MOLECULAR AND CELLULAR BIOLOGY, Jan. 1993, p. 358–366
0270-7306/93/010358-09$02.00/0

Vol. 13, No. 1

Molecular Characterization of a Thyroid Tumor-Specific Transforming Sequence Formed by the Fusion of *ret* Tyrosine Kinase and the Regulatory Subunit RIα of Cyclic AMP-Dependent Protein Kinase A

I. BONGARZONE,* N. MONZINI, M. G. BORRELLO, C. CARCANO, G. FERRARESI, E. ARIGHI, P. MONDELLINI, G. DELLA PORTA, AND M. A. PIEROTTI

Division of Experimental Oncology A, Istituto Nazionale Tumori, Via G. Venezian 1, 20133 Milan, Italy

Received 6 August 1992/Returned for modification 10 September 1992/Accepted 21 October 1992

The *ret* oncogene frequently has been found activated in papillary thyroid carcinomas. A previous characterization of *ret* activation revealed recombination of its tyrosine kinase domain and sequences derived from an uncharacterized locus (D10S170). The mechanism leading to this recombination was identified as a paracentric inversion of the long arm of chromosome 10, inv(10)(q11.2q21), with the breakpoints occurring where *ret* and D10S170 were mapped. To further characterize the activation of *ret* in papillary thyroid carcinomas, we have now isolated and sequenced a second type of *ret* oncogenic rearrangement not involving the D10S170 locus. The nucleotide sequence indicated that the transforming activity was created by the fusion of the *ret* tyrosine kinase domain with part of the RIα regulatory subunit of protein kinase A (PKA). This is the first example of an oncogenic activity involving a PKA gene. PKA is the main intracellular cyclic AMP receptor, and its RIα subunit gene is located on chromosome 17q. RIα-*ret* transcripts encode two isoforms of the chimeric protein (p76 and p81), which display constitutive tyrosine phosphorylation as well as a tyrosine kinase enzymatic activity. Under nonreducing conditions, both isoforms are found in a dimeric configuration because of both homo- and heterodimer formation. Thus, the in vivo activation of *ret* in human papillary thyroid carcinomas is provided by the fusion of its tyrosine kinase domain with different genes and can be mediated by different mechanisms of gene rearrangement.

Tumor-specific rearrangements of the proto-oncogenes *ret* and *trk*, encoding tyrosine kinase membrane receptors, have been detected with a high frequency in human papillary thyroid carcinomas (3, 10, 11, 24). The oncogenic activation of these genes is accomplished by the fusion of their tyrosine kinase domain with unlinked amino-terminal sequences following intrachromosomal rearrangements involving chromosome 10 and chromosome 1 in the case of *ret* and *trk*, respectively (10, 24). In particular, a paracentric inversion of the long arm of chromosome 10, inv(10)(q11.2q21), was shown to provide the structural basis for the fusion between *ret* and D10S170 (19), a locus formerly designated H4 (11) and coding for an as-yet-uncharacterized protein which leads to the generation of the transforming sequence *ret*/PTC. Following a transfection assay and Southern blot analysis, we have detected oncogenic activation, due to a DNA rearrangement, of the *ret* proto-oncogene in 14 of 42 cases of papillary thyroid carcinomas. The involvement of the D10S170 locus was found in nine cases forming the same *ret*/PTC chimeric transforming sequence (hereafter designated *ret*/PTC1). In the remaining five cases, Southern blot analysis of the respective NIH 3T3 transformants failed to reveal the presence of the D10S170 sequence (2). To further characterize the mechanisms leading to oncogenic activation and to increase our knowledge of the properties of the sequences fused with the tyrosine kinase domain of *ret*, we cloned and sequenced a cDNA related to a first novel transforming rearrangement of the *ret* proto-oncogene. Analysis of the cDNA sequence indicated that the tyrosine kinase domain of the *ret* proto-oncogene was fused with sequences belonging to the gene encoding regulatory subunit RIα of cyclic AMP (cAMP)-dependent protein kinase A (PKA) (22). A preliminary biochemical analysis of the transformation product indicated that, in NIH 3T3 transformants, two isoforms of the hybrid RIα-*ret* protein were present in dimeric forms and that they displayed constitutive tyrosine phosphorylation.

