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Single missense mutation in the tyrosine kinase catalytic domain of the *RET* protooncogene is associated with multiple endocrine neoplasia type 2B

(medullary thyroid carcinoma/receptor protein tyrosine kinase/dominant oncogene/pheochromocytoma)

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ABSTRACT **Multiple endocrine neoplasia type 2B (MEN 2B) is a human cancer syndrome characterized by medullary thyroid carcinoma (MTC), pheochromocytomas, mucosal neuromas, ganglioneuromas of the intestinal tract, and skeletal and ophthalmic abnormalities. It appears both as an inherited disorder and as *de novo* disease. Sequence analysis of germ-line DNA from MEN 2B patients revealed the existence of the same point mutation in the *RET* protooncogene in 34 unrelated individuals. This sequence difference was not observed in 93 unaffected individuals, including the normal parents of 14 *de novo* MEN 2B patients. The mutation (ATG → ACG) results in the replacement of methionine with threonine within the catalytic core region of the tyrosine kinase domain. We propose that this amino acid replacement effects substrate interactions and results in dominant oncogenic activity by the RET protein. Missense mutations in the extracellular ligand-binding domain of the RET protooncogene previously have been associated with two other disorders [MEN 2A and familial MTC (FMTC)] in which MTC is observed. MEN 2B represents the third form of heritable MTC known to be an allele of *RET*. Alterations in two different functional domains of the putative receptor protein tyrosine kinase are implicated in development of MTC.**

Multiple endocrine neoplasia type 2B (MEN 2B) is the most severe form of the autosomal dominant MEN 2 syndromes (1–3). Often detected early in life by the presence of mucosal neuromas, skeletal and ophthalmic abnormalities, and ganglioneuromas of the intestinal tract, MEN 2B progresses rapidly with the development of metastatic medullary thyroid carcinoma (MTC). Pheochromocytomas (adrenal medulla tumors) are present in ≈50% of cases. Without surgical removal of the thyroid gland prior to metastasis, individuals affected by MEN 2B seldom survive past the age of 40 years. The MEN 2A syndrome (multiple endocrine neoplasia type 2A) also includes MTC and pheochromocytomas (3). MTC is detected at a much later age, commonly in the second decade of life with pheochromocytomas detected about 10 years after the appearance of MTC. In addition to MTC and pheochromocytomas, MEN 2A is characterized by parathyroid hyperplasia in approximately one-third of the cases. A third disorder, familial MTC (FMTC), is characterized by MTC alone, usually appearing between 40 to 50 years of age. The inherited defects responsible for all three disorders map to the pericentromeric region of chromosome 10 (4–7). Recently, numerous point mutations in the *RET* protooncogene have been identified in association with MEN 2A and FMTC (8, 9).

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The *RET* protooncogene [4726 nucleotides in length (10, 11)] is a member of a large class of protein tyrosine kinases that function as signal transduction molecules to regulate cellular growth and proliferation and in some cases to arrest growth and promote differentiation (12, 13). Similar in structure to the epidermal growth factor receptor, the RET protein [1114 amino acids long (10, 11)] consists of a relatively large putative extracellular ligand-binding domain with two cysteine-rich regions, a single transmembrane region, and an evolutionarily conserved intracellular tyrosine kinase domain (10–14). The extracellular domain of the mouse and human *RET* protooncogenes also contains a region with homology to cadherins, a superfamily of transmembrane proteins that mediate calcium-dependent cell–cell adhesion (15, 16). Although the ligand for the RET protein has yet to be identified, studies of similar receptor tyrosine kinases suggest the likelihood that ligand binding results in conformational changes that initiate a cascade of events that include receptor dimerization, transmittal of signal through the transmembrane domain, autophosphorylation, and tyrosine phosphorylation of substrate molecules.

All of the mutations identified thus far in cases of MEN 2A and FMTC are contained within the extracellular ligand-binding domain of the *RET* protooncogene and result in nonconservative substitutions for four different cysteines. Despite searches of this region and elsewhere in the RET coding sequence using single-strand conformational polymorphism (SSCP) analysis, mutations associated with MEN 2B were not found. We now report the identification of a single point mutation in the tyrosine kinase domain of RET associated with MEN 2B.¶ This observation provides evidence that these three divergent clinical disorders are allelic. The analysis presented in this paper supports a model of dominant oncogenic activity for the RET protein. When applied to RET protein activity during embryological development, the model also explains other clinical manifestations of MEN 2B. The mutation, which is located at the catalytic site for tyrosine phosphorylation, offers empirical evidence for a new perspective on the regulation and activity of protein kinases.

