Localization of the Gene for MEN 2A

Jay B. Lichter,* Michael Difilippantonio,* Jingshi Wu,† Diane Miller,‡ David C. Ward,* Paul J. Goodfellow,‡ and Kenneth K. Kidd*

The search for the gene that causes the multiple endocrine neoplasia type 2A (MEN 2A) syndrome is entering a new phase. Genetic linkage studies have localized the gene to the pericentromeric region of chromosome 10. The statistical portion of mapping the gene for MEN 2A is nearly complete and now classical molecular biological/gene mapping techniques will be employed. We have used fluorescence in situ hybridization to estimate the size of the MEN2A region to be about 2 to 5 mb, using some liberal assumptions; at worst the region should contain no more than about 10 mb of non-alphoid DNA. Our mapping panels (meiotic recombinant and radiation reduced hybrid) give consistent orders of markers in this small region. We describe our initial attempts to clone the region using yeast artificial chromosomes. (Henry Ford Hosp Med J 1992;40:199-204)

The positional cloning approach has been extremely successful in recent years in identifying and cloning several genes causing inherited disorders. In such studies genetic linkage provides the initial regional position of the disease gene, followed by molecular work to identify and clone the specific gene. Duchenne muscular dystrophy was an early success aided by numerous deletion mutants (1); cystic fibrosis was another success aided by linkage disequilibrium and a biochemical defect in a specific tissue (2-4); and polyposis coli is a recent success aided by a rare deletion mutant (5,6). The hope/expectation is that multiple endocrine neoplasia type 2A (MEN 2A) will soon join this list of success stories, but there are also cautionary notes sounded by the difficulties in identifying and cloning the gene for Huntington disease (7,8), since deletions have not been found and linkage disequilibrium does not provide much help in localization. The work on MEN 2A is now focused largely on molecular efforts to localize and clone the gene; that search is concentrated in a small region of chromosome 10 that has been defined by the genetic linkage studies that have been done over the last decade. We wish to review briefly those initial studies that localized MEN2A to the pericentromeric region of chromosome 10 as an introduction to our current efforts to narrow the region even further, clone the region, and identify the gene.

Regional Localization of MEN2A

At the first MEN 2A workshop in Kingston, Ontario, the initial linkage studies with DNA markers were presented (9) but little of the genome had been studied, much less excluded. The initial positive linkage findings for MEN 2A occurred at the second workshop with the chromosome 10 marker D10S5; by that time, one-third of the genome had been excluded (10). The initial LOD score was not significant (less than 3) but more extensive typing confirmed the positive finding. A second marker, RBP3, documented to be near the centromere of chromosome 10, was fortuitously closer to the MEN2A locus, allowing conclusive evidence for the location of MEN2A near the centromere of chromosome 10 (11,12). Initially, there was no linkage map for chromosome 10 and few markers. The demonstration that RBP3 was linked to MEN2A and to D10S5 constituted the first map of part of chromosome 10. Our progressive building of the chromosome 10 linkage map since these initial findings is illustrated in Fig 1.

Initially, the orientation of RBP3 and D10S5 (and hence the orientation of MEN2A) along the chromosome was unknown but the order became clear when we mapped three markers, D10S3, D10S1, and D10S4, distal to D10S5 (13). We subsequently added four other markers to the linkage map of chromosome 10: D10S20 (14), D10S24 (15), the OAT locus (16), and EGR2 (17). Others were also working on mapping chromosome 10 (18-20) and in 1990 the first CEPH consortium map was published (21). That map, however, had no markers closer to MEN2A than RBP3. At the same time, several polymorphisms were identified for a clone for the beta subunit of the human fibronectin receptor, FNRB (22), and the FNRB locus became our closest flanking short arm marker, about the same distance from MEN2A that RBP3 was. Another short arm marker, D10S34, had been characterized by Nakamura et al (19). This marker also appeared to be roughly the same distance away from RBP3 as FNRB and, therefore, a close flanking marker on the short arm of chromosome 10. We identified several new polymorphisms closer to MEN2A at the centromeric alpha satellite locus D10Z1

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*Department of Genetics, Yale University, New Haven, CT.
†Harbor/UCLA Medical Center, Torrence, CA.
‡Department of Medical Genetics, University of British Columbia, Vancouver, BC.
Address correspondence to Dr. Kidd, Department of Genetics, Yale University School of Medicine, I-311 SHM, 333 Cedar Street, New Haven, CT 06510.