MATERIALS AND METHODS

Nucleic acid extraction and blot hybridization. Genomic DNA extraction, Southern blotting, and hybridization were performed as previously described (3). Total RNA was extracted by the method of Chomczynski and Sacchi (6). For Northern (RNA) blotting, poly(A)$^+$ selection was performed with Amersham Hybond m-Ap paper as suggested by the manufacturer. Ten micrograms of total RNA was electrophoresed, blotted on a nitrocellulose membrane (MSI), subjected to hybridization at 42°C in a buffer containing 50% formamide, 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 1× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 100 μg of single-stranded salmon sperm DNA per ml, and washed at 60°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS. ^{32}P-labeled probes were prepared by the multiprime DNA labeling system (9).

cDNA cloning and sequencing. cDNA synthesis was performed by use of an Amersham kit with oligo(dT)-primed NIH 3T3 transformant poly(A)$^+$ mRNA. The cDNA was ligated into the *Eco*RI site of λ-gt10 (Stratagene). Phage DNA was packaged by use of packaging extracts (GIGA

* Corresponding author.

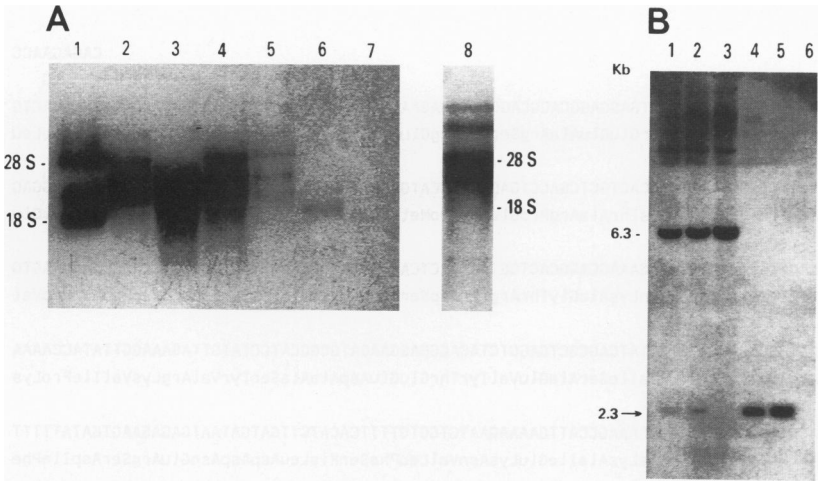


FIG. 1. Detection of human *ret*-related transforming sequences in NIH 3T3 transformants. (A) Northern blot analysis of total RNAs from NIH 3T3 cells transformed by DNAs from different papillary thyroid carcinomas (lanes 1 to 6). The results obtained with mouse (untreated NIH 3T3 cells, lane 7) and human SK-N-SH neuroblastoma cell line (lane 8) control RNAs are also shown. The blot was hybridized with a *ret* tyrosine kinase domain-specific probe (11). (B) Southern blot analysis of *Eco*RI-digested DNAs from a primary tumor (lane 1), a lymph node metastasis sample (lane 2), normal peripheral blood cells of patient 65 (lane 3), NIH 3T3 transformants of the primary tumor (lane 4) and the metastasis sample (lane 5), and an NIH 3T3 control cell line (lane 6). The blot was hybridized with a genomic *ret* proto-oncogene probe (probe 2 of reference 19). Human *ret*-related rearranged bands are indicated.

gold; Stratagene). A total of 5×10^5 PFU was plated onto each of 10 plates (243 mm). GeneScreen Plus (Dupont, NEN) filters from each plate were probed with an *Eco*RI-*Bgl*II fragment derived from the *ret*/PTC1 cDNA sequence (11) and washed under high stringency. Positive clones were purified, and the longest cDNA was isolated and inserted into plasmid pBS (Stratagene) by standard protocols (17). Nucleotide sequencing was performed by the dideoxy chain termination method (23) with a Sequenase kit (U.S. Biochemicals Corp.) primed with the T3 and T7 promoters or with primers specific for the *ret* and RIα sequences, synthesized by use of an Applied Biosystems (Foster City, Calif.) synthesizer. A computer-assisted homology search of the EMBL data bank was performed for known DNA sequences.