MATERIALS AND METHODS

Pedigree Resources and Preparation of Genomic DNA. The MEN 2B family resources available for this study and diag-

Abbreviations: MEN 2A and MEN 2B, multiple endocrine neoplasia type 2 A and B; MTC, medullary thyroid carcinoma; FMTC, familial MTC; SSCP, single-strand conformational polymorphism; SSCV, single-strand conformational variant.

¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U05016 and U05017).

nostic procedures have been described elsewhere (1–5). Altogether 34 families were investigated. Eighteen families demonstrate transmission of MEN 2B, and 16 are new cases with no affected offspring. In 27 of the 34 families, we were able to determine that MEN 2B arose *de novo* on the basis of information that the parents of the first affected pedigree member were unaffected. DNA was available from both normal parents of 14 patients with *de novo* disease. Normal reference pedigrees were obtained from the Centre d'Etude du Polymorphisme Humain (Paris). Methods used for preparation of genomic DNA from lymphocytes, lymphoblastoid cell lines, and tumor tissue have been reported (17, 18).

PCR Assays and SSCP Analysis. The genomic organization of the RET protooncogene was determined by using a PCR and sequence analysis approach (D.C., unpublished data). Oligonucleotide primer sequences were devised based on published cDNA sequences (refs. 10 and 11; GenBank accession numbers X12949 and X15262), and PCR assays were developed with cloned genomic sequences. PCR assays for SSCP were designed so that the product size was kept to about 150 base pairs (bp) when possible according to the recommendations of Sheffield *et al.* (19). The complete genomic structure, sequences of the intron/exon boundaries for all exons, and primer sequences will be reported elsewhere (D.C., unpublished data). PCR products were uniformly labeled and amplified by using the following conditions: exon 16 PCR primers 16F (5′-AGAGAGTAGAGTAACTTCAATGTC-3′) and 16R (5′-CTACATGTATAAGGGTGTTT-3′) (2 μM of each primer) in a total reaction volume of 5 μl containing 50 ng of genomic DNA, 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 5 mM NH_4Cl, 0.25 unit of *Taq* polymerase (AmpliTaq, Perkin–Elmer/Cetus), 200 μM of each dNTP, 15 μCi of [α-^{32}P]dCTP (1 μCi = 37 kBq) at 94°C (1 min), 55°C (2 min), and 72°C (2 min) for 25 cycles with an automatic thermocycler (Biometra, Emertson Instruments, Ontario). The expected product size is 175 bp. Reaction mixtures were diluted 1:4 with 5 μl of quenching buffer (95% formamide/2 mM EDTA/0.005% bromophenol blue/0.005% xylene cyanol) and 10 μl of distilled H_2O. SSCP analysis was done essentially as described (9).

DNA Sequencing. Template for DNA sequencing was prepared as follows: genomic DNA (≈100 ng) was amplified in a 25-μl reaction volume in 1× buffer (above) containing 1.5 mM $MgCl_2$, 125 μM dNTP, 0.5 unit of AmpliTaq, and 0.5 μM each of 16F and 16R primer. Buffer composition, denaturing, annealing, and extension conditions were as described for SSCP. Products were purified by using 1% low-melt agarose gels (SeaPlaque, FMC). Sequencing was performed essentially as described with ^{32}P-end-labeled 16F primer (20).

RESULTS

SSCP Analysis of RET Protooncogene Genomic Sequences. Genomic DNAs from MEN 2B family members and reference individuals were tested for sequence variation within the coding region of the *RET* protooncogene by using SSCP analysis. We studied 18 families in which transmission of MEN 2B has been observed and 16 cases demonstrating the first appearance of MEN 2B (*de novo* disease) with no affected offspring. A total of 18 different PCR assays developed for 17 exons (including intron boundaries), for portions of 3 other exons, and for the 5′ untranslated region of RET (D.C., unpublished data) were utilized in this study. PCR amplification products ranged in size from 100 to 270 bp, but most were about 150 bp in length in order to maximize the likelihood of finding single base-pair differences. We had previously identified seven single-strand conformational variants (SSCVs) that detected MEN 2A or FMTC mutations and three SSCVs that were not associated with disease and were therefore designated as neutral variants (9). Three new SSCVs were detected in exons 12, 14, and 15 in MEN 2B families, but these variants were also found to be present in normal individuals and in MEN 2A families [data not shown; Mulligan *et al.* (8) have also reported an exon 15 SSCV].

