DNA Sequence

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cDNA Sequence and Genomic Structure of the Rat Ret Proto-Oncogene

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The *RET* proto-oncogene, a member of the Receptor Tyrosine Kinase family, plays a crucial role during the development of the excretory system and the enteric nervous system, as demonstrated by in *vivo* animal studies and by its involvement in the pathogenesis of several human neurocristopathies like Hirschsprung disease and Multiple Endocrine Neoplasia type 2. Using a multistep RT-PCR approach we have isolated and sequenced the cDNA of the whole rat RET proto-oncogene, reporting the deduced amino acid sequence in comparison with the human and mouse counterparts. Moreover, two different isoforms (RET9 and RET51) have been confirmed in the rat, while a third RET isoform demonstrated in human (RET43) has not resulted to be conserved in this species. Finally, we have determined the genomic structure of the rat RET proto-oncogene comparing the exon-intron boundaries and intron sizes with the known structure of the human homologous gene. Our findings will facilitate the molecular study of appropriate rat models of RET related human diseases.

Keywords: RET proto-oncogene, rat, RT-PCR, DNA sequence, genomic structure

INTRODUCTION

The *RET* proto-oncogene belongs to the Receptor Tyrosine Kinase (RTK) family. The extracellular domain is homologous to the cadherin superfamily of transmembrane proteins that mediate Ca^{2+}-dependent cell-cell adhesion, a crucial cellular function in differentiation and tumor suppression (Pasini et al., 1996). The *RET* extracellular portion is involved in the formation of a complex with two other proteins, the Glial cell-line Derived Neurotrophic Factor (*GDNF*) and the *GDNF* Receptor-alpha (*GFRA1*), resulting in the activation of the intracellular TK activity (Robertson and Mason, 1997). Additional *RET* ligands, namely Neurturin, Artemin and Persephin have been identified, which are able to activate the RET-TK activity after binding specific co-receptors, *GFRA2*, *GFRA3* and *GFRA4* respectively (Saarma and Sariola, 1999). All these molecules require Ret to transduce their signals to a variety of intracellular pathways which, depending on

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the cell type involved, may lead to morphological changes in the cytoskeleton, cell scattering, proliferation and differentiation during embryo development (van Weering and Bos, 1998).

The human full length *RET* cDNA consists of 5225 bp, including an open reading frame of 3342 bp which encodes a protein of 1114 amino acids. An alternative splicing has been described at the 3'end of the gene which originates a protein of 1072 amino acids, whose 9 terminal residues are generated by the prolonged translation of intron 19 (RET9), instead of the 51 codified by exon 20 (RET51) (Tahira et al., 1990). In addition to these RET ends, the last 43 amino acids of a third form (RET43) are encoded by exon 21 (Myers et al., 1995). The genomic structure of the human *RET* gene consists therefore of 21 exons spanning a total of 53 Kb, with a very large first intron of about 23 Kb. Exon-intron junctions have also been identified along with the intronic sequences flanking each exon (Ceccherini et al., 1993). The complete sequencing of a YAC clone (214H10) encompassing the whole *RET* has recently confirmed the estimated length and the genomic structure of this human gene (GenBank AL022344).

In human, heterozygous mutations of the *RET* proto-oncogene can result in different disease phenotypes. Gain-of-function mutations are associated with Medullary Thyroid Carcinoma (MTC) and Phaeochromocytoma in Multiple Endocrine Neoplasia type 2A (MEN2A) and type 2B (MEN2B). On the other hand, loss-of-function of the *RET* gene may lead to a congenital disorder of the innervation of the distal intestine known as Hirschsprung (HSCR) disease (Pasini et al., 1996; Eng and Mulligan, 1997). The *RET* proto-oncogene is also involved in the pathogenesis of those Papillary Thyroid Carcinomas (PTC) characterized by the presence of somatic rearrangements, often induced by environmental factors as proven by their high frequency in the population exposed to the fall-out of the Chernobyl accident (Pasini et al., 1996; Rabes and Klugbauer, 1998).

No other mammalian *RET* homologue has been isolated so far, with the exception of the mouse cDNA (Iwamoto et al., 1993). Moreover, both the chicken and the zebrafish *RET* homologous genes have also been cloned (Schuchardt et al., 1995; Marcos-Gutiérrez et al., 1997), and a functional homologue of mammalian ret believed to play a role in neurogenesis has been identified in Drosophila (Sugaya et al., 1994).

As deduced by the expression pattern observed both in mouse and in rat, the RET gene drives, during early stages of the embryogenesis, the migration and/or maturation of specific neural crests cell lineages, thus leading to the development of several cell types among which enteric ganglia, thyroid C cells, pigmentary cells, Schwann cells and medullary cells of the adrenal gland (Pachnis et al., 1993; Tsuzuki et al., 1995; Taraviras et al., 1999). In human, *RET* expression has been confirmed in the developing kidney, enteric neuroblasts, cranial ganglia and in the motor neurons of the spinal cord (Attiè-Bitach et al., 1998). A high degree of conservation of the *RET* expression pattern has also been demonstrated in the zebrafish and in the chicken (Schuchardt et al., 1995; Marcos-Gutiérrez et al., 1997). In agreement with these observations *Ret* knockout homozygous mice showed a lack of enteric ganglia throughout the digestive tract in addition to renal agenesis or dysgenesis (Schuchardt et al., 1994).

