locus centromeric to *D10S102* and defines the orientation of the physical map on the chromosome. The orientation of the contig and order of the markers are centromere–*RET*–*D10S94*–*D10S102*–telomere. In addition, a microsatellite repeat polymorphism with a heterozygosity of 71% at the *RET* locus and a restriction fragment length polymorphism with a heterozygosity of 42% detected by a λ clone from the *D10S94* locus have been developed for high-resolution genetic linkage mapping and predictive diagnostic testing. These data place three important markers on a contiguous physical map, narrow the MEN2 disease locus interval, and provide a framework for further candidate gene identification efforts. Placement of these genetic loci along a clone-based map and continued expansion of the contig will also facilitate efforts to determine the relationship of physical to genetic distance near the centromeres of human chromosomes.**

Multiple endocrine neoplasia type 2A (MEN2A) is a dominantly inherited cancer syndrome consisting of medullary thyroid carcinoma, pheochromocytomas, and parathyroid hyperplasia. Genetic linkage studies have assigned the *MEN2A* locus to the centromere region of chromosome 10 in the interval between *RBP3* (10q11.2) and *D10S34* (10p11.2) (1–4). The mutation(s) causing the related multiple endocrine neoplasia type 2B (MEN2B) and familial medullary thyroid carcinoma (MTC1) syndromes have also been assigned to this region by similar linkage studies (5–7), thus raising the possibility that the three phenotypes could arise from mutations either in a single gene or in several closely linked genes.

Although a variety of restriction fragment length polymorphism (RFLP) markers have been mapped within the *RBP3*–*D10S34* interval, estimated to span approximately 12 centimorgans (cM), no recombinants have yet been reported between the *MEN2A* locus and at least six loci, which include *D10S94* (8), *D10S97* (9), *D10S102* (10), the *ret* protooncogene (*RET*) (11, 12), the centromere marker *D10Z1* (4, 13), and *D10S176* (14). Therefore, in the absence of more highly informative markers and/or larger pedigree resources, it appears that meiotic mapping has reached a limit for resolution of the disease locus. Isolation of genes that lie within this interval by positional cloning strategies will provide candidate genes for MEN2A, MEN2B, and MTC1 and reveal whether these phenotypes are caused by mutations in a single gene or multiple genes.

We have utilized a number of entry points, including *RBP3*, *D10S102*, *D10S94*, *RET*, the *ZNF22*/*Kox15* zinc finger gene locus (15), *D10Z1*, and *D10S176* to identify yeast artificial chromosome (YAC) clones from the interval for genomic walking and physical mapping. Our group, like others in the field, has focused its efforts on proximal 10q11.2 as the most likely position for the *MEN2A* gene because a number of markers from this region have not been observed to recombine with the disease locus. Although a single recombinant has been described which supports placement of the *MEN2B* locus on the long arm of chromosome 10 (6), it has not been clearly established where the *MEN2A* gene maps with respect to the centromere.

The RFLP locus D10S94, detected by probe (pC11/A1S-6-23), maps in proximal 10q11.2 between *RBP3* and the centromere marker *D10Z1* and is closely linked to the *MEN2A* mutation, with no recombinants yet observed (8). A 160 kb-bidirectional cosmid walk from *D10S94* has been reported (16), revealing a clustering of CpG islands at this locus and a putative gene (mcs94-1) (17). However, sequence analysis of mcs94-1 exons from an *MEN2A* mutation chromosome and its wild-type homologue did not reveal differences between the two alleles, suggesting that it is unlikely to be the gene responsible for MEN2A.

The *ret* protooncogene, which also maps to 10q11.2 (11), is a candidate gene for MEN2A. Preliminary investigation of the *ret* protooncogene for germ-line chromosomal rearrangements in patients with MEN2 by conventional Southern blot and pulsed-field gel electrophoresis (PFGE) analysis has not revealed evidence of alterations, nor have allelic losses been detected in MEN2 tumor DNAs (12). Linkage of *RET* to the *MEN2A* locus has been demonstrated (18), but the RFLP systems detected by probes at this locus have limited informativeness. The *ret* protooncogene is activated in some papillary thyroid carcinomas by a gene rearrangement leading to the substitution of the 5′ end of *ret* with a distant sequence. The oncogenic papillary thyroid carcinoma (PTC) sequence results from the juxtaposition of this region with the 3′ tyrosine kinase domain of the *ret* protooncogene (19).

