

(Fig. 1a). Thus BII may encode the granule cell current.

The functional properties of *doe-1* present an interesting contrast with aspects of previously characterized  $\text{Ca}^{2+}$  channels. Although these channels inactivate rapidly and are quite sensitive to  $\text{Ni}^{2+}$ , like T-type channels<sup>24,29</sup>, they differ markedly from T-type channels with regard to their voltage-dependence and their sensitivity to  $\text{Cd}^{2+}$ . The channels described here differ from L-type and P-type channels by virtue of their kinetics and their insensitivity to DHPs and  $\omega$ -Aga-IVA, respectively. Thus, *doe-1* and its putative mammalian counterpart can be readily distinguished from T-, L- and P-type channels.

*Doe-1* and the granule cell current share more functional properties with N-type channels than with the other channel types. But these channels are distinguished from *rbB*, a previously expressed N-type  $\text{Ca}^{2+}$  channel<sup>14</sup>, by the rapidity of their inactivation and their susceptibility to  $\text{Ni}^{2+}$  block. At least some of these distinguishing characteristics have already been reported for currents broadly classified as N-type in mammalian neuronal membranes. Rapid inactivation ( $\tau \sim 10\text{--}20\text{ ms}^{30}$ ) is an earmark of the  $\text{N}_1$  channel of peptide-releasing mammalian nerve terminals<sup>30,31</sup>. A component of N-type current that is reversibly blocked by  $\omega$ -CTx-GVIA has been described in peripheral neurons<sup>32</sup>. Together, these results highlight the diversity of  $\text{Ca}^{2+}$  channels that have been or could be considered within the general category of N-type. Some of these channels may be found to arise from *doe-1* or BII-like clones, rather than the *rbB* gene.

Until now, functional classes of  $\text{Ca}^{2+}$  channels seemed to map neatly onto a structural family tree, with separate limbs for N-type, P-type and various L-type channels<sup>2,6</sup>. In this context, the resemblance of *doe-1* to various N-type channels is particularly noteworthy because there is less structural homology between *doe-1* and *rbB* (clearly N-type<sup>14</sup>) than between *rbB* and BI (clearly not N-type<sup>11</sup>). Thus,  $\text{Ca}^{2+}$  channel classifications based on kinetics and pharmacology can cut across categories based on sequence homology. A similar principle is emerging for  $\text{K}^+$  channels<sup>33</sup>. Structurally, *doe-1* is most akin to the BII channel of mammalian brain<sup>15</sup> (68% identity). Once BII has been expressed, it will be interesting to compare it to *doe-1* and the cerebellar granule cell current.

The rapid inactivation of *doe-1*-like channels may have important consequences for neuronal function. Such inactivation would strongly influence the ability of a channel to contribute to  $\text{Ca}^{2+}$  entry during repetitive depolarizations. This might reduce the activation of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and thereby preserve the excitability of the neuron. At a nerve terminal, such properties could decrease synaptic efficacy during a train of impulses. Our results with *doe-1* emphasize the extent to which  $\text{Ca}^{2+}$  channels have diversified to allow neuronal responses to be tuned to particular patterns of electrical activity. □

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## Germ-line mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A

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**MULTIPLE endocrine neoplasia type 2A (MEN 2A) is a dominantly inherited cancer syndrome that affects tissues derived from neural ectoderm. It is characterized by medullary thyroid carcinoma (MTC) and pheochromocytoma<sup>1</sup>. The *MEN2A* gene has recently been localized by a combination of genetic and physical mapping techniques to a 480-kilobase region in chromosome 10q11.2 (refs 2,3). The DNA segment encompasses the *RET* proto-oncogene, a receptor tyrosine kinase gene expressed in MTC and pheochromocytoma and at lower levels in normal human thyroid<sup>4</sup>. This suggested *RET* as a candidate for the *MEN2A* gene. We have identified missense mutations of the *RET* proto-oncogene in 20 of 23 apparently distinct MEN 2A families, but not in 23 normal controls. Further, 19 of these 20 mutations affect the same conserved cysteine residue at the boundary of the *RET* extracellular and transmembrane domains.**

We searched for gross alterations of the *RET* locus, using pulsed field and standard Southern blots hybridized with a *RET* complementary DNA<sup>5</sup> and probes derived from a *RET* cosmid contiguous sequence (contig; J.B.J.K., manuscript in preparation) but detected no altered DNA fragments in a panel of 50 unrelated MEN 2A patients (data not shown). To search for smaller mutations, we designed polymerase chain reaction (PCR) primers for an overlapping set of 16 amplicons spanning the entire *RET* coding sequence<sup>6,7</sup>. Because *RET* transcripts are not detected in lymphocytes, we screened RNA from four MTCs and three pheochromocytomas from six MEN 2A patients and from a sporadic MTC and pheochromocytoma, all of which were known to express *RET* transcripts. First-strand cDNA was prepared, PCR-amplified for each amplicon and the products analysed for sequence variations by the chemical cleavage mismatch procedure (CCM)<sup>8,9</sup>.