In 1995 we isolated a fragment of 300bp of the rat RET cDNA and took advantage of a single nucleotide polymorphism to map this gene on rat chromosome 4, in a region known to share conserved syntheny with mouse chromosome 6, where the same gene had already been localized (Canzian et al., 1995). More recently, a fragment of the rat cDNA encompassing the transmembrane domain and part of the cysteine-rich domain has been isolated and used to study the regulation of RET expression by retinoic acid in rat metanephros (Moreau et al., 1998).

In this paper we report the cDNA sequence of the whole rat RET proto-oncogene, the characterization of two isoforms, described also in human and mouse, due to 3' alternative splicing and the genomic organization in terms of

exon-intron boundaries and intronic sequences flanking each exon.

MATERIALS AND METHODS

RT-PCR and RACE

PCR reactions were set up in a total volume of 50 µl by adding five µl of cDNA diluted at 1:100 to 50 pmoles of each primer, 1X buffer with 1.5 mM $MgCl_2$, 200 µM of each dNTP and 1.25U AmpliTaq DNA Polymerase (Perkin Elmer).

The primers used to carry out RT-PCR and RACE (Rapid Amplification of CDNA Ends) (Frohman, 1993) are listed in Table I.

Reverse transcription of adult rat brain total RNA (Sprague-Dawley rat strain) was performed using the Advantage RT-for-PCR Kit (Clontech) according to the manufacturer's instructions, except that first strand cDNAs were synthetised using primers appropriately designed for each gene portion to be amplified.

The cDNA resulting from reverse transcription with a degenerate primer designed on the basis of the human exon 14 (RR14R), was amplified with nested degenerate oligonucleotides designed on the basis of human exons 10 (RR10F) and 11(RR11F) as forward primers and of human exon 12 (RR12.5R; RR12R) as reverse primers, thus obtaining a fragment of exon 12 of the rat RET proto-oncogene (Figure 1). In particular, we used 40 cycles for the first round and 30 cycles for the second one, both at 94°C (1 min), 65°C (1 min) and 72°C (1 min), with a final extension at 72°C (10 min). The availability of the sequence just obtained, specific for rat RET exon 12, together with a sequence specific for exon 2 (GenBank U22514), allowed us to design rat specific primers to be used to synthetize a first strand cDNA (RRS12.1R) and to amplify the rat RET transcript encompassing exons 2 to 12 (RRS2.1F/RRS12R). The amplification procedure described above was used with an annealing temperature of 60°C and an extension time of 4 min.

TABLE I Sequence of the primers used in RT-PCR and RACE experiments'

name	*sequence (5'->3')*	*sense*	*position within the gene*
Degenerate primers[a]			
RR10	GGAATTCATTAAAGCNGGNTAYGG	forward	exon 10
RR11	GGAATTCGGAGAACCAGGTNYCNGT	forward	exon 11
RR12	GAAGCTTACTTTTCCRAAYTCNCCYTC	reverse	exon 12
RR12.5	GAAGCTTAGCATYTTCACRGCNACNGT	reverse	exon 12
RR14	GAAGCTTTTGGCRTACTCNACDAT	reverse	exon 14
Rat specific primers[b]			
RRS2.1	ATAGAGCAGAGGTGTGCC	reverse	exon 2
RRS2.1	GCTCTATGTCCATGCCCTAC	forward	exon 2
RRS2.2	CACTTCTCCAGGGGCATCC	reverse	exon 2
RRS2.3	CGCCATAGAGATACTGGCCCA	reverse	exon 2
RRS12	GTGGGAATTTCCTCGGAAGA	forward	exon 12
RRS12	TCTTCCGAGGAAATTCCCAC	reverse	exon 12
RRS12.1	TTCTTGGGAAAACCCTGGGA	forward	exon 12
RRS12.1	TCCCAGGGTTTTCCCAAGAA	reverse	exon 12
RRS19.1	TCCCCTCCCGCGCTCCC	forward	exon 19
RRS19.2	CTCCCTTCCACATGGATT	forward	exon 19

a. designed on the basis of the sequence of the human *RET* proto-oncogene
b. designed on the basis of the segments of rat RET fragments already isolated

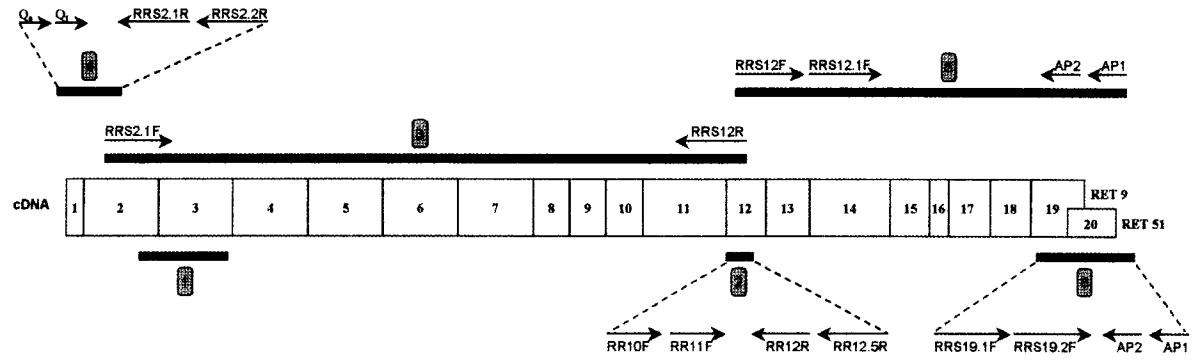

FIGURE 1 Schematic representation of the RT-PCR multistep approach undertaken to clone the rat RET proto-oncogene. Six cDNA fragments (numbered from 1 to 6 and depicted as thick black bars) were combined to reconstruct the whole coding region of this gene. Fragment number 1 had already been reported (Canzian et al, 1995). Arrows flanking each of the fragment indicate the combination of forward and reverse primers used for its amplification (see Table I for the primers sequences)