Abbreviations: MEN, multiple endocrine neoplasia; MTC, medullary thyroid carcinoma; RFLP, restriction fragment length polymorphism; YAC, yeast artificial chromosome; PFGE, pulsed-field gel electrophoresis; Mb, megabase; STS, sequence-tagged site; SSRP, simple sequence repeat polymorphism; CEPH, Centre d'Etude du Polymorphisme Humain.

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Furthermore, the *ret* protooncogene is abundantly expressed in human pheochromocytomas and medullary thyroid carcinomas (20).

Several zinc finger protein genes, including the *ZNF22/Kox15* locus, have been physically mapped to 10q11.2 (15, 21) but have not been genetically mapped with respect to other loci. Zinc finger proteins, which bind to DNA and often act as regulatory elements for gene expression, have been implicated in the development of several inherited disorders such as Greig syndrome (22) and Wilms tumor (23, 24), suggesting their possible importance in the development of the MEN type 2 syndromes.

The genetic locus defined by the RFLP probe MEN203 (*D10S102*) also maps to 10q11.2 and is tightly linked to the *MEN2A* locus. A 360-kb cosmid contig and a physical map of a 950-kb YAC contig have been developed at this locus (25). Previous to this report, no recombinants have been reported between *MEN2A* and *D10S102*.

We have considerably extended the genomic map at *D10S94* and physically connected two other genetic loci (*D10S102* and *RET*) to it within a 1.5-megabase (Mb) YAC contig. The identification of a critical crossover event within this interval establishes *D10S102* (MEN203) as a new flanking marker, narrowing the *MEN2* disease gene interval, and allows orientation of the contig with respect to the centromere and telomere of the chromosome. We also describe two new genetic markers, a simple sequence repeat polymorphism (SSRP) at the *RET* locus and a new RFLP at the *D10S94* locus.

MATERIALS AND METHODS

YAC Library Screening. YAC libraries from the Center for Genetics in Medicine (26) (Washington University, St. Louis) or the Centre d'Etude du Polymorphisme Humain (27) (CEPH, Paris) were screened by using a PCR-based method (28). The PCR amplifications were performed from 33–50 ng of template DNA in a reaction volume of 5 μl including (for the *D10S94* PCR assay) 50 mM KCl, 10 mM Tris·HCl at pH 8.3, and 2.5 mM $MgCl_2$ or (for the other PCR assays) 50 mM KCl, 10 mM Tris·HCl at pH 8.3, 1.5 mM $MgCl_2$, 5 mM NH_4Cl, and 200 μM for each dNTP, 10 pmol of each primer, and 0.5 unit of *Taq* DNA polymerase (AmpliTaq, Perkin–Elmer). The reactions were carried out for 30–35 cycles of 94°C for 1 min, 58–63°C for 2 min, and 72°C for 2 min in a Perkin–Elmer thermal cycler. The final step in screening was a filter colony hybridization using the PCR product (uniformly labeled with [α-^{32}P]dCTP during the PCR amplification) as the probe.

Oligonucleotide primers amplifying a 165-bp product at *D10S94* (5′-AGCCAAAAGAGGGTCTTC-3′, 5′-CAGAGAACGGATCTGAGG-3′) were synthesized from sequence information obtained from the plasmid probe at the locus. Oligonucleotide primers amplifying a 122-bp product at *RET* (5′-ATCAGGGCCAGCATTTTTC-3′, 5′-ACCTGGTGTATGAAATTGGACC-3′) were chosen from the published sequence of the *ret* protooncogene (GenBank accession no. M57464). The MEN203 STS-1 and STS-2 primer sequences at *D10S102* were previously described (25).