Identification of a RET Protooncogene Missense Mutation by DNA Sequencing. SSCVs associated with the MEN 2B phenotype were not evident; therefore, we directly sequenced part of the *RET* protooncogene to determine whether sequence differences not detectable by SSCV were present. Exon 10 and 11 PCR amplification products [previously shown to contain MEN 2A and FMTC mutations (8, 9)] from two MEN 2B individuals were analyzed, and the normal RET allele was found in both cases. However, DNA sequence analysis of exon 16 PCR amplification products from a MEN 2B patient revealed a single base-pair difference in one allele and the expected sequence for the other allele. All 58 individuals studied who were affected with MEN 2B were found to share this sequence difference. For 14 patients with *de novo* disease, DNA was available from both unaffected parents. In all 28 normal parents, only the normal exon 16 sequence was observed. The sequence difference was found to be transmitted from MEN 2B-affected parents to affected children in the 14 families from whom DNA was available. This alteration (ATG → ACG) results in the substitution of threonine for methionine at codon 918 according to the sequence reported by Takahashi *et al.* (11) (Figs. 1 and 2). The mutation appears to have arisen independently in germline DNA in the 34 families and sporadic cases examined. These individuals are not known to be biologically related according to historical records, and they are widely dispersed throughout North America and Europe. In 27 of the families, MEN 2B had arisen *de novo*. Haplotypes that were constructed for 6 MEN 2B families by using closely linked flanking markers (e.g., sTCL-1 [*D10S176*], sTCL-2 [*RET*], sKMC-2 [*ZNF22*]) were found to be nonidentical, providing additional evidence for the independent origins of the mutation (data not shown).

We also sequenced MTC tumor genomic DNA from a MEN 2B patient with *de novo* disease. Sequence analysis of exon 16 PCR amplification products revealed the same T → C missense mutation that had been found in the patient's

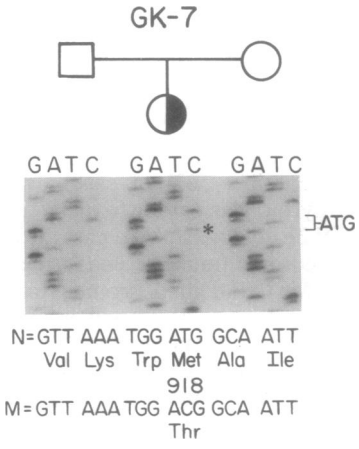

FIG. 1. *RET* tyrosine kinase domain point mutation in germ-line DNA from a MEN 2B patient. DNA sequence analysis of exon 16 PCR amplification products from genomic DNA of unaffected parents (○ and □) and a MEN 2B-affected individual (◑) from family GK-7. The DNA and corresponding amino acid sequence are shown below the DNA sequencing ladders. N, normal sequence; M, mutant sequence. Both parents have the normal sequence for both alleles (ATG at codon 918), whereas the affected daughter has one normal allele and a mutant allele with a T → C change (indicated on the DNA sequencing gel by an asterisk) at codon 918, which replaces methionine with threonine.

germ-line DNA. The normal *RET* coding sequence for the other allele was also present. Genomic DNAs from 93 individuals not affected with MEN 2B were found to contain only the normal sequence. These individuals included 42 unaffected parents and siblings of MEN 2B patients, 22 MEN 2A patients, 3 FMTC patients, 2 patients with sporadic MTC, 9 patients with sporadic pheochromocytomas, 1 neuroblastoma patient, and 14 individuals from the Centre d'Etude du Polymorphisme Humain reference pedigrees.

DISCUSSION

MEN 2A, MEN 2B, and FMTC Are Alleles of the *RET* Protooncogene. Three clinically distinct disorders, MEN 2A, MEN 2B, and FMTC, have now been shown to be associated with mutations in different functional domains of the RET protooncogene. For all three disorders, we found that patient's germ-line and corresponding tumor DNAs were found to contain a mutant and a normal *RET* allele, suggesting that *RET* exerts its effect in the heterozygous state. While the same missense mutation in the tyrosine kinase domain has been found for every case of MEN 2B, the 13 different mutations reported thus far in association with MEN 2A and FMTC result in nonconservative substitution of four of the six cysteine residues located in the extracellular domain adjacent to the transmembrane segment of the RET protein (8, 9). In addition, we have identified a new missense mutation for another cysteine (at codon 609) in three MEN 2A families (D.C., unpublished data) and a 6-bp deletion in tumor DNA from a sporadic case of MTC that removes the sixth cysteine in the cluster (codon 630) (9). Thus, mutations have been identified in all six codons that specify cysteine and are located immediately adjacent to the transmembrane domain (Fig. 2).