Rapid amplification of 5′ cDNA ends (5′ RACE) was performed according to the method of Frohman (1993). A first strand cDNA was synthetized, using a primer specific for rat exon 2 (RRS2.3R), and then tailed with addition of ddATP to the 3′-OH end by using terminal transferase (Roche). By using primers RRS2.2R/Q_T, Qo for the first PCR reaction and primers RRS2.1R/Q_I for the second nested PCR reaction, a fragment including the start codon was successively obtained (Figure 1).

Rapid amplification of 3′ cDNA ends (3′ RACE) was performed using the Marathon cDNA Amplification Kit (Clontech). According to the manufacturer's instructions the synthesis of the cDNA was achieved with the modified lock-docking oligo(dT) primer. The successive nested PCR reactions were set up using primers AP1 and AP2 coupled with rat specific primers. In particular primers RRS12F and RRS12.1F were used to generate the rat RET short isoform (RET9), while the primers RRS19.1F and RRS19.2F allowed us to amplify the rat RET long isoform (RET51) (Figure 1).

DNA sequencing

Each cDNA fragment obtained as described above was subcloned by using the TA Cloning Kit (Invitrogen). The clones were sequenced using the Dye primer cycle sequencing kit (Perkin Elmer Applied Biosystem) and analysed on an ABI model 373A DNA automated Sequencer. The sequences were assembled into contiguous fragments and combined to reconstruct the whole rat RET cDNA, whose deduced aminoacid sequence is reported in Figure 2.

Genomic Structure

A YAC clone, isolated from rat YAC library pools (Research Genetics) to contain the whole rat RET proto-oncogene, was used to determine the rat RET intron-exon boundaries by a PCR based strategy. Starting from the rat RET cDNA sequence, primers designed on adjacent exons were used to amplify all the introns (Table II). PCR reactions were set up in 50 μl of total volume containing 20 ng of YAC DNA, 1 μM primers, 1X buffer with 1.5 mM $MgCl_2$, 200 μM of each dNTP and 1.25U AmpliTaq DNA Polymerase (Perkin Elmer) and subjected to 35 cycles at

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Human   MAKATSGAAGLRLLLLLLPLLGKVALGLYFSRDAYWEKLYVDQAAGTPL   50
Mouse   MAKATSGAAGLGLKLILLLPLLGEAPLGLYFSRDAYWERLYVDQPAGTPL   50
Rat     MAKARSGAAGLGLKLFLLLPLLGEAPLGLCFSRDAYWERLYVDQPAGTPL   50

Human   LYVHALRDAPEEVPSFRLGQHLYGTYRTRLHENNWICIQEDTGLLYLNRS   100
Mouse   LYVHALRDAPGEVPSFRLGQHLYGVYRTRLHENDWIRINETTGLLYLNQS   100
Rat     LYVHALRDAPGEVPSFRLGQYLYGVYRTRLHENDWIHIDAGTGLLYLNQS   100

Human   LDHSSWEKLSVRNRGFPLLTVYLKVFLSPTSLREGECQWPGCARVYFSFF   150
Mouse   LDHSSWEQLSIRNGGFPLLTIFLQVFLGSTAQREGECHWPGCTRVYFSFI   150
Rat     LDHSSWEQLSIRNGGFPLLTVFLQVFLGSTAQREGECHWPGCARVYFSFI   150

Human   NTSFPACSSLKPRELCFPETRPSFRIRENRPPGTFHQFRLLPVQFLCPNI   200
Mouse   NDTFPNCSSFKAQDLCIPETAVSSRVRENNPPGTFYHFHMLPVQFLCPNI   200
Rat     NDTFPNCSSFKARDLCTPETGVSFRIRENRPPGTFYQFRMLPVQFLCPNI   200

Human   SVAYRLLEGEGLPFRCAPDSLEVSTRWALDREQREKYELVAVCTVH-AGA   249
Mouse   SVKYSLLGGDSLPFRCDPDCLEVSTRWALDRELREKYVLEALCIVAGPGA   250
Rat     SVKYKLLEGDGLPFRCDPDCLEVSTRWALDRELQEKYVLEAECAVAGPGA   250

Human   REEVVMVPFPVTVYDEDDSAP-TFPAGVDTASAVVEFKRKEDTVVATLRV   298
Mouse   NKETVTLSFPVTVYDEDDSAP-TFSGGVGTASAVVEFKRKEGTVVATLQV   299
Rat     NKEKVAVSFPVTVYDDEDDSPPTFSGGVGTASAVVEFKRKEGTVVATLQV   300