Identification of YAC Terminal Insert Sequences and STS Assay Development. The terminal sequences of the human insert of each YAC were obtained by a ligation-mediated PCR strategy described elsewhere (29). Briefly, yeast genomic/YAC DNA was digested with a variety of blunt-cutting restriction enzymes and then ligated to the restriction fragments with a specially designed linker (30). The terminal insert sequences were then specifically amplified by PCR using a vector-derived primer and a linker-derived primer and were further purified by dilution and reamplification using a nested vector-derived primer. Sequencing of the products was performed directly in low-melting-temperature agarose by using a modified dideoxynucleotide sequence method with a ^{32}P end-labeled primer and *Taq* DNA polymerase temperature-cycled reactions (31).

Sequence-tagged sites (STSs) were developed from the end-fragment sequences by using oligonucleotide primers chosen with the aid of the computer program PRIMER (provided by E. Lander, Whitehead Institute). The STSs for each YAC end-fragment and the primer sequences that define them are as follows: yWME31-R (5′-TAAGGTGAAGCAGGAACTCG-3′, 5′-TGTAGTGTAGCCCCCACTCC-3′), yWME31-L (5′-GATGGAAGTGAAGAGCCTGC-3′, 5′-TCACCTGTGTGAGGTAGGCA-3′), yCME1-R (5′-GAGAGAAACCACAAGACACTGG-3′, 5′-CAGCTGTTAACTCTTCCTTGGT-3′), yCME1-L (5′-TATTCCACTGTCTCTTGGCTCA-3′, 5′-AACCCGAAGAATCTCAAGCA-3′), yWME28-R (5′-CATGCCTATTGTGCAGCAAC-3′, 5′-TACCCTTCACCATCCCACAT-3′), yWME28-L (5′-ACCTTTGCCAGCTTGACTTT-3′, 5′-AGGGCATGGTTTAGTCAACTG-3′), yWME34-R (5′-GCCCAGCCCGATATTATTTT-3′, 5′-CCAAGATACCACATGGTCATAGC-3′), yWME34-L (5′-GCCTCTGCTAGCTTTTGAATG-3′, 5′-TCTGGGACACATTCAAAGCA-3′), yWME36-R (5′-TTCATAGGGTTGCTCCCTTG-3′, 5′-ACATCAGAGAACCCACACAGG-3′), yWME36-L (5′-GTGGCCCAAAGCACATAATT-3′, 5′-GAGTTTGGCACATAATAGTTGCC-3′), and yCME2-R (5′-TTGCTGATGAGGAAACTGAGG-3′, 5′-CCCAACACCTCCACTCTGAT-3′). An STS assay was not developed for the left end of yCME2.

The chromosomal origins of the end-fragment sequences were determined by amplification of the STSs against genomic DNAs from a rodent/human somatic cell hybrid panel including cell lines available from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ) and the human chromosome-10-containing somatic cell hybrid line R342-A4 obtained from C. Jones (Eleanor Roosevelt Institute for Cancer Research, Denver).

Long-Range Restriction Mapping of YAC Clones. High molecular weight yeast genomic/YAC DNA was prepared from yeast cells embedded in agarose plugs or beads and digested with rare-cutting restriction endonucleases according to the conditions specified by the manufacturer (Boehringer Mannheim). PFGE was performed through 1% agarose gels in 0.5× TBE buffer (1× TBE = 90 mM Tris borate, pH 8.3/2 mM EDTA) at 200 V and 14°C, using a Bio-Rad CHEF-DR II apparatus. Phage λ DNA concatamers were used as molecular size markers. The size-fractionated DNAs were depurinated by treatment of the gels with 0.25 M HCl for 10 min followed by neutralization in 0.5 M NaOH and 1.5 M NaCl and alkaline transfer in 0.25 M NaOH and 1.5 M NaCl to a nylon membrane (MagnaGraph, Micron Separations, Inc., Westboro, MA). Hybridizations were carried out in 30% (vol/vol) formamide/10% dextran sulfate/1 M NaCl/100 mM Tris·HCl, pH 7.5, at 50°C overnight. Washes were performed to a stringency of 0.1× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0) and 0.2% SDS at 65°C.

DNA Probes and Genetic Marker Characterization. Clones were provided for the loci *D10S94* [plasmid clone pc11/A1S-6-c23 from P. J. Goodfellow; University of British Columbia, Vancouver (8)] and *D10S102* [cosmid MEN203 from Y. Nakamura; Cancer Institute, Tokyo (10, 25)] and the *ret* protooncogene locus [genomic and cDNA λ clones from G. M. Cooper; Harvard Medical School, Boston (32, 33)].