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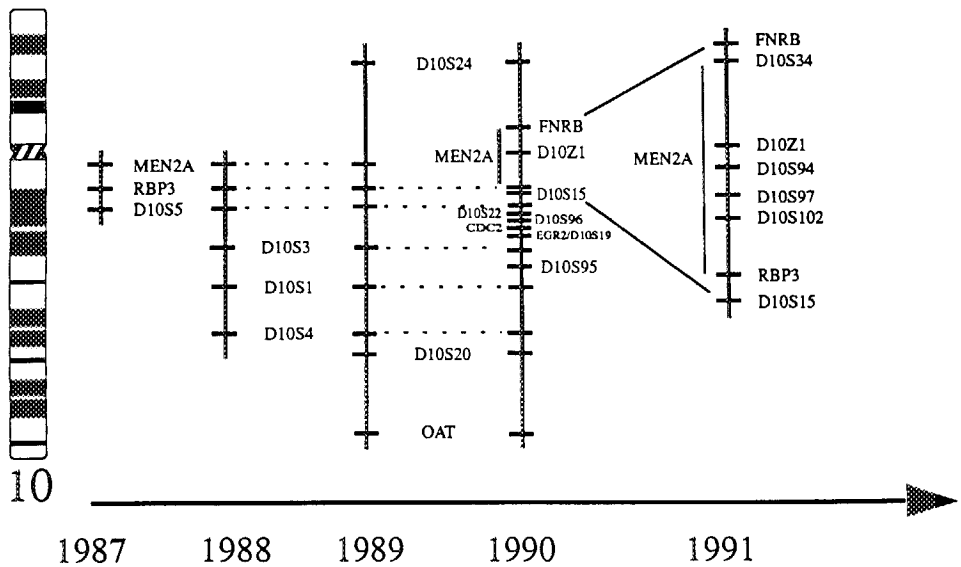

Fig 1—Progressive building of the chromosome 10 linkage map. Vertical lines are roughly proportional representations of the linkage maps. Horizontal lines mark the position of the loci in the map. The MEN2A region is labeled. The arrow at the bottom shows the rough time scale.

of chromosome 10 (23,24). We showed MEN2A to be completely linked to D10Z1 in our sample and definitely flanked by FNRB on the proximal short arm and RBP3 on the proximal long arm (25,26).

These markers now make DNA-based diagnosis feasible. Data on FNRB, RBP3, and D10S15 allowed us to obtain probabilities greater than 95% for diagnosis in 77% of patients and probabilities greater than 90% for a diagnosis in 90% of patients (27). This study showed that difficulties in the clinical diagnostic criteria for MEN 2A lead to difficulties in the genetic diagnoses: we feel that C-cell hyperplasia alone, in the absence of a clear breach of the basal membrane, is not sufficient to conclude that a patient has MEN 2A (27).

The position of the short arm marker D10S34 with respect to FNRB has not previously been determined. Our recent analysis (27A) shows D10S34 is proximal to FNRB. The LOD scores between FNRB and D10S34 are a maximum at $\theta_{m=f} = 2.4\%$, LOD = 34.19, with no significant sex difference observed in our data. We have found four meiotic recombination events between FNRB and D10S34 that support the order of FNRB, D10S34, D10Z1. This order is in agreement with preliminary data from other groups (28) and shows that D10S34 is about 2.4 cM closer to D10Z1 than FNRB, making D10S34 our most proximal polymorphic short arm marker definitely flanking MEN2A.

Fine Structure Linkage Mapping of the Region

The same logic of identifying crossovers to order markers has been applied to MEN2A. We have identified seven crossovers between MEN2A and various nearby markers. One of those crossovers is one of the four mentioned above that occurs between D10S34 and FNRB. This panel of seven meioses will continue to be instrumental in locating the gene to the smallest possible region of DNA that cosegregates with the disease, greatly reducing the molecular size of the region from the full D10S34-RBP3 interval currently being studied. (The one confirmed crossover between D10Z1 and MEN2B [29,30] already narrows the region to the proximal long arm if we assume MEN 2A is caused by the same locus as MEN 2B or another extremely close one; while plausible, it is presently merely an assumption.)