Human   FDADVVPASGELVRRYTSTLLPGDTWAQQTFRVEHWPNETSVQANGSFVR   348
Mouse   FDADVVPASGELVRRYTNTLLSGDSWAQQTFRVEHSPIETLVQVNNNSVR   349
Rat     FDADVVPASGELVRRYTSTLLSGDSWAQQTFRVEHTPNETLVQSNNNSVR   350

Human   ATVHDYRLVLNRNLSISENRTMQLAVLVNDSDFQGPGAGVLLL-HFNVSV   397
Mouse   ATMHNYKLILNRSLSISESRVLQLAVLVNDSDFQGPGAGGILVLHFNVSV   399
Rat     ATMHNYRLVLNRSLSISESRVLQLVVLVNDSDFQGPG-SGFLFLHFNVSV   399

Human   LPVSLHLPSTYSLSVSRRARRFAQIGKVCVENCQAFSGINVQYKLHSSGA   447
Mouse   LPVTLNLPRAYSFPVNKRARRYAQIGKVCVENCQEFSGVSIQYKLQPSSI   449
Rat     LPVTLNLPMAYSFPVNRRARRYAQIGKVCVENCQEFSGVSIQYKLQPSST   449

Human   NCSTLGVVTSAEDTSGILFVNDTKALRRPKCAELHYMVVATDQQTSRQAQ   497
Mouse   NCTALGVVTSPEDTSGTLFVNDTEALRRPECTKLQYTVVATDRQTRRQTQ   499
Rat     NCSALGVVTSTEDTSGTLYVNDTEALRRPECTELQYTVVATDRQTRRQTQ   499

Human   AQLLVTVEGSYVAEEAGCPLSCAVSKRRLECEECGGLGSPTGRCEWRQGD   547
Mouse   ASLVVTVEGTSITEEVGCPKSCAVNKRRPECEECGGLGSPTGRCEWRQGD   549
Rat     ASLVVTVEGTYIAEEVGCPKSCAVNKRRPECEECGGLGSPTGRCEWRQGD   549

Human   GKGITRNFSTCSPSTKTCPDGHCDVVETQDINICPQDCLRGSIVGGHEPG   597
Mouse   GKGITRNFSTCSPSTRTCPDGHCDAVESRDANICPQDCLRADIVGGHERG   599
Rat     GKGTTRNFSTCSPSTRTCPDGHCDALESRDINICPQDCLRGPIVGGHERG   599
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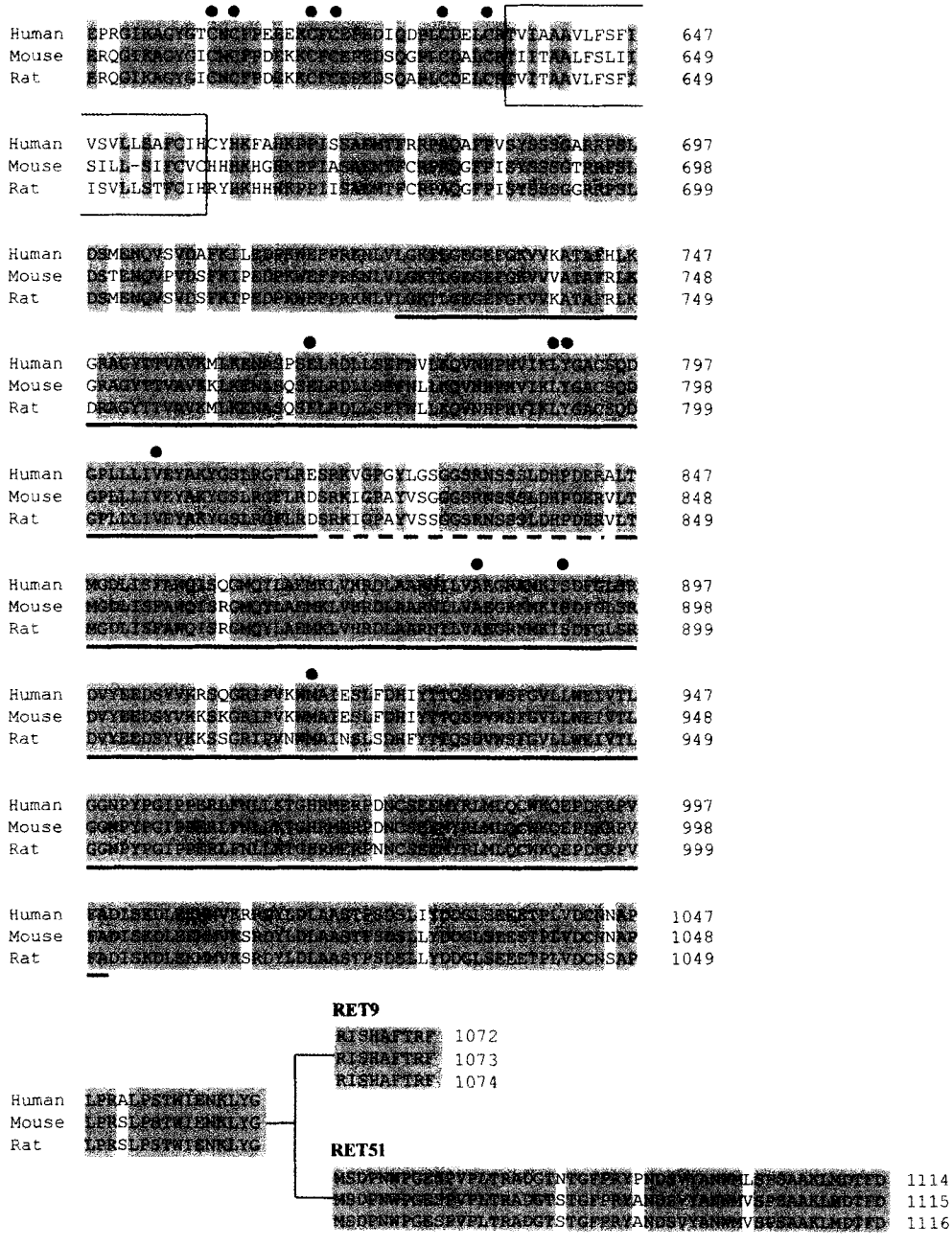