λ subclone libraries were constructed from YAC clones and tested for RFLPs by Southern hybridization. Polymorphisms identified were further characterized by genotyping using the CEPH reference pedigree collection.

The simple sequence repeat element at the *RET* locus, sTCL-2, was PCR amplified by using primers flanking the repeat (A, 5′-CCAGACTCTCAAAGACCAGG-3′; and B,

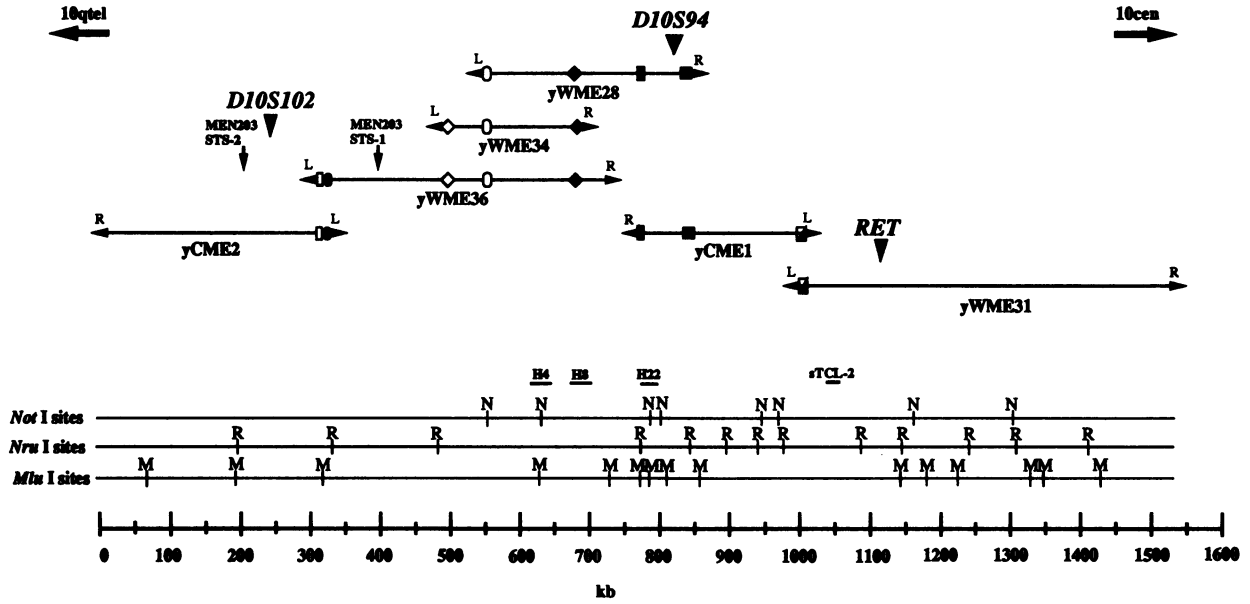

FIG. 1. Physical map of the contig of YAC clones from 10q11.2. The six overlapping YAC clones spanning 1.5 Mb of DNA are shown, with the orientation of the map on the chromosome indicated above. The localizations of the various end-fragment STSs within each of the YACs are indicated by the open or solid symbols. L and R indicate the left and right YAC vector arms. The positions of the *D10S102*, *D10S94*, and *ret* protooncogene loci are indicated by ▼. The RFLP locus identified by MEN203 (*D10S102*) is between the two STSs (STS-1 and STS-2). The restriction sites for *Not* I (N), *Nru* I (R), and *Mlu* I (M) that are present in the cloned YAC DNA are indicated at the bottom. The map positions of some DNA probes used for restriction mapping (H4, H8, and H22) and the SSRP marker sTCL-2 are also shown.

5′-CATAATACTGGCCTATAG-3′) from 50 ng of genomic DNA in a reaction volume of 5 μl including 50 mM KCl, 10 mM Tris·HCl at pH 8.3, 1.5 mM $MgCl_2$, 5 mM NH_4Cl, 200 μM each dNTP, 0.625 pmol of end-labeled primer B, 1.8 pmol of unlabeled primer B, and 2 pmol of unlabeled primer A for 30–35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min.