An understanding of the effect of mutations responsible for the MEN 2A phenotype comes from domain-switching and protein-crosslinking experiments using a very similar protein tyrosine kinase, epidermal growth factor receptor. These studies show that the ligand for the epidermal growth factor receptor binds to a region flanked by two cysteine-rich regions, one of which is immediately adjacent to the transmembrane domain. It has been proposed that the cysteine-rich regions fold up to form a ligand-binding pocket (21, 22). The extracellular domain for RET is similar in configuration. It is possible that ligand binding also occurs in this analogous region. Mutations that result in the replacement of cysteines by other amino acids could diminish or abolish the regulatory effect of ligand-binding on the activity of the tyrosine kinase. A missense mutation in the extracellular domain of a related receptor tyrosine kinase, colony stimulating factor −1 receptor, provides evidence supporting this hypothesis. This mutation results in constitutive autophosphorylation in the absence of ligand and a transformed phenotype. Moreover, the deletion of the extracellular domain in a v-*erbB* mutant results in constitutive tyrosine kinase activity (23). The *RET* protooncogene is expressed at a high level in MTCs and pheochromocytomas from MEN 2A patients but is scarcely evident in normal postnatal thyroid and adrenal tissues (24, 25), suggesting an uncoupling of the normal regulatory control process and that overexpression of the *RET* protooncogene could have oncogenic effects. These observations also argue against an alternative hypothesis that the observed RET extracellular domain mutations could interfere with correct dimerization of the molecules or otherwise interrupt signal transduction, producing a dominant negative effect.

A Single *RET* Allele in MEN Type 2B. In view of the fact that MEN 2A and FMTC are characterized by allelic heterogeneity, the identification of a single point mutation that is the same for all 34 independent occurrences of MEN 2B was unanticipated. Furthermore, virtually all heritable diseases including tyrosine kinase disorders such as insulin resistance (due to a defective insulin receptor), agammaglobulinemia (the result of a defective SRC-like protein atk), and piebaldism (caused by c-*kit* alleles) (26–28) are characterized by allelic heterogeneity. The only known exception is sickle cell anemia, which is due to a single point mutation in β-globin [reviewed by Orkin and Kazazian (29)]. It is possible that other mutations in the *RET* tyrosine kinase domain are not compatible with life, even in the heterozygous state, or that other mutations produce non-MEN 2B phenotypes. For example, one form of Hirschsprung disease has been localized by deletions that include the *RET* locus and genetic linkage analysis to the pericentromeric region of chromosome 10 (30, 31). This phenotype is in some ways the opposite