Cloning of *ret*/PTC2 in the pMAM-neo expression vector and transfection. To synthesize *ret*/PTC2 cDNA with a flanking *Xba*I restriction site suitable for cloning into the pMAM-neo (Clontech) vector, we performed a polymerase chain reaction (PCR) with an F2 phage DNA clone and synthetic oligonucleotide primers complementary to the left (N7) and right (N8) cohesive termini of λ-gt10 and containing the *Xba*I restriction site. The primers used were as follows: 5′-GCTCTAGAAGCAAGTTCAGCCTGGTTAAG-3′ (N7, left) and 5′-GCTCTAGACTTATGAGTATTTCTTCCAGG GTA-3′ (N8, right); the added linkers are underlined. PCR was performed by amplifying inserted cDNA with the N7 and N8 primers for 30 cycles (1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min of elongation at 72°C). Amplified fusion cDNA was visualized by ethidium bromide staining. After elution from the gel, the amplified product was digested with restriction enzyme *Xba*I and cloned into the *Xba*I site of dephosphorylated pMAM-neo. Multiple independent recombinant clones were analyzed to isolate plasmids with sense or antisense orientations of the insert.

NIH 3T3 cells were transfected by $CaPO_4$ coprecipitation as previously described (3). Ten nanograms of plasmid DNA and 20 μg of mouse DNA per 78-cm^2 plate were used. Transfected cells were selected for resistance to G418, and G418-resistant clones were treated with 1.5 mM dexamethasone to induce the expression of the *ret*/PTC2 insert. Foci of transformed cells appearing 3 weeks later were quantitated after Giemsa staining.

Antibodies. Anti-*ret* antibodies were raised in rabbits immunized with the following synthetic peptides conjugated to ovoalbumin (Neosystem, Strasbourg, France) (see Fig. 3B): KRRDYLDLAASTPSDSL, derived from amino acids 1011 to 1027 of the C-terminal sequence common to the two isoforms, for the anti-common peptide serum (anti-RET common); ENKYGRISHAFTRF, derived from the 9-amino-acid C-terminal isoform, for the anti-RET 9 serum (anti-RET 9); and finally LSPSAAKLMDTFDS, derived from the 51-amino-acid C-terminal isoform, for the anti-RET 51 serum (anti-RET 51) (26). The sera were purified on Affigel columns to which the respective synthetic peptides were covalently bound (30). Antisera operationally defined as antiphosphotyrosine sera were raised in rabbits immunized with azobenzylphosphonate conjugated to hemocyanin from *Megathura crenulata*. Azobenzylphosphonate was synthesized by the method of Landt et al. (15) and linked to the carrier as described by Ross et al. (21). The antiphosphotyrosine antibodies were affinity purified on columns containing immobilized phosphotyrosine-bovine serum albumin as reported by Cirillo et al. (7); their specificities have been reported (16). Monoclonal antibodies directed against the product of the RIα gene expressed in human testis were kindly provided by B. Skälhegg (University of Oslo, Oslo, Norway).

Immunoprecipitation and Western blotting (immunoblotting). Cell lysates and immunoprecipitates were prepared as follows. The cultures were washed twice with cold phosphate-buffered saline and solubilized on ice in lysis buffer (10 mM sodium phosphate [pH 7], 100 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml). The cells were scraped, and the lysates were collected into Eppendorf tubes and clarified by centrifugation at 15,000 × *g* for 30 min at

CAGAGAACC 9
G
ATGGAGTCTGGCAGTACCGCCGCCAGTGAGGAGGCACGCAGCCTTCGAGAATGTGAGCTCTACGTCCAGAAGCATAACATTCAAGCACTG 99
MetGluSerGlySerThrAlaAlaSerGluGluAlaArgSerLeuArgGluCysGluLeuTyrValGlnLysHisAsnIleGlnAlaLeu 30

CTCAAAGATTCTATTGTGCAGTTGTGCACTGCTCGACCTGAGAGACCCATGGCATTCCTCAGGGAATACTTTGAGAGGTTGGAGAAGGAG 189
LeuLysAspSerIleValGlnLeuCysThrAlaArgProGluArgProMetAlaPheLeuArgGluTyrPheGluArgLeuGluLysGlu 60

GAGGCAAAACAGATTCAGAATCTGCAGAAAGCAGGCACTCGTACAGACTCAAGGGAGGATGAGATTTCTCCTCCTCCACCCAACCCAGTG 279
GluAlaLysGlnIleGlnAsnLeuGlnLysAlaGlyThrArgThrAspSerArgGluAspