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The mapping of the locus for multiple endocrine neoplasia type 2A (MEN 2A) to chromosome 10 using linkage is briefly reviewed including a discussion of linkage strategy and reference to some of the exclusions before the assignment. The subsequent development of the map of the centromeric region of the chromosome and the linking of what appear to be the four closest flanking markers and the centromeric alphoid sequence to the disease locus are reviewed. To date no recombination has been observed between the centromeric marker and the MEN2A locus among, at least, 26 informative meioses, 11 of which are phase known. While no obligate recombination has been observed between the markers FNRB, D10S34, and RBP3 and the MEN2A locus in males, it has been observed in females and is as much as 10% for the marker RBP3. The sex difference in recombination frequency is significant. The four polymorphic flanking markers, FNRB, D10S34, RBP3, and D10S5, along with the centromeric marker D10Z1 will prove to be useful for management of the families with the disease. It will be possible in most families to give a very high (or low) probability for "at risk" members of the families and in some cases the DNA results will be virtually diagnostic. (Henry Ford Hosp Med J 1989;37:100-5)

The relation between the apparent allele for multiple endocrine neoplasia type 2A (MEN 2A) and the development of the three characteristic tumors, medullary thyroid carcinoma (MTC), pheochromocytoma, and parathyroid adenomas, is completely unknown. The understanding of diseases that have a clear mendelian inheritance suggesting that a single allele is responsible for the disease, at least in a given family, has been the focus of human genetic research since Mendel's theory was rediscovered at the turn of the century. Many diseases usually inherited in a recessive fashion are the result of the lack of or an inactive enzyme coded for by the mutation of a normal allele and often are recognized by the excess of enzyme substrate. The diseases are referred to as inborn errors of metabolism (1). The understanding of the biochemistry of these diseases has led to cures or prevention of their clinical manifestations. The etiology of some, but not all, of the known recessive diseases has been solved in this manner. The identification of the protein product of dominant autosomal alleles responsible for human diseases, however, has not been as successful. Because the individual diseases are relatively rare, it can be assumed that those individuals with the disease are heterozygous, ie, they have one allele for the disease and one for the normal protein product, and although a reduction in an enzyme or protein might be recognized, it is usually more difficult than discovering an absolute lack of an enzyme when no normal allele is present as in the case of the recessive diseases. One strategy for dealing with this problem, possible now that the DNA code is understood and molecular techniques have been developed, is first to find the gene by linkage and then use reverse genetics to isolate the gene and thereby clone it and find the protein product. As MEN 2A is apparently dominantly inherited, the linkage approach seemed a suitable method toward understanding how a mutation could determine this particular cancer syndrome.

Prevalence

Although MEN 2A is considered rare among all cancers, it is probably not as rare as was thought in the 1960s and 1970s after it was first described by Sipple (2). When we became interested in the disease in the mid 1970s, we knew of only two families with MEN 2A in Canada. Since the disease locus has been assigned to a chromosome and thereby received considerable publicity, we have become aware of at least 16 families in Canada. In countries in which active case finding has occurred (Great Britain, France, and Holland), an even greater increase in the number of families has been noted. Dr. W. J. Simpson (personal communication, 1989) at the Princess Margaret Hospital in Toronto estimates that 25% of patients with MTC have the MEN 2A syndrome and thus a family history of this disease.

Linkage Strategy

There are two important prerequisites for linkage studies of diseases. The first is the availability of several large families in

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which the disease occurs. Our studies began with two Canadian families in which MEN 2A was segregating (3-5) and soon included an additional Canadian (6,7) and three large American families (8-10). A second prerequisite is variant forms of the protein coded by a gene or of a DNA sequence even when its protein product is unknown; the latter are known as anonymous sequences and both are known as "markers." The markers are usually not associated with disease, and the variants need to be common to be useful for linkage studies. They are more useful if their location on the chromosome is known. Some clue is desirable but not always available.