Two-point genetic linkage analyses were performed by using the program package LINKAGE (34) and the *MEN2A* pedigree resource (14).

EXPERIMENTAL RESULTS

YAC Library Screening, End-Fragment Characterization, and Contig Assembly. We developed an STS from the *D10S94* locus and used it to identify and isolate clones from two independent recombinant human YAC libraries (Washington University, yWME clones; CEPH, yCME clones). To develop new STSs for contig assembly and further chromosome walking, the terminal sequence of the human insert of each YAC was determined from vector-insert junction sequencing by a ligation-mediated PCR strategy (29, 31). Because of the relatively high frequency of chimeric clones in YAC libraries, we first determined the chromosomal origin of each YAC end fragment by PCR amplification from a panel of somatic cell hybrid DNAs to identify YACs derived entirely from chromosome 10. Although a number of YAC clones were isolated during the library screenings, efforts were focused on characterization of the minimum set of nonchimeric clones representing the maximum distance walk for each round.

A map of the YAC contig constructed during the walk is shown in Fig. 1. Two nonchimeric clones, yWME28 (345 kb) and yCME1 (275 kb), served as the nucleus for contig building and mapping of the *D10S94* region. Two end-fragment STSs and the primary screening STS from *D10S94* were found to be shared between these YACs; the remaining two end-fragment STSs were then used to rescreen the libraries. Two rounds of walking were performed in one direction. Screening with the STS from the left end of yWME28 resulted in the identification of two nonchimeric clones, yWME34 (245 kb) and yWME36 (450 kb), and subsequent screening with the STS from the left end of yWME36 resulted in the identification of yCME2 (350 kb). A single round of walking was performed in the opposite direction, using the STS at the right end of yWME28 (as well as the original *D10S94* assay in a separate library), resulting in the identification of yCME1 (275 kb). The five overlapping YAC clones isolated in this manner form a contig covering approximately 1 Mb of DNA.

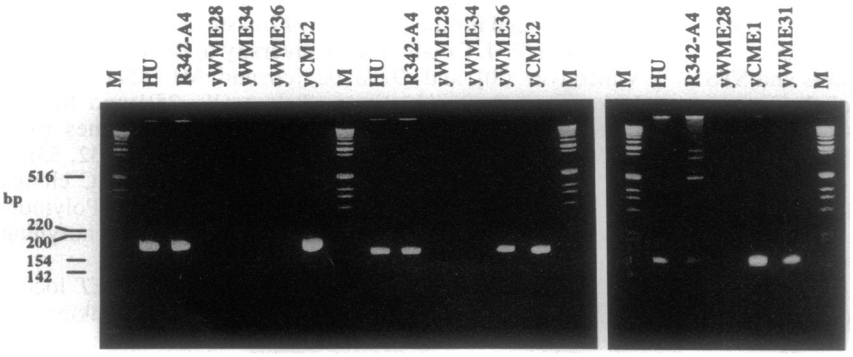

FIG. 2. STS content mapping of YAC clones. Polyacrylamide gel electrophoresis of products from the MEN203 STS-2 (*A*), yWME36-L (*B*), and yCME1-L (*C*) STS assays tested against positive controls and selected YAC clones is shown. The templates utilized are total human genomic DNA (HU), DNA from a chromosome 10-specific somatic cell hybrid (R342-A4), and DNA from the individual YAC clones indicated. M, molecular length markers.

Table 1. Characteristics of two genetic markers

Marker	Locus	Type	Alleles	Het., %	PIC	IM	vs. MEN2A Z_{max}	vs. MEN2A θ
sTCL-2	*RET*	SSRP	8	71	0.66	524	33.30	0.00
H8/*Pvu* II	*D10S94*	RFLP	2	42	0.33	246	12.0	0.00

Het., heterozygosity; PIC, polymorphism information content; IM, informative meioses; Z_{max}, maximum logarithm of odds (lod) score; q, recombination fraction.