Analysis of the entire *RET* coding sequence in these nine tumour samples identified CCM variants in three regions of the

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TABLE 1 *RET* mutations in MEN 2A

Codon	Position* Base pair	Family	Base pair changes	Amino-acid changes
364	1,783	A34	TGC→GGC	Cys→Gly
378-380	1,827-1,831	A33†, M01 M04, M09, M10 M11, M14, M19 M32, A12, 165, 167	GAG CTG TGC→GAC GTG CGC	Glu Leu Cys→Asp Val Arg
380	1,831	123, M18, F193	TGC→GGC	Cys→Gly
	1,832	M06, F060 A11 M07	TGC→TAC TGC→TCC TGC→TTC	Cys→Tyr Cys→Ser Cys→Phe

\* Amino-acid and codon position refer to the *ret* sequence of ref. 6.

† Cys 380 sequence was investigated in tumour material only for this family.

gene (Fig. 1). PCR products identified by cleavage of variant sequence were isolated and sequenced directly. Two of these variants represented conservative base-pair substitutions at positions 2,238 and 2,643 (numbered according to the published sequence of ref. 6) in the tyrosine kinase domain and are probably normal sequence polymorphisms (E.G. *et al.*, manuscript in preparation).

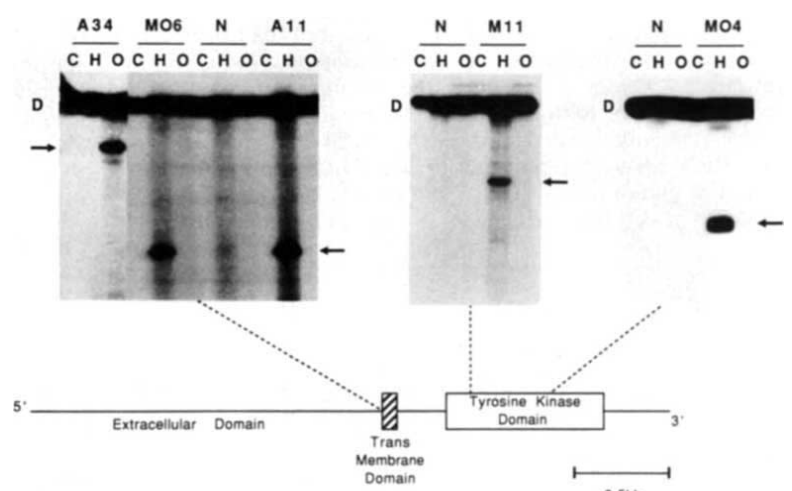
The remaining CCM variants were detected in a 413-base-pair (bp) PCR amplicon spanning the *RET* transmembrane domain and 5' portion of the tyrosine kinase domain (Fig. 1). Sequence analysis of these PCR products identified an altered sequence in all seven tumour samples from six patients with MEN 2A (Table 1; A12, A33, A34, 167, M04, M11). The samples from the two sporadic tumours had no sequence alterations. Six of the MEN 2A samples, including an MTC and pheochromocytoma from the same individual, proved to have an identical, three-base sequence change (GAGCTGTGC→GACGTGCGC) resulting in the substitution of three amino acids in codons 378-380 (Glu-Leu-Cys→Asp-Val-Arg). The presence of all three changes on a single allele was confirmed by sequencing cloned *RET* cDNAs from an MEN 2A pheochromocytoma cDNA

library (Fig. 2a). The library was prepared from an individual (A12) heterozygous for the mutant allele. The codon for cysteine at position 380 (Cys 380) is one of 27 cysteine residues in the *RET* extracellular domain that are conserved between man and mouse<sup>6,7,10</sup>. In the remaining MEN 2A tumour sample (A34) there was no mutation in Cys 380. But a T→G conversion on codon 364 again led to replacement of a conserved cysteine residue, in this case by a glycine. In all but one tumour, heterozygosity of the DNA sequence indicated that both normal and mutant *RET* alleles were expressed. The exception was in the MTC of individual 167-302, which expressed only the mutant *RET* allele (Table 1).