FIGURE 2 The amino acid sequence of the rat RET proto-oncogene, deduced from the nucleotide sequence obtained as described in Figure 1, is shown in comparison with the mouse (Iwamoto et al., 1993) and the human (Takahashi et al., 1988; 1989) homologous proteins. Amino acids (aa) are numbered in the right-hand margin. The transmembrane domain (aa638–660) is boxed and the cadherin like region (aa204–316) is double underlined. The tyrosine kinase domain and its insertion of 27 amino acids (aa821–847) are indicated by single and broken lines, respectively. The amino acid residues, whose mutations are involved in MEN2A, MEN2B, FMCT and sporadic MTC, are indicated by dots. An "X" is present in correspondence of an aminoacid uncertainty. Residues shared by all the three species are boxed in grey. The 3'-end isoforms RET9 and RET51 of rat, mouse and human are shown separately at the end of the sequence

94°C (1 min), 60°C (1 min) and 72°C (3 min). Intron 18 was amplified by long PCR using the DyNAzyme EXT DNA Polymerase Kit (Finzymes) according to the manufacturer's instructions. To isolate the 3′ boundary of intron 1 an inverse PCR method already reported (Ceccherini et al., 1995) was used with primers RATRET-2bF (5′-GCACACCTCTGCTCTATG-3′) and RATRET-2R (5′-TCCACATACAGCCTCTCC-3′). The PCR products thus obtained were subcloned and sequenced as described above. The intron-exon junctions were identified by comparing the sequences obtained from the YAC fragments with the sequence already known of the rat RET cDNA and by recognizing, as further confirmation, the consensus sequences of the donor and acceptor splice sites.

TABLE II Sequence of the primers used to characterize the genomic structure of the rat RET proto-oncogene'

name	*sequence (5′->3′)*	*sense*	*position within the gene*
Rint2	CTGGATCCACATCGATGCG	forward	exon2
Rint2	ACACTCTCCCTCTCTCTGG	reverse	exon3
Rint3	TGGCACCTTCTACCAGTTCC	forward	exon3
Rint3	TCTCCTGAAGCTCACGATCC	reverse	exon4
Rint4	GGTGTATGATGATGAAGACG	forward	exon4
Rint4	GTAGTGTGCTTGTGTACCG	reverse	exon5
Rint5	GCACACACCCAACGAGACC	forward	exon5
Rint5	TGGAAGTCTGAGTCATTGACC	reverse	exon6
Rint6	CCATTTCAACGTGTCTGTGC	forward	exon6
Rint6	AGGGCACTGCAGTTGGTGC	reverse	exon7
Rint7	CGAGCTTCAGTACACAGTGG	forward	exon7
Rint7	CCACACTCCTCACACTCAG	reverse	exon8
Rint8	CTGAGTGTGAGGAGTGTG	forward	exon8
Rint8	GCATCACAGTGGCCATCAGG	reverse	exon9
Rint9	CCACTGTGATGCTCTGGAGA	forward	exon9
Rint9	ACCCTGGCTGTCCTCTGG	reverse	exon10
Rint10	CCAGAGGACAGCCAGGGT	forward	exon10
Rint10	CGAGGAAGAATAGCTGATTG	reverse	exon11
Rint11	CAATCAGCTATTCTTCCTCG	forward	exon11
Rint11	TCTTCCGAGGAAATTCCCAC	reverse	exon12
Rint12	GGAAAAGTAGTCAAGGCCAC	forward	exon12
Rint12	ACCATCCTGGCTGCAAGC	reverse	exon13
Rint13	GCTTGCAGCCAGGATGGT	forward	exon13

name	*sequence (5'->3')*	*sense*	*position within the gene*
Rint13	CAGCTAAGTCTCGATGTACG	reverse	exon14
Rint14	ACCGAGGTTGGGCCTGAC	forward	exon14
Rint14	CAGCTAAGTCTCGATGTACG	reverse	exon15
Rint15	CGTACATCGAGACTTAGCTG	forward	exon15
Rint15	TGAGTGGTATAGAAGTGATCGG	reverse	exon16
Rint16	CCGATCACTTCTATACCACTCA	forward	exon16
Rint16	GGTTGAAGAGTCGTTCAGGA	reverse	exon17
Rint17	TCCTGAACGACTCTTCAACC	forward	exon17
Rint17	GCTCCTGCTTCCAGCACTG	reverse	exon18
Rint18	CTGACATCAGCAAGGATCTGG	forward	exon18
Rint18	AGAGCAGTGAGTCCGAAGGG	reverse	exon19
Rint19	CTCCCTTCCACATGGATT	forward	exon19
Rint19	TTAACTATCAAATGTGTCCAT	reverse	exon20