In the last decade, particularly in the past five years, the number of useful markers has increased exponentially (11). The use of recombinant DNA techniques and the Southern blot (12) for the detection of common DNA variation or restriction fragment length polymorphisms (RFLPs) and the recognition that RFLPs could be treated as alleles and used as landmarks on the map by Botstein et al (13) are responsible for this increase. RFLPs have become a powerful tool for the detection of linkages between each other and between their loci and those of disease alleles that code for unknown protein products. Technology has now made it possible for the generation of a large data base of markers, ie, landmarks on the genetic map (11). The linkages, if close, can provide a starting point for reverse genetics which begins with the mapping of the disease allele to a small region of a chromosome through linkage to loci that can be physically mapped. At this stage much more satisfactory risks compared to mendelian risks can be given to members of families with genetic diseases. The precise location of the disease allele follows from short walks and the use of denaturing gradient gel electrophoresis. Once the gene is found it can be isolated, cloned, and sequenced. Identification of the disease mutation(s) and the development of allele specific oligonucleotide probes for direct diagnosis from DNA derived from a small blood sample will often be possible. For example, direct diagnosis is now possible for the Duchenne and Becker muscular dystrophies. The gene has been isolated and the mutations studied (14-16) which has led to the identification of the product dystrophin (17) that can now be studied for the ultimate understanding of the disease pathology.

Linkages are detected by studies of the transmission of the variants of the markers from parents to their children. To recognize a linkage it must be possible to identify at least two forms or variants at each locus. As an example, we shall designate as alleles A1,A2 at the first and B1,B2 at the second marker locus. The variation may just as well be at a locus that codes for disease or normal. In the first example, if the loci A and B are linked and A1B1 are together on one and A2B2 are on the other parental chromosome, they will occur together in their offspring more often than 50% as expected by Mendel's second law of independent assortment. If the loci are not close (not linked) on the same chromosome or are on different chromosomes, then A1 and B1 will occur together in the offspring 50% of the time. The frequency of reassortment of two variants when the loci are on the same chromosome is dependent on the frequency of crossing over between the two loci in the gametes resulting in the exchange of bits of the chromosome; in our example, crossover would result in A1B2 or A2B1 being on the same chromosome. This phenomenon is known as recombination, and its frequency is a function of the distance between the loci and often is referred to as theta. The best estimate of theta is measured by the maximum log of the probability (lod score or Z) of linkage versus no linkage, and when $Z \geq 3$ the odds for linkage are 1000:1 and considered significant. A score of $Z \leq -2$ is considered grounds for exclusion of linkage.

History of the Search for the MEN2A Locus

Attempts to find a linkage between the locus for MEN 2A and a marker gene predated the RFLP marker innovation, but a successful linkage of the MEN2A locus to two DNA markers that had been mapped to chromosome 10 awaited the use of the new technology (18,19). Searching for a linkage without a clue is like looking for a needle in a haystack. The search for a MEN2A linkage began by looking for the needle. First, linkage with the histocompatibility locus antigen complex was excluded (20,21) which was followed by the exclusion of a number of loci for protein markers that were already mapped and have been previously reviewed (22). We then continued the linkage search by pursuing the clue of a putative deletion on chromosome 20 in patients with MEN 2A reported by Babu et al (23,24). A deletion was not unexpected as the familial cancer with specific organ sites was analogous to that of retinoblastoma and Wilms tumor in which constitutive chromosomal deletions and chromosomal deletions or rearrangements in the primary tumors had been reported in some patients (25-31). Our studies led to exclusion of linkage in the MEN 2A families using two anonymous sequences D20S5 and D20S6 that mapped to the site of the putative deletion, thereby excluding the deletion site as a possible map position for the MEN2A locus and the eventual exclusion of most of chromosome 20 (32,33). Other exclusions were reviewed by us (34) by which time 32% of the genome was excluded.