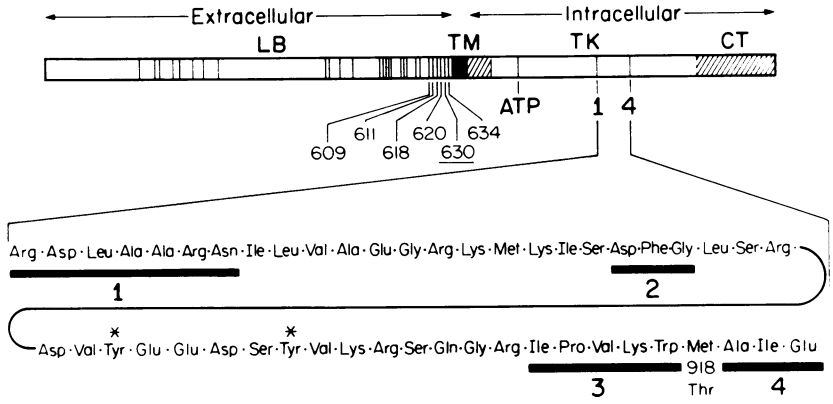

FIG. 2. RET Protein domains and mutations observed in MEN 2 and sporadic MTC. A schematic drawing of the *RET* protooncogene cDNA sequence is shown at the top of the figure and indicates the coding sequence and the relative sizes of domains (10, 11). LB is the extracellular ligand binding domain (open rectangle). Vertical bars within this rectangle designate the positions of cysteine codons (25 total). TM is the transmembrane domain (filled-in box), TK is the intracellular tyrosine kinase domain, with the site for ATP binding indicated (open rectangle). Hatched areas indicate the juxtamembrane region adjacent to the TK domain and the carboxyl-terminal region (CT). Mutations in cysteine codons for MEN 2A, FMTC, and a sporadic MTC tumor (630), underlined (refs. 8 and 9; Chi *et al.* and Dou *et al.*, unpublished), are indicated (609–634) under their relative positions in the extracellular domain. Region 1–4 indicates a portion of the tyrosine kinase catalytic core. Underlined amino acid sequences 1 and 3 are conserved sequences that distinguish serine/threonine kinases from tyrosine kinases. Region 1 also distinguishes the two subfamilies of tyrosine kinases (32). Region 2 (Asp-Phe-Gly) and region 4 (Ala-Ile-Glu) indicate other highly conserved sequences among protein kinases. The MEN 2B mutation at codon 918 is indicated with the substituted amino acid, threonine, shown below the normal sequence. Possible sites for tyrosine autophosphorylation are indicated with asterisks. Codons are numbered to include the additional 5′ cDNA sequence encoding 254 amino acids (11) reported subsequent to ref. 10.

to that observed for MEN 2B with respect to intestinal ganglia being absent in Hirschsprung disease and hyperplastic in MEN 2B. It is conceivable that the lack of RET activity in one case (Hirschsprung disease) and inappropriate activity in the other (MEN 2B) account for the phenotypes.

The MEN 2B Mutation Predicts a Structural Alteration at the RET Tyrosine Kinase Catalytic Site. The MEN 2B missense mutation at codon 918 lies within the catalytic core region of the intracellular tyrosine kinase domain of the RET protein and is flanked by two amino acid sequences that are conserved in all tyrosine kinase receptors (Fig. 2). One of these sequences Ala-Pro-Glu (Ala-Ile-Glu in RET, region 4 in Fig. 2) has been shown to be essential for catalysis, since a mutation in any one of these residues, for example in v-src, destroys enzyme activity (33, 34). The other flanking sequence (region 3 in Fig. 2) is one of the two sequences that distinguish serine/threonine and tyrosine kinases (35). Computer modeling studies of epidermal growth factor receptor based on the crystallographic molecular model for cAMP-dependent protein kinase suggest that region 3 forms a β-turn and serves as a binding surface for proper positioning of the tyrosine in the substrate molecule (35). Substitution of a threonine for methionine at position 918 in RET would be expected to be of considerable significance for enzyme catalysis because the amino acid at 918 is predicted to interact with the amino acid located one residue to the carboxyl-terminal side of the tyrosine residue undergoing phosphorylation in the protein substrate (D. Knighton, personal communication). This prediction is based on observations of the crystal structure of the cAMP-dependent protein kinase complexed with a substrate-analog peptide inhibitor. In this complex the residue leucine-205 (which corresponds to methionine-918 in RET) contacts isoleucine in the peptide inhibitor. The isoleucine residue immediately follows alanine, which occupies the position of the phosphate-acceptor residue in authentic cAMP-dependent protein kinase substrates (36, 37). This same alanine occupies, by analogy, the position of the phosphate-accepting tyrosine in protein tyrosine kinase substrates. Residue 918 participates in formation of the substrate recognition pocket in this region. The smaller size and other differences in characteristics of threonine compared with methionine might enhance the rate or change the specificity of RET phosphorylation (D. Knighton, personal communication).