RESULTS

Isolation of the rat RET cDNA

Starting from a previously obtained fragment of the rat RET gene (Canzian et al., 1995), we have cloned the whole cDNA through a multiple step RT-PCR approach. As depicted in Figure 1, we first isolated two rat RET cDNA fragments including exons 2 and 12 respectively, the cDNA portion lying in between these two fragments was then amplified and, finally, 3' and 5' RACE allowed the isolation of rat RET cDNA fragments containing the stop and the start codons respectively. Two different terminal fragments were isolated by 3'RACE, each corresponding to one of the isoforms (RET9 and RET51) expected on the basis of the human *RET* gene (Tahira et al., 1990). Despite several attemps, we could not isolate the isoform homologous to human RET43.

CDNA sequence of the rat RET proto-oncogene

The complete coding portion of the rat RET proto-oncogene has been reconstructed by combining and ordering the sequences, each determined on both strands, of the different cDNA clones obtained as reported above (EMBL accession numbers from AJ298999 to AJ 299017). The deduced amino acid sequence is reported in Figure 2, in comparison with the human and murine counterparts. The rat RET is highly conserved with respect to the mouse (92%) and the human (84%) homologous genes and, as already noticed in the mouse (Iwamoto et al., 1993), the similarity is higher for the intracellular domain with respect to the extracellular domain. The most evident differences between the rat RET proto-oncogene and its mouse and human counterparts reside in five residues (positions 247, 265, 272 and 654 of the rat sequence and 388 of the mouse sequence) at which we could detect either loss or gain of amino acids (Figure 2). Overall, the rat Ret protein results in one and

two additional amino acid residues with respect to the mouse and the human receptors.

The comparison between our rat RET sequence and the fragment obtained by Moreau et al (1998) from the same gene (nucleotides from 1528 to 2018 corresponding to amino acids from 527 to 672) has revealed differences at codons 553, 621, 629, 657 and 658 (data not shown). They might reflect either disparities in the nucleotide sequence or, most probably, single nucleotide polymorphisms arisen among different rat strains and functionally tolerated in the RET extracellular region.

To verify the presence of exon 21 in the rat RET gene we have compared the rat genomic region corresponding to the 3'UTR of RET51 with the sequence of human exon 21. Figure 3 reports the result of such an alignment, showing a degree of identity between these two sequences of 67%, which is much lower than that calculated in the rest of the gene. The AG donor splice site seems conserved at the 5'end of the putative rat exon 21, while the stop codon is shifted 60bp downstream with respect to the human sequence. Such a DNA exonic segment would encode for an isoform 20 amino acids larger than that expected, whose expression infact has not been detected by our RT-PCR approach.