Finding a Linkage and Chromosomal Assignment

The first hint of a positive linkage came from the thesis of Goodfellow (35) in which a lod of 1.5 at theta = 0 was reported for linkage between the disease locus and an anonymous sequence at D10S5 in one family but not in two other families. When the data were expanded to include those from five large families, the significant lod score of 3.6 was reached at theta = 0.19. By this time the DNA sequence had been mapped to chromosome 10 by *in situ* hybridization (36), and studies with the cDNA probe for interstitial retinol binding protein (RBP3), which had been mapped to chromosome 10 (37) in a region similar to that for the D10S5 locus, indicated that the locus for MEN 2A was clearly linked to both chromosome 10 sequences, nearer to the RBP3 locus than to the D10S5 locus (18,19). Since RBP3 had been mapped to a fairly broad pericentric area and D10S5 to a single q band, it could be inferred that the MEN2A locus was also in the pericentric region of the chromosome. At this stage it was also clear that both of the marker loci were on one side of the disease locus.

The Map of Chromosome 10

The chromosome assignment and linkages of MEN2A stimulated interest in chromosome 10, and 55 and 73 polymorphic

Table 1
Chromosome 10 Pericentric DNA Markers and their Polymorphisms*

Locus	Probe	Location	Restriction Enzymes	PIC
D10S24	p7A9	10p12.2-p13	MSPI	0.55
			TaqI	
FNRB	pGEM-32	10p11.2	BanII	0.71
			KpnI	
			HinfI	
			BG1II	
			SacI	
	pB/R2		MspI	
D10Z1	p10RP8	10 cen	PstI	0.50
			EcoRV	
			HincII	
RBP3	H.4IRBP	10q11.2	Bg1II	0.67
			MspI	
	TBIRBP9	10q11.2	TaqI	
D10S15	pMCK2	10q11.2	PvuII	0.35
			RsaI	
D10S5	p9-12A	10q21.1	TaqI	0.49
			HincII	
			DraI	

*From Wu J, Carson NL, Myers S, et al. The genetic defect in multiple endocrine neoplasia type 2A maps next to the centromere of chromosome 10. Am J Hum Genet (in press) (44).
PIC = polymorphic information content.

Table 2
Recombination Frequencies (θ) Between the Loci for MEN2A and Six Pericentric DNA Markers*

	θ			θ	
MEN 2A versus	Male	Female	Zmax†	Male = Female	Zmax
D10S24	0.14	0.18	5.98	0.15	5.92
FNRB	0.06‡	0.09	13.21	0.06	13.16
D10Z1	0.00	0.00	12.02	0.00	12.02
RBP3§	0.00	0.10	14.05	0.05	13.05
D10S15	0.05	0.05	17.29	0.05	17.29
D10S5	0.05	0.15	7.54	0.11	7.05

*From Wu J, Carson NL, Myers S, et al. The genetic defect in multiple endocrine neoplasia type 2A maps next to the centromere of chromosome 10. Am J Hum Genet (in press) (44).
†Maximum lod score.
‡No obligate recombinants were observed.
§Sex difference significant at $P < 0.05$.

markers have been isolated and mapped, respectively, to chromosome 10 (38). Some have reasonably refined positions on the map (38). Furthermore, the Centre d'Etude du Polymorphisme Humain (CEPH) consortium chose chromosome 10 for their first consensus map (39). The general interest in the chromosome has made it easier to close in on the MEN2A locus. In addition, the original localization of 10p11.2-q11.2 for the RBP3 locus was reduced to q11.2 from studies of a somatic cell hybrid line in which the only human chromosome was 10q (40), from in situ data localizing it to 10cen-q24 (41) and from dosage studies of several human cell lines (42). The reduced localization of RBP3 suggested that we look for flanking markers on the p arm near the centromere.

Table 3
Recombination Frequences (θ) Between Pairs of Six Pericentric DNA Markers on Chromosome 10*

	θ			θ	
Locus Pair	Male	Female	Zmax†	Male = Female	Zmax
D10S24-FNRB‡	0.06	0.22	19.5	0.14	17.7
D10S24-RBP3‡	0.04	0.35	25.5	0.16	18.2
FNRB-D10Z1‡	0.00	0.07	30.3	0.04	28.2
FNRB-RBP3‡	0.00	0.18	36.3	0.10	30.3
D10Z1-RBP3‡	0.00	0.07	31.1	0.04	29.3
D10Z1-D10S15	—	—	—	0.05	11.93
RBP3-D10S15	0.03	<0.01	46.6	0.02	45.9
RBP3-D10S5	0.07	0.07	19.0	0.07	19.0

*From Wu J, Carson NL, Myers S, et al. The genetic defect in multiple endocrine neoplasia type 2A maps next to the centromere of chromosome 10. Am J Hum Genet (in press) (44).
†Maximum lod score.
‡Sex difference significant at $P < 0.01$.

Closing in on the Disease Locus with Flanking Markers

The first flanking marker that we found is at D10S24 (43) but was rather far from the disease locus (15% recombination) (44). Two flanking markers, the gene for the beta subunit of the fibronectin receptor (FNRB) (45) and an anonymous sequence at D10S34 (46), were soon found to be on the short arm and to be approximately the same distance from the disease locus (about 6% recombination) (44,47), but their order has not yet been established because both of the markers have not been studied in the same data set. FNRB was physically localized to q11.2 by Goodfellow et al (45) and shown to be linked to the MEN2A locus with a lod score of 13.16 at theta = 0.06 (44). Nakamura et al (47) have shown from linkage studies that the anonymous sequence cTBQ14.34 at D10S34 also flanks the disease locus with a lod score of 3.61 at theta = 0.07, although they have physically localized the sequence only to 10p in somatic cell hybrid and monosomic cell lines. Linkage studies between D10S34 and MEN2A and the relevant markers are currently under way in our families. As well as the first two markers RBP3 and D10S5 on the q arm, Lathrop et al (40) and Nakamura et al (46,47) have reported an anonymous sequence pMCK2 at D10S15 which physically maps to q11.2 (42). The D10S15 marker is linked to MEN2A with a lod score of 19.48 at theta = 0.01 (47) and in our families with a lod of 17.29 at theta = 0.05 (44). Our multipoint analysis maps D10S15 distally to RBP3 (44). All of the flanking markers are reasonably informative, and at least some of the markers will be useful in most of the families, as indicated by their polymorphic information content value (13) in Table 1.

The Centromeric Marker

More recently and more importantly, no recombinants have been found between the centromeric alphoid sequence (D10Z1) on chromosome 10 isolated by Devilee et al (48) and the MEN2A locus in two studies (44; C.G. Mathew and B.A. Ponder, personal communication, 1989). Our data represent eight male and 18 female informative meioses, of which three male and eight female are phase known. This linkage is impor-

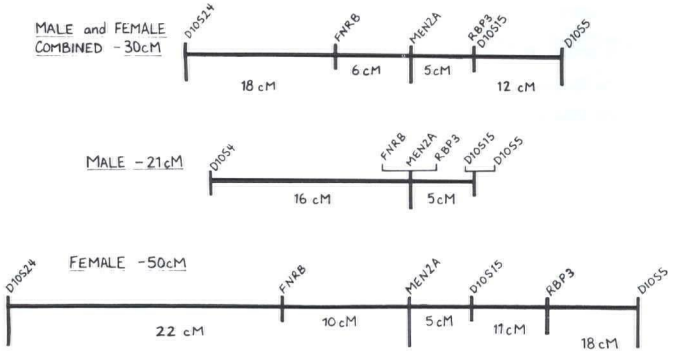

Fig 1—A genetic map of five markers flanking the MEN2A locus showing the shorter male than female map. The distances are derived from the recombination frequencies in six large families with MEN 2A (Table 2) and are converted to centimorgans (cM) using Haldane's function (50). To the left of the MEN2A locus is the p arm and to the right is the q arm of chromosome 10.