To test for physical continuity of the contig with other markers from 10q11.2, STS assays from *D10S102, RET, D10Z1*, and *ZNF22* (including STSs derived from the end fragments of YACs at these loci) were tested against the YACs forming the *D10S94* contig. Conversely, the most distal end-fragment STSs from the contig were tested against the YAC clones from each of these loci. Examples of the STS content mapping experiments are shown in Fig. 2. Physical overlap of the contig was detected with two of the three loci tested (Figs. 1 and 2). The STS defining the most rightward end fragment of the *D10S94* contig (yCME1 left end fragment, see Fig. 1) was found to be present on a 560-kb YAC (yWME31) isolated independently by using a specific STS assay developed from the *ret* protooncogene sequence, extending the total YAC contig to over 1.5 Mb in size. In addition, two STSs from *D10S102* (25), which are separated by approximately 200 kb, were found to be contained within YAC clones from the opposite end of the contig (STS-1 mapped to yWME36 and STS-2 mapped to yCME2). Confirmatory evidence for physical continuity in each case was provided by the demonstration that the expected end fragments were mutually present on overlapping YACs and by Southern blot analysis of DNA from each YAC clone digested with *Eco*RI (data not shown). A number of YACs were isolated and characterized during the mapping experiments, but only the minimum six clones are depicted in Fig. 1. Except for the case of a single overlap between yCME1 and yWME31 (which may extend less than 50 kb), the contig is more than two YACs deep throughout the contig. Similar mapping experiments using STSs from the *ZNF22/Kox15* zinc finger protein locus (in 10q11.2) (15) and the *D10Z1* locus (at 10p11–10q11) (35) did not reveal evidence of overlap with this 1.5-Mb contig.

Long-Range Restriction Map of the 1.5-Mb Contig. Southern blots of YAC restriction fragments size-fractionated by PFGE were probed with radiolabeled PCR products specific for the right and left YAC vector arms, and with λ subclones from the YACs (including H4 and H22) previously found to contain *Not* I restriction sites. The positions of the *Not* I, *Nru* I, and *Mlu* I restriction sites in the YAC DNA are indicated in Fig. 1. The proposed orientation of the physical map with respect to the centromere and telomere of the chromosome is indicated at the top of the figure.

New Genetic Markers and Identification of a Critical Crossover. A $(CA)_{14}GA(CA)_3$ dinucleotide repeat element was identified near the left end of YAC clone yWME31, a region adjacent to the *RET* locus (Fig. 1). A PCR assay using primers flanking the repeat element (sTCL-2) was developed, and a highly polymorphic locus was detected (heterozygosity 71%) which substantially improves the informativeness of the *RET* locus for linkage mapping of the *MEN2A* gene region. Two-point linkage data in six large *MEN2A* kindreds using the sTCL-2 marker confirm very close linkage to the *MEN2A* mutation with a maximum lod score (Z_{max}) of 33.3 at $\theta = 0.00$ (Table 1). In addition, cosmid and λ subclones from the YACs were tested for RFLPs, and a two-allele *Pvu* II polymorphism was identified (Table 1).

Using these newly developed markers, we identified an important crossover chromosome in an individual with MEN2A. A well-characterized recombination event between *D10S102* and *D10S94* is depicted in Fig. 3. The phases of the markers in the affected grandfather are supported by haplotype analysis of the remainder of the pedigree. To minimize the possibility of sample mix-up, new Southern blots of the relevant pedigree members were prepared and rehybridized, and the same genotype data were obtained. In addition, the relevant individuals have been genotyped with a great number of markers without evidence of noninheritance. The affected status of each recombinant individual was established by pathologic evidence of MTC. This crossover is, to our knowledge, the first reported between *D10S102* and the disease locus, supporting the placement of *MEN2A* centromeric to *D10S102*. Based on these data, the orientation of the physical map and order of the markers are cen–*RET*–*D10S94*–*D10S102*–tel (Fig. 1).