To determine whether the *RET* mutations found in the tumours were germ-line or somatic in origin, we used PCR primers derived from *RET* intronic sequences to analyse normal tissue DNA from 21 unrelated MEN 2A individuals, including 4 of the 6 MEN 2A individuals with tumour mutations and from 23 normal controls (Fig. 2b). *RET* mutations of codon 380 were detected in 17/21 germ-line MEN 2A samples and the codon 364 mutation was detected in one (A34). Normal *RET* sequence was observed in all normal controls. In every case in which a *RET*

FIG. 1 Chemical cleavage mismatch analysis (CCM) of *RET*. Autoradiograms showing variations in CCM cleavage pattern of PCR-amplified *RET* transcripts. MEN 2A family members are indicated together with normal (N) controls. C, Control lanes containing uncleaved heteroduplex. H and O, Hydroxylamine- and osmium tetroxide-modified heteroduplexes, respectively, cleaved by treatment with piperidine. The positions of the heteroduplex is marked by D and the *RET* cleavage products by arrows. The positions of the cleaved bases relative to the *RET* extracellular, transmembrane and tyrosine kinase domains are shown.

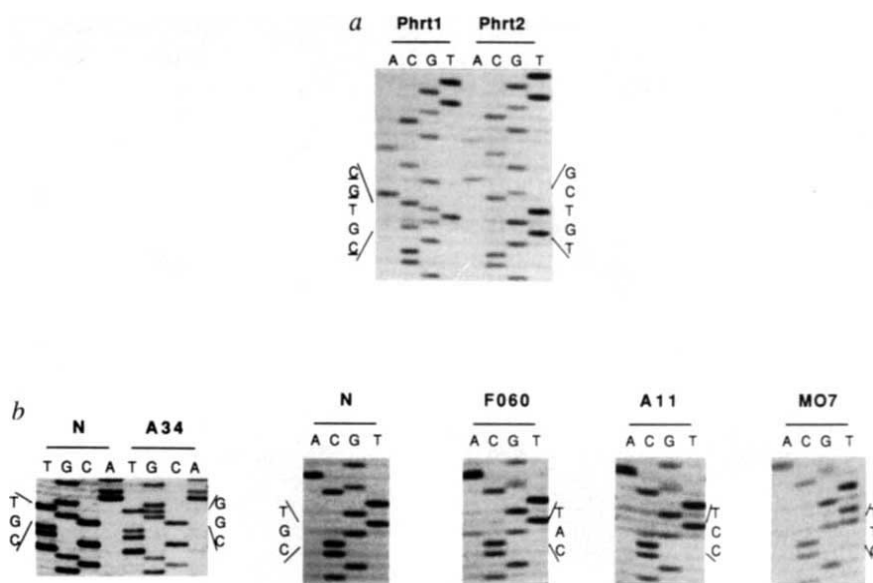
METHODS. PCR primers used in analysis of *RET*. Transmembrane domain (codons 262 to 399): CRT2S (5'CCCGTTTCCTGTGCAGTCAGCAAG3') and CRT2A (5'GACAGCACCGAGACGAT3'); 5' tyrosine kinase domain (codons 480 to 604): CRT4S (5'GAAAAAGTGGTCAAGGC-AAC3') and CRT4A (5'GATCTGCCAGGCAAATGAGATG3'); 3' tyrosine kinase domain (codons 590 to 716): CRT5S (5'GGGCCCTCACCATGGGCGACCT3') and CRT5A (5'CCATCCGGTGGCCGGTCTTCAAG3'). PCR amplification of poly(T)-primed first-strand cDNA was done for 55 cycles of 1 min each at 95 °C, and 55 °C and 72 °C. A normal control fragment was gel-purified in low-melting-point agarose, end-labelled using [<sup>32</sup>P-γ]ATP and T4 polynucleotide kinase and hybridized to the corresponding PCR fragment from each sample. The heteroduplex was modified by treatment with either 2.5 M hydroxylamine or



0.025% osmium tetroxide and modified bases cleaved by addition of 10% piperidine. The cleavage products produced by sequence variations were visualized by separation on 5% polyacrylamide gels and autoradiography.