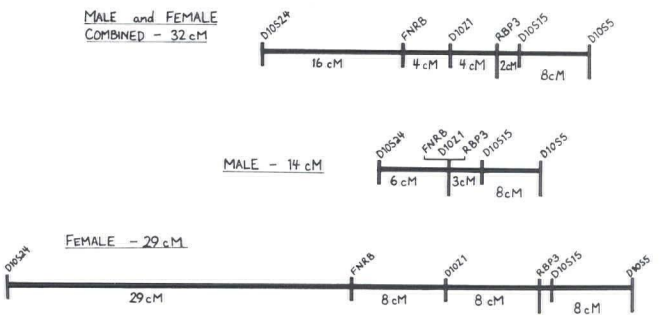

Fig 2—A genetic map of five markers flanking the D10Z1 locus showing the shorter male than female map. The distances are derived from the recombination frequencies in 15 large families (Table 3) including the six with MEN 2A in Fig 1 and are converted to centimorgans (cM) using Haldane's function (50). To the left of the D10Z1 locus is the p arm and to the right is the q arm of chromosome 10.

tant for identifying those "at risk" members of the MEN 2A families. The polymorphisms, however, are often noninformative which has led to considerable effort, with some success, to find more of them (49). The linkage is also important because it indicates that the MEN2A locus must be very close to the centromere, and possibly the mutation lies in the alphoid sequence, but we have no evidence of this yet. Moreover, if the disease locus is not a part of the alphoid DNA (the more likely hypothesis), the finding of a recombinant between it, the disease locus, and one of the close flanking markers will enable us to determine on which side of the centromere the disease allele lies, which will make it easier to identify the MEN 2A gene.

Sex Difference in Recombination

A remarkable sex difference is seen in the recombination frequencies in the pericentromeric region of chromosome 10 (Tables 2 and 3), resulting in quite different male and female maps (Figs 1 and 2 [50]). In the six MEN 2A families there were no obligate male recombinants between the closest flanking markers FNRB and RBP3 and the MEN2A locus (Table 2). The lack of recombination between RBP3 and MEN2A in males has also been reported by Ponder's group (47) and is evident in the data from Yamamoto et al (51). It is presumably the case in the data in which no recombination was reported by Sobol et al (52), but the data were not separated by sex. The lack of recombination between the markers FNRB, D10Z1, and RBP3 in males but not in females is significant in our data set with 15 families that includes the MEN 2A families (Table 3) from Wu et al (44). Lathrop et al (40) and Nakamura et al (46) have also reported sex differences in the recombination frequencies in this area of the chromosome. These differences have important implications for genetic counseling for members of the families at risk for having the MEN 2A gene and mean that the sex of the affected parent has to be considered. The sex difference in recombination is apparent from D10S24 on the short arm to RBP3 just below the centromere on the long arm of the chromosome but interestingly not immediately distal on the long arm (53).

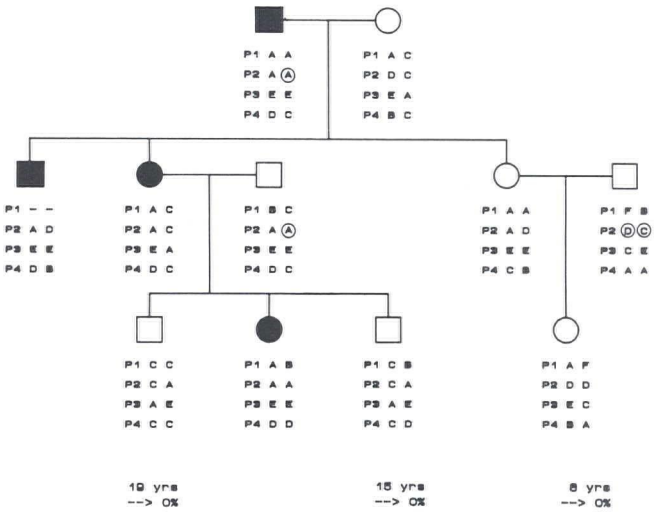

Fig 3—P1, P2, P3, and P4 represent loci FNRB, D10Z1, RBP3, and D10S15, respectively. The letters A-F represent haplotypes derived from several restriction enzyme digests for each locus. The restriction enzymes are BqlII, BanII, and HnfI for FNRB; PstI and EcoRV for D10Z1; TaqI, BglII, and MspI for RBP3; and RsaI for D10S15 (see Table 1). Circled haplotypes are the most likely ones. See text for a discussion of the probabilities for the unaffected members of generations II and III.