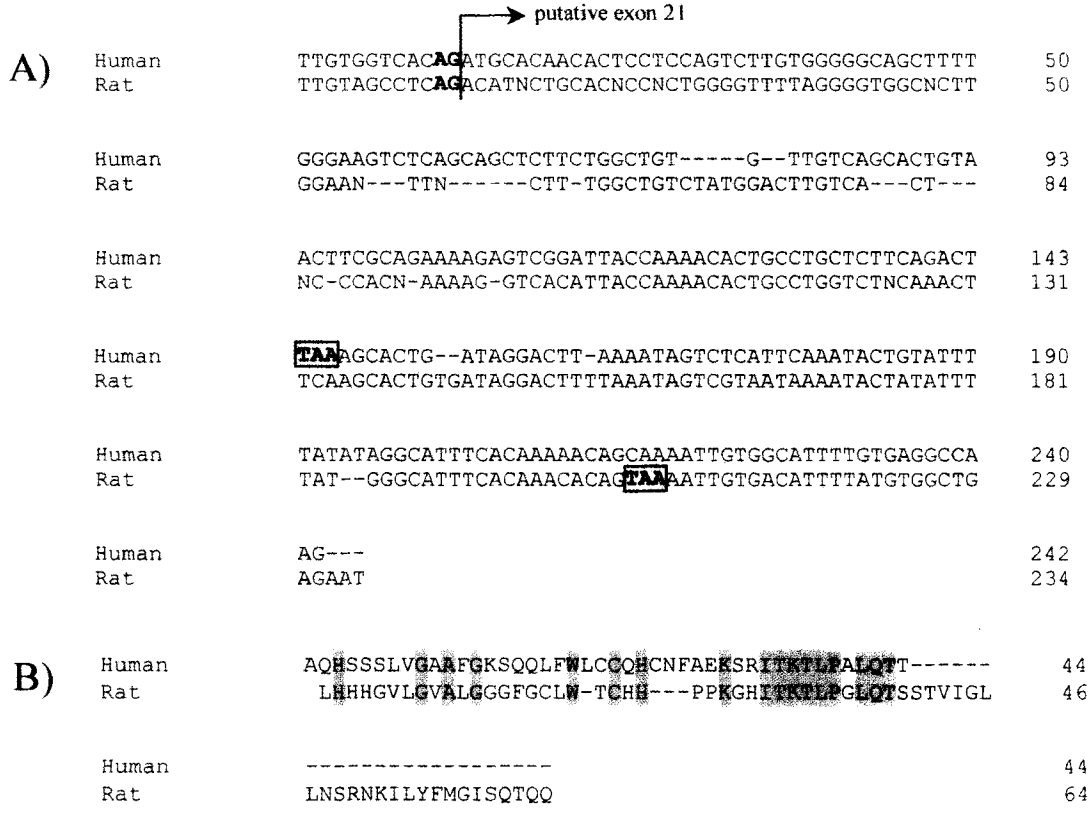

FIGURE 3 Alignment of human and rat putative RET exon 21. **A)** nucleotide sequence corresponding to the exon 21 region of the human and rat RET proto-oncogenes are compared and putative splice sites and stop codons are shown in bold. **B)** the amino acid sequences obtained by translating the above nucleotide sequences are aligned to maximize their similarities, which are restricted to the grey boxes

Characterization of the genomic structure of the rat RET proto-oncogene

Taking advantage of the availability of both the rat RET cDNA sequence and a YAC clone containing the whole rat RET proto-oncogene, we have determined the rat RET intron-exon boundaries by a PCR based strategy. In particular, all introns were amplified using primers designed on adjacent exons, cloned and intron/exon junctions sequenced on one strand only, following an approach already described (Ceccherini et al., 1993). Of all the intronic DNA sequences thus obtained (EMBL accession numbers from AJ298999 to AJ299017), fifteen basepairs located at the 5′ and 3′ends of each intron are reported in Table III, together with introns approximate sizes. Introns boundaries and sizes are all conserved with respect to the human RET genomic structure (Ceccherini et al., 1993), with the exception of the 5.2Kb of intron 18 which could be amplified by using a long PCR method. As expected on the basis of the RET organization in human, intron 1 could not be obtained probably because exceding the size amplifyable. However, its 3′ boundary was amplified through inverse PCR while, despite several attempts, the same approach failed to amplify the 5′, exon 1-intron1 junction.

TABLE III Intronic sequences flanking the 20 exons of the rat RET proto-oncogene with position and estimated length of each intron within its coding sequence

Exon number	*exon size (bp)*	*3′ end of the exon*				*5′ end of the intron*[a]	*approximate size (bp)*	*3′ end of the intron*[a]	*5′ end of the next exon*			
1	73	CTG Leu	GGA Gly	GAA Glu	G	nd	nd	tcatgtctcccacag	CC Ala	CCG Pro	CTG Leu	GGT Gly
2	264	AGC Ser	ATC Ile	CGA Arg	A	gtaagagaacagcc	2100	cttctattcatgcag	AT Asn	GGC Gly	GGC Gly	TTC Phe
3	288	CTC Leu	TTA Leu	GAA Glu	G	gtgagtgccagccc	1300	tctgcgtggtgacag	GG Gly	GAC Asp	GGT Gly	CTG Leu
4	248	AAG Lys	CGG Arg	AAG Lys	GAG Glu	gtttgtccgcagtcg	800	ctctgatacctgcag	GGC Gly	ACT Thr	GTG Val	GTA Val
5	196	CAC His	AAT Asn	TAC Tyr	A	gtaaggagccgacag	500	gtcgcccacctacag	GG Arg	CTG Leu	GTT Val	CTC Leu
6	200	CGT Arg	TAT Tyr	GCC Ala	CAG Gln	gtgagcccatggccc	1900	gatctcccccctccag	ATT Ile	GGG Gly	AAA Lys	GTT Val
7	259	GAG Glu	GGG Gly	ACA Thr	T	gtaagtgtcaggctc	335	tccggcccctcccag	AC Tyr	ATT Ile	GCA Ala	GAA Glu
8	126	GAT Asp	GGT Gly	AAA Lys	G	gtaggttcgggagct	700	tgtactccatgnaag	GG Gly	ACC Thr	ACC Thr	AGG Arg
9	111	GAC Asp	TGT Cys	CTC Leu	C	gtaagcccaggctag	675	ccacatatgtcctcag	GT Arg	GGC Gly	CCC Pro	ATT Ile
10	120	GAC Asp	AGC Ser	CAG Gln	G	gtaaggagcacctct	670	ctctgccctgccacag	CC Ala	CCA Pro	TTG Leu	TGC Cys
11	257	TTC Phe	AAG Lys	ATC Ile	CCG Pro	gtaaggggccccaag	2800	tctacccccaatatag	GAG Glu	GAT Asp	CCG Pro	AAG Lys
12	148	ATG Met	CTG Leu	AAA Lys	G	gtacctgtttagggg	1600	atgtgctgcgtttcag	AA Glu	AAC Asn	GCC Ala	TCC Ser