In a similar fashion, we have identified several recombinant chromosomes that are not informative for MEN2A. These come from unaffected individuals in both MEN 2A kindreds and in other non-MEN 2A kindreds. These crossovers allow us to use a larger meiotic mapping panel for ordering markers in this region. A subset of these crossovers was used to determine the order of FNRB and D10S34. We have refined the positions of 36 crossovers in our family material and there are additional crossovers in the CEPH families that we have not yet studied. Our crossovers divide the region from FNRB to D10S15 into 37 regions for high resolution meiotic mapping.

Three markers that have so far not been seen to be separated from MEN2A by crossovers have been mapped using the meiotic mapping panel (27A). Each of these markers (D10S94, D10S97, D10S102) has already been shown to map into the region between D10Z1 and RBP3 (31-33); however, the relative order of these loci was not known. Data from the meiotic mapping panel suggest the order of these loci to be (D10Z1, D10S94)-D10S97-D10S102-RBP3. Each interval is defined by at least two crossovers except the D10Z1,D10S94 interval, in which no recombinants have been detected. Although every effort is made to insure that the typing data are free of errors, errors cannot be absolutely ruled out for any particular crossover. Moreover, some "crossovers" represent only the most likely interpretation of the data; other interpretations may be possible. Thus, until this order can be confirmed either by a large number of crossovers or by physical cloning, we consider this order the most likely but not the definitive ordering.

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More Polymorphisms

The meiotic mapping panel is only as valuable as the number of informative polymorphic loci that have been mapped in it. We would ultimately like to have at least one locus in each of the 37 intervals from FNRB to D10S15. However, an immediate goal is to make the 20 crossovers that are definitely between D10S34 and RBP3 fully informative for a series of loci that will be spaced at about 1 mb intervals. Several new clones are being typed by standard restriction fragment length polymorphism technology for segregation in these meiotic mapping panels. However, this technology may be limited in the extent of informativeness of any given locus.

Several new techniques based on the polymerase chain reaction (PCR) (33,34) are capable of detecting more informative polymorphisms. The short tandem repeats (STR) or microsatellites (35) are blocks of di-, tri-, or tetranucleotides (sometimes more) with the most common being repeating dinucleotides. Estimates of the number of STR loci in human DNA range from about 35,000 to 130,000 (36) for CA repeats alone and Edwards and associates (37) estimate a like number for the total of the various tri- and tetranucleotides, making STRs one of the most abundant sources of polymorphisms. With over 200 of these repeats having already been identified as being polymorphic, it is rapidly becoming possible to efficiently scan the genome as well as chromosome 10 using these markers (35).

Both single stranded conformational polymorphisms (SSCP) and denaturing gradient gel electrophoresis (DGGE) are extremely sensitive in detecting polymorphisms in the genome, but because of the technical difficulty of these techniques, it is not generally practical to use them to generate LOD scores from several hundred individuals. Furthermore, our interest now is making each recombination event fully informative for evenly spaced loci, so a complete statistical analysis is unnecessary. The use of SSCP and DGGE on the donors of recombinant chromosomes is quite feasible. We have developed a way to streamline this technique further. We have developed "family" tubes to rapidly test our meiotic panel for informativeness of particular PCR products using DGGE. Each tube contains pooled DNA from five individuals who are meiotic recombinant donors; we amplify the pooled DNA for a segment of DNA within the region of interest. The resulting product is subjected to DGGE. We can detect most DNA variation in this fashion and will be able to scan the entire meiotic panel on one single gel. The sensitivity of this technique has been fully explored and the approach used successfully in our population studies (unpublished data). Our studies in the meiotic mapping panel have so far not yielded any new polymorphisms but large amounts of newly cloned material and DNA sequence are just becoming available, as yeast artificial chromosome (YAC) cloning gains momentum.