Probabilities and Diagnosis

We have reached a stage whereby the RFLPs of closely linked markers can determine a very high (or low) probability for "at risk" members of families possessing the allele for this hereditary cancer. If the centromeric sequence is informative, probabilities of virtually 0% or 100% can be given and thereby screening could be more selective. For example, in Fig 3 the affected mother II-2 is informative for the centromeric haplotype A/C. Since her father had the A/A haplotype and her mother had C/D,

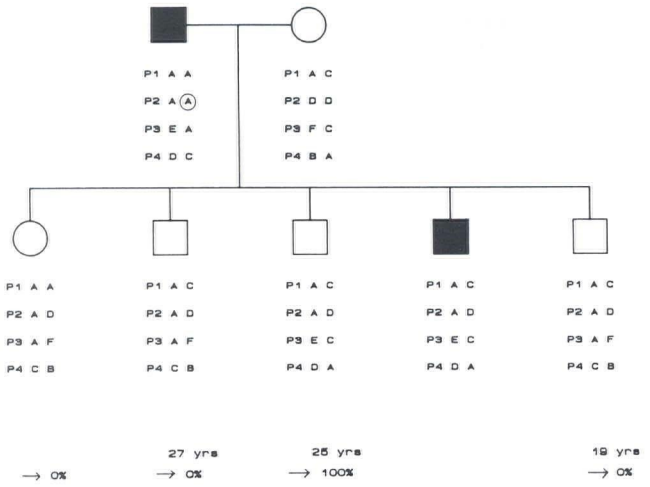

Fig 4—P1, P2, P3, and P4 represent loci FNRB, D10Z1, RBP3, and D10S15, respectively. The letters A-F represent haplotypes derived from several restriction enzyme digests for each locus. The restriction enzymes are BglII, BanII, and MsPI for FNRB; PstI and EcoRV for D10Z1; TaqI, BglII, and MspI for RBP3; and RsaI for D10S15 (see Table 1). Circled haplotypes are the most likely ones. See text for a discussion of the probabilities for the unaffected members of generation II.

the disease allele must be on the chromosome with the A allele at D10Z1. Both of her two teenaged sons received her C allele and are therefore most unlikely to have received the disease allele. The conclusion is further supported since the mother could only have received the chromosome with the three loci haplotypes C,C,A from her mother and the two sons also received this chromosome, ie, their normal maternal grandmother's chromosome.

In the above case, the flanking markers still gave a very good probability before we had the centromeric marker information. Since the haplotypes for FNRB and RBP3 came from the maternal grandmother, the only way that the sons III-1 and III-3 could have received the disease allele is from a double crossover between the two flanking markers. The probability of the double crossover in a female is $0.09 \times 0.10 = 1\%$ (Table 2).

The probability for the daughter II-5 in Fig 3 as deduced from the DNA results is not as satisfactory. Her father I-1 is informative only for the D10S15 locus, and, even for males, the probability of a recombinant child is 5%. However, the daughter is 30 years old and has had negative calcitonin tests. For this reason, the probability at her age that she has the disease allele is even less than 5%.

Probabilities of almost certainty can also be deduced when one or another of the markers FNRB or RBP3 are informative in an affected father but not when in an affected mother. Fig 4 illustrates an example of RBP3 and D10S15 being the only informative markers. The affected parent is the father, and since no male recombination between RBP3 and the MEN2A locus has been found to date, the probabilities approach 0% or 100% for the children who still have negative screening tests. The son II-3 was recently found to have a positive pentagastrin-stimulated test and had a thyroidectomy with node dissection. He had MTC with extension to the lymph nodes.

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