FIG. 2 Sequence of codons 378 to 380 of the *RET* proto-oncogene in MEN 2A individuals. *a*, Sequence of the 378 to 380 codons in two copies of *RET* isolated from a pheochromocytoma cDNA library prepared from patient A12 who is heterozygous for the 378–380 *RET* mutation. PHRT2 contains the normal *RET* sequence. PHRT1 has three base-pair changes (underlined) showing that all three changes are present in a single *RET* allele. *b* Germ line sequence of normal individuals (N) and MEN 2A patients from families A34, F060, A11 and M07. The mutant sequence for the relevant codons is shown to the right of each sample. The *RET* normal DNA sequence is shown to the left.

**METHODS.** PCR primers used were CRT2B (5'CGTATGCGTCGG3') and CRT2A. PCR amplification and product purification were as described in the legend of Fig. 1. Amplification products were sequenced using the CRT2A primer with the  $\Delta$ TaqI sequencing kit (US Biochemicals). Plasmid DNAs were sequenced directly with the Sequenase kit (US Biochemicals).



mutation had been observed in tumour samples, the same sequence variant was present in the germ-line, indicating that the mutations did not arise somatically during tumorigenesis.

If *RET* mutations are responsible for the MEN 2A phenotype, the mutant alleles should segregate with the disease in MEN 2A families. Confirmation of this was obtained in 8 families (M04, M06, M07, M11, M14, 167, M18, 123) which had the same mutation of codon 380 in two or more affected family members but not in unaffected family members, confirming that the mutation was on the disease chromosome in each case.

In total, 19 distinct mutations at codon 380 were detected, representing 5 different amino-acid changes (Table 1). Despite sharing the same *RET* codon 380 mutation, some families (M09 and M19; 123 and M18) did not share haplotypes for other polymorphisms<sup>11–13</sup> within the *RET* locus (<30 kb away; J.B.J.K. *et al.*, manuscript in preparation). This suggests that at least some of these similar mutant alleles have arisen by independent mutation.

The MEN 2B syndrome, similar to MEN 2A but characterized by earlier tumour onset, ganglioneuromatosis of the intestine and Marfanoid habitus, also maps to the 10q11.2 region<sup>2</sup>. We did not find mutation of codon 380 in any of 10 MEN 2B patients examined. This may not be surprising, considering the differences in disease phenotype. We cannot, as yet, predict whether MEN 2B is due to a different mutation in the *RET* gene or to mutation of a closely linked gene.

Taken together, our data show that 20/23 unrelated patients with MEN 2A are heterozygous for *RET* missense mutations in germ-line and/or tumour DNA. In 19 out of 20 cases (95%) the mutation affects Cys 380. The *RET* cysteine residues are highly

conserved between species and 27 of 28 cysteines, including Cys 380, are present in the mouse *RET* extracellular domain<sup>6,7,10</sup>. These data suggest that replacement of the Cys 380 residue may disrupt normal *RET* protein conformation, altering *RET* signal transduction and leading to the MEN 2A phenotype. The remaining three MEN 2A patients do not have sequence changes in codon 380, however, so it seems likely that mutation of positions elsewhere in the *RET* extracellular domain may be responsible for MEN 2A in these cases.

The *RET* gene is structurally rearranged in 25% of papillary thyroid carcinomas by juxtaposition of *RET* transmembrane and tyrosine kinase domains with other 5' expressed sequences<sup>14</sup>. Clearly, similar activating mechanisms do not occur in MEN 2A. If replacement of Cys 380 and heterozygous expression of the mutant *RET* isoform is responsible for MEN 2A, a dominant or dominant-negative mechanism for the disease seems more probable than the classic tumour suppressor model. This is consistent with the absence of allele loss reported for chromosome 10q in MTC and pheochromocytoma<sup>15–17</sup>. The specificity of the *RET* mutants observed in Cys 380 is reminiscent of the codon specificity of oncogenic point mutations of other tyrosine kinase receptor genes such as *c-fms*, *neu* and *trk*<sup>18–20</sup>. *In vitro* analyses have shown that substitution of specific amino acids in the extracellular (*fms*, *trk*<sup>18–19</sup> or transmembrane (*neu*<sup>20</sup>) domains of these proteins can induce cellular transformation. If the *RET* Cys 380 mutations observed in MEN 2A are functionally analogous, then this would be the first example, to our knowledge, of dominantly acting oncogenic point mutations as the initiating events in human hereditary neoplasia. □

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