Other Methods Used to Order Markers into a Region

As mentioned above, the statistical portion of the linkage phase of mapping MEN2A is reaching its limits. Current focus is on the refinement of the linkage map and detecting and mapping crossovers. Although we are continuing to exploit the meiotic mapping panel, the majority of current work involves the molecular characterization and actual cloning of the region. While the meiotic mapping panel will continue to be a valuable and powerful tool for ordering clones from the region, other, more molecular mapping techniques will also be invaluable in the search for MEN2A by independently confirming the order of loci mapped using the meiotic mapping panel.

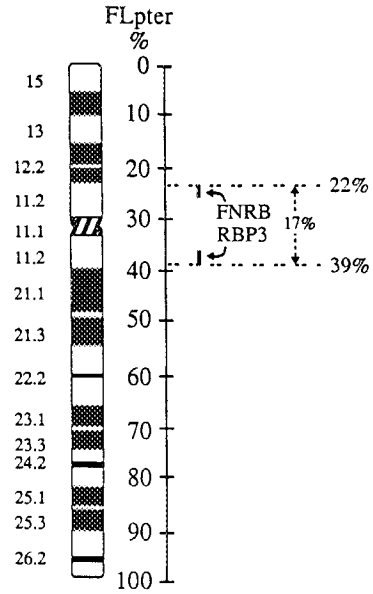

Fig 2—Cytogenetic limits of the MEN2A region. FLpter % is the percent fractional length from the end of the p arm of the chromosome. The FNRB and RBP3 bars represent the error in measurement using FISH. Dotted lines are drawn at the outer limits of the region defined by FNRB and RBP3.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH), as the name suggests, utilizes fluorescent-labeled DNA molecules as hybridization probes on human metaphase chromosomes (38). This technique is rapid and highly specific. The positions of probes along the chromosome are measured as the fractional length of the chromosome from the tip of the short arm. Although the resolution of this technique is limited to about 1 mb, it can be useful to determine whether a new marker falls into the region of interest. We have mapped 37 chromosome 10 clones using FISH on metaphase spreads. Several of these clones are "anchor" markers that are also in the linkage map (39), but most of these probes are new clones. We have found three new clones that map in the pericentromeric region using this technique.

We are using our FISH results for the flanking markers D10S34 and RBP3 to estimate the amount of DNA in the MEN2A region. Our current estimation for the maximum size of the region is 17% of chromosome 10 using the most distal FISH locations for D10S34 and RBP3 (Fig 2) on metaphase chromosomes. Chromosome 10 has been estimated to contain 4.4% of total genomic DNA. If we assume that mitotic chromosome length is proportional to the length of DNA, this region contains

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about 22 mb of DNA ($3 \times 10^9 \times 4.4\% \times 17\% = 2.2 \times 10^7$ base pairs). We believe we can use additional clones in combination with our MEN2A meiotic mapping panel to reduce the region cosegregating with MEN2A to less than 10% of the entire chromosome. (Assuming a random, uniform distribution of crossovers in the region, this is a conservative estimate. However, our data suggest crossovers occur more frequently as one moves away from the centromere and closer to D10S34 or to RBP3.) Thus, the meiotically defined MEN2A region will be about 10 to 15 mb of DNA ($3 \times 10^9 \times 4.4\% \times 10\% = 13 \times 10^6$ base pairs). Of this, 2 to 5 mb will be alpha satellite sequences (40). Therefore, we expect the entire region cosegregating with MEN2A to be not more than 10 mb of nonalphoid DNA. If we further assume that MEN2A is on the proximal long arm of chromosome 10 using the MEN2B crossover data (29,30), then the region is only about 5% of chromosome 10 or about 2 to 5 mb of DNA. This estimate of the cytogenetic limits of the MEN2A region suggests that the region is now small enough to consider complete cloning.