DISCUSSION

The 1.5-Mb YAC contig connects three important genetic loci (*D10S102, D10S94*, and *RET*) which are closely linked to the *MEN2A* locus. The map defines the physical distance between the loci and uniquely places the *ret* protooncogene, the precise genetic or physical position of which was previously unknown. The *D10S102* (MEN203) and *D10S94* loci are separated on the physical map by a distance of approximately 600 kb, while *D10S94* and the *ret* protooncogene locus are separated by approximately 300 kb. The order of the markers and the orientation of the physical map (cen–*RET*–*D10S94*–*D10S102*–tel) on chromosome 10q are provided by a combination of the physical and meiotic mapping data reported. The proposed order is also consistent with the order of the centromere marker *D10Z1, D10S94*, and *D10S102* determined by genetic linkage analysis and hybrid panel mapping (36, 37). However, the placement of *RET* centromeric to *D10S94* by our data is in discrepancy with the order proposed by Miller *et al.* (37). These authors reported a deletion hybrid which retains the centromere marker *D10Z1* as well as *D10S94* but does not contain *RET*. It is possible that a

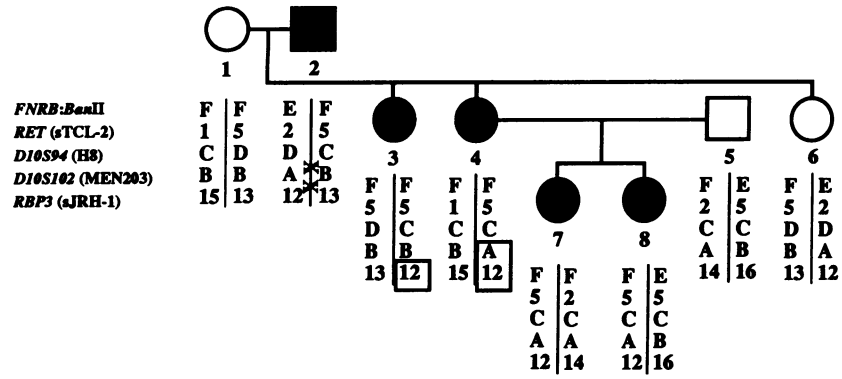

FIG. 3. Crossover chromosomes in a section of an MEN2A disease pedigree. Shown are two recombinant chromosomes (open boxes) which originated during meiosis of the affected male individual 2. Individual 3 is recombinant for *RBP3*. More importantly, a crossover between *D10S94* (H8) and *D10S102* (MEN203) is evident in the affected individual 4, and the new disease haplotype is transmitted to the affected children in the subsequent generation. (Affected individuals are indicated by solid shading; the position of each meiotic recombination event is indicated in the parent by an X, with the new crossover chromosome appearing in the offspring.)

rearrangement or deletion of small fragments within this hybrid accounts for the absence of the *ret* protooncogene sequence. We are not aware of any reported crossovers between *RET* and *D10S94*, nor have we observed recombination between these markers in six large *MEN2A* kindreds and the CEPH reference pedigrees.

The YAC contig and physical map reported here provide an important framework for further efforts to identify the gene(s) responsible for the MEN type 2 syndromes. However, further genomic PFGE mapping is needed to establish which rare-cutting restriction sites are methylated in genomic DNA and to verify that the YAC DNA does not contain deletions. Probes developed from these YAC clones may be used to search for allelic losses in tumor DNA or to search for genomic chromosomal rearrangements detectable by conventional Southern blot or PFGE analysis. In addition, new highly informative genetic markers present within the cloned DNA may be used for high-resolution genetic linkage mapping in the *MEN2A* gene region. Two such genetic markers, including a highly informative dinucleotide repeat marker (sTCL-2) between *RET* and *D10S94* reported in this study, have allowed the identification of a critical recombination event between *RBP3, D10S102* and *MEN2A, D10S94, RET*. This recombination event, while recognized to be a single observation, is, as far as we know, the only reported crossover between *MEN2A* and *D10S102* (MEN203) and supports the localization of the *MEN2A* locus centromeric to *D10S102*.

Because no recombination events have yet been reported between the *MEN2A* locus and either *D10S94* or *RET*, the contiguous 1.5-Mb segment of DNA contained within the YAC contig represents a valuable resource in which to conduct a systematic search for candidate gene(s) causing these familial cancer syndromes. Recently, new strategies have been described for the identification of transcriptional units within large regions of genomic DNA, including functional strategies such as exon trapping (38, 39) and hybridization-based strategies such as direct selection (40, 41). Further efforts to identify transcribed sequences from this region should be facilitated by these methods.

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