Clinical Manifestations of MEN 2B and the Proposed Normal Function of RET. Recent studies by Larsson *et al.* (38), Schuchardt *et al.* (39), and Pachnis *et al.* (40) have elucidated a central role for the RET protooncogene in mammalian embryogenesis. Schuchardt *et al.* (39) used gene targeting to produce mutant mice that were homozygous for a deletion of RET tyrosine kinase activity and found that development of the kidney and the enteric nervous system was severely affected, demonstrating an absolute requirement for functional RET protein. Heterozygotes for the mutation were normal in appearance. Furthermore, Pachnis *et al.* (40) studied in detail the expression of the *RET* protooncogene during mouse embryogenesis and found that transcripts were temporally expressed predominantly in the developing nervous and genitourinary systems. Their finding of *RET* expression in the endoderm of the posterior branchial arches, from which parathyroid progenitor cells are derived, appears to resolve a major ambiguity about the MEN 2A phenotype—namely, how cells that are not directly derived from the neural crest can be involved in MEN 2A. The results of Pachnis *et al.* (40) also provide a developmental context for interpreting the clinical manifestations of MEN 2B. RET transcripts are temporally expressed in all of the cell lineages that give rise to the syndrome. Evidence previously reported indicated that *RET* is expressed in rat embryos, but the sites of expression were not delineated (41). Pachnis *et al.* (40) found that *RET* is highly expressed in the neural crest cells (which give rise to the thyroid C cells and adrenal medulla) and in the developing enteric nervous system. The enteric nervous system progenitor cells give rise to tissues that develop into mucosal neuromas and ganglioneuromas of the intestine and to cells that result in corneal nerve thickenings characteristic of MEN 2B. It is also possible that the MEN 2B-associated skeletal abnormalities are due to *RET* expression in the neural crest cells, which contribute to the cranial mesenchyme and elsewhere. Inappropriate expression or activation of *RET* during embryonic and fetal development that continues during the postnatal years could also explain the rapid onset of metastatic disease, a hallmark of MEN 2B. Definitive evidence of the role for these various *RET* mutations in the pathogenesis of MEN 2 may come from studies with transgenic mice and by continued analysis of the function of the normal gene during development.

Model for Dominant Oncogenic Activity by the RET Protein in MEN 2A, MEN 2B, and FMTC. Because the MEN 2B mutation lies within the catalytic site of the tyrosine kinase domain of *RET*, the question arises as to whether the mutation causes a decrease or increase in catalytic activity, alters enzyme specificity, or affects the regulation of catalytic activity. Methionine-918 is conserved in the mouse *RET* protooncogene (15, 16) and for 13 of 14 receptor tyrosine kinases (leucine replaces methionine in DER), but threonine is found in this position in 12 of 13 closely related cytoplasmic tyrosine protein kinases (alanine replaces threonine in Dsrc28), which have no extracellular receptor domain (the Src and Abl subfamilies) (32), indicating that the enzyme is unlikely to be inactivated by the threonine substitution mutation that we have identified. Furthermore, the experiments of Schuchardt *et al.* (39) indicate that loss of function of RET produces a phenotype that is not analogous to the manifestations of MEN 2B. Because the tyrosine kinases that do not rely on extracellular signals to regulate enzyme activity contain threonine at this position, like the mutant that we have identified, it is possible that this position is important in either the ligand-dependent or another regulatory pathway for RET. For example, with Src, removal of the carboxyl-terminal tyrosine that normally represses Src when it is phosphorylated results in activation of the tyrosine kinase [reviewed by Cooper and Howell (42)], and a similar activating event might occur when threonine replaces methionine in RET. Alternatively, substitution of the smaller threonine residue for methionine might simply allow other molecules to become substrates for phosphorylation or result in increased enzyme activity. Replacement of methionine by threonine appears in some way to enhance the activity of the tyrosine kinase and confers the phenotype observed in MEN 2B. Such an effect accounts well for the dominant phenotype of this mutation. It is of interest that other clinically recognized mutations in the *RET* protooncogene are characterized by dominance. For example, the *RET* protooncogene can become activated by somatic DNA rearrangements that replace various portions of the extracellular domain with other sequences. Hybrid RET transcripts and their protein products have been observed in papillary thyroid carcinomas, leukemias, and stomach and colon cancers (10, 14, 43–47). Dominant oncogenic activity has also been produced by fusing the *RET* oncogene to metallothionine gene regulatory sequences, which resulted in neuroblastoma and malignant melanoma in transgenic mice (48, 49). Thus, the loss of the extracellular domain or mutations in this region appear to cause a loss of regulation of receptor tyrosine kinase activity, resulting in overproduction of the gene product. However, the MEN 2A, MEN 2B, and FMTC phenotypes differ in their clinical expression. These differences may be explained by the timing of expression of the *RET* gene during development. Experiments that test whether the *RET* mutations function as

dominant transforming genes in NIH 3T3 cell transfection assays should provide additional insight as to the effect of *RET* mutations on cellular transformation and oncogenesis.

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