Exon number	*exon size (bp)*	*3' end of the exon*				*5' end of the intron*[a]	*approximate size (bp)*	*3' end of the intron*[a]	*5' end of the next exon*			
13	108	AGC Ser	CAG Gir	GAT Asp	G	gtaaggctaatcaca	1000	ccccttttgtccccag	GG Gly	CCA Pro	CTT Leu	CTT Leu
14	215	GCT Ala	GAG Glu	ATG Met	AAG Lys	gtgagagccacagat	93	ctgcttcctttctgcag	CTC Leu	GTA Val	CAT His	CGA Arg
15	123	AAG Lys	AAA Lys	AGC Ser	ANG ?	gtacctacccataatt	2100	ttgctcaccaccctttag	GGC Gly	CGG Arg	ATT Ile	CCC Pro
16	71	CAA Gln	AGT Ser	GAT Asp	GT Val	gtaagtatgggagttg	1700	cccttccttgccacag	G	TGG Trp	TCC Ser	TTT Phe
17	138	AGC Ser	GAG Glu	GAA Glu	AT Met	gtgagtntggcttttt	1900	tatgctaccctccag	G	TAC Tyr	CGC Arg	CTG Leu
18	100	GTC Val	AAA Lys	AGC Ser	AGA Arg	gtgagtcccagcgtc	5200	ctctctctcttccag	GAC Asp	TAC Tyr	TTG Leu	GAC Asp
19	148	AAA Lys	CTC Leu	TAT Tyr	G	gtcaacgcagtccc	1300	tctgttttcatttttag	GC Gly	ATG Met	TCA Ser	GAC Asp
20	158	TTT Phe	GAT Asp	AGC Ser	TAA Stop							

nd: not determined

a. these sequences have been determined only on one strand

DISCUSSION

The complete coding portion of the rat RET proto-oncogene has been isolated, sequenced and compared with the human and murine homologous genes. A very high degree of conservation has been confirmed among these three mammalian species at both the nucleotide and the amino acid level (Figure 2). With the exception of residue Leu654 which is included in the TM domain, all the other differences lie in the extracellular domain, and in particular within or near the cadherin homology domain. This may reflect the fact that in this region there is no strict need for specific amino acids or spacing among crucial residues, to mantain a correct folding and therefore a normal function of the Ret receptor, while effective catalytic Ret function may have tolerated no structural change. Accordingly, codons whose mutations are associated with MEN2A, MEN2B and Familial Medullary Thyroid Carcinoma (FMTC) (Pasini et al., 1996; Eng and Mulligan 1997), as well as codons whose mutations cause HSCR disease (Hofstra et al., 1997), are unchanged in the three species considered, with the only exception of one HSCR mutation reported to change codon Glu251 to Lys (Attiè et al., 1995). Since a Lys residue is present at the same position both in the mouse and in the rat, a role of such substitution in the loss of function of *RET*, leading to a defect of intestinal innervation, is unlikely.

A high degree of homology has also been observed between the rat and human RET genes in the localization and sizes of their intervening sequences. Only intron 1 could not be isolated from the rat gene, probably because as large as its human counterpart (about 23 Kb), while intron 18 has resulted much larger than the human homologous intervening sequence (5.2Kb vs 1.6Kb), suggesting that a loss of meaningless DNA must have occurred during evolution.

The high degree of conservation of both the cDNA sequence and the genomic organization

of the rat RET proto-oncogene, with respect to different mammalian species, confirms the critical role played by this receptor in basic developmental functions. The conservation in the rat of the RET9 and RET51 isoforms (Tahira et al., 1990; Iwamoto et al., 1993) is also suggestive of the great importance they must have in RET mediated development. Conversely, we have failed both to isolate the rat RET43 isoform by RT-PCR and to identify the rat homologue of human exon 21 by comparative sequence analysis (Figure 3). On the basis of our results we therefore conclude that exon 21 is not conserved in the rat genome and postulate that the RET43 isoform observed in human (Myers et al., 1995) might have a not fundamental functional meaning, if any. The availability of the genomic sequences corresponding to different evolutionary versions of a gene can facilitate the identification of regions playing relevant functional or regulatory roles (Oeltjen et al, 1997; Hardison et al, 1997). Starting from such a consideration, we have performed a comparison of intronic sequences between the human and the rat RET genes by using a BLAST2 Software (http://www.ncbi.nlm.nih.gov/gorf/wblast2), and found a very high degree of identity, ranging from 79% to 88%, in specific stretches of both the 5′ and 3′ end of intron 19 and the 3′UTR of the RET51 isoform (data not shown). This is suggestive of a non random conservation during evolution of these distal DNA portions, which might be due to the need of avoiding loss of sequences crucial for the generation of the differently spliced 3′ isoforms. No other non coding portion of the rat RET gene sequenced so far has shown any similarity to its human counterpart. A similar comparative sequence analysis, performed on chromosome regions flanking the RET gene, will allow to correlate genomic sequences to gene function and basic biology thus identifying, for istance, the still unknown sequences driving the fine regulation of *RET* expression.

The characterization of the rat RET genomic structure represents a useful mean to perform mutation screening for defining the possible role of the RET proto-oncogene in the spontaneous and induced development of rat thyroid cancer. Different rat strains have been studied so far as in *vivo* models of thyroid carcinogenesis (Fernandez Rodriguez et al., 1991; Hiasa et al., 1992; Kitahori et al., 1997) but many of them still awaits a molecular characterization and RET represents one of the most promising candidate genes to test.

Finally, the isolation of the rat RET gene described here may contribute to the genetic characterization of one of the most important experimental organism, to the development of its genomic map and sequence, and to the collection of molecular tools and data, which are projects in rapid expansion as documented by the large amount of data regarding the genetics and the molecular genomics of the rat, currently released both in the literature and in public databases (Jacob 1999; Steen et al., 1999; Watanabe et al., 1999).

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