Radiation reduced hybrid mapping

Another approach to mapping and ordering probes in a small region is the use of radiation reduced hybrids. These hybrid cell lines are developed by radiation treatment of somatic cell hybrid lines in which the chromosome of interest is the only human material, in this case human chromosome 10. The hybrids are scored by Southern blot hybridization of chromosome specific probes or by PCR using specific primers. A positive hybridization signal or a PCR product of the right size and sequence indicates that a hybrid line contains that target sequence. These hybrid lines tend to accumulate complex rearrangements and deletions that can make interpretations difficult. One can use either a least number of breaks hypothesis or a likelihood analysis to make a radiation hybrid panel powerful for ordering probes (41).

We have recently used a radiation reduced hybrid panel with several of our chromosome 10 probes (41A). The order of probes that we have determined for the pericentromeric region of chromosome 10 using the radiation reduced hybrid panel agrees with the order determined by the meiotic mapping panel: D10Z1-D10S94-D10S97-D10S102-RBP3 (41A). These two independent methods for determining the order of loci complement each other to help refine the map of the entire region. However, neither method is infallible. The radiation hybrid and the meiotic mapping techniques both rely on determining the most likely map based on the data. The most likely map might not be the actual map.

Contig mapping—cloning the region

The ultimate test of order is the complete cloning of the part of the region from D10S34 to RBP3 that cosegregates with MEN2A. Once the region is completely cloned with an ordered set of overlapping clones, each "new" clone can be precisely mapped with the "exact" number of base pairs known between several loci. Given the currently estimated size of the region as 10 to 15 mb (including the centromere), standard plasmid, phage, or cosmid clones would not be an efficient means of cloning the region. To cover 2 to 5 mb with cosmids, with an average insert size of 35 kb, would take a minimum of 150 clones if they were minimally overlapping; in reality at least 500 clones would have to be studied.

YACs offer an excellent source of clones with large inserts. We have begun a YAC screening effort with the CEPH YAC library (42). An average YAC in this library contains human DNA inserts of about 400 kb. Using this size insert reduces the number of independent clones needed to cover the entire region by a factor of about 10 relative to cosmids. Fifteen to 50 clones is more feasible than 150 to 500. We are using a PCR-based screening approach using sequence-tagged sites for clones that we have isolated and mapped to the region. We hope to be able to establish that the region is only about 2 to 5 mb of DNA.

We first attempted to find YACs for the D10S97 locus (32,43) using sequence for the clone KW6. That clone detects two loci on chromosome 10 by FISH, and we believe that the clone itself derives from the middle of the short arm (32). The polymorphism, mapped by the linkage and radiation hybrid panels, exists in 10q11.2 sequences recognized by cross hybridization. We have found three YACs for the probe KW6 (CEPH YAC numbers 27B11, 185E2, and 261B8). Unfortunately, but not unexpectedly, these YACs map by FISH to the 10p locus (D10F38S2) that is detected by the probe KW6 (32,43). These YACs, although not in the region, show that the PCR-based screening method for the YAC library is feasible. Though KW6 is a known special case of "duplicated" segments of homologous sequences, the result illustrates the need to confirm that YACs map into the region and are not isolated because of some shared homologous sequence elsewhere in the genome. We are now working to identify YACs at D10S94.

Cloning the MEN2A Gene

The ultimate question is how will the gene for MEN2A actually be cloned? The cloning and mapping of the entire region will be relatively easy, compared to the task of actually finding the gene. Small deletions or rearrangements in the DNA of affected individuals will point towards candidate regions. Linkage disequilibrium may be helpful in identifying a candidate region. However, except within families, linkage disequilibrium will only exist if there were relatively few MEN2A mutations. In the absence of either of these guides, systematic testing of each gene in the region for point mutations is the only current recourse. The now "classical" approach of positional cloning has been successful in the cloning of several disease-causing genes (4,6,44). We expect that this strategy will also lead to the identification of the gene responsible for MEN2A.

Summary

We now have excellent tools for the mapping and cloning of the MEN2A region. The mapping tools have their individual strengths and serve as cross-checks for each other. Furthermore,

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we have the means necessary to clone the region using YACs derived from the CEPH YAC library. Complete cloning of the region will provide the DNA in which other techniques can be used to identify the gene responsible for MEN 2A. In the meantime, a by-product of this work will be new, highly informative markers that will not only help the research by refining the limits of the relevant region using the mapping panels but also increase the diagnostic capabilities of the DNA-based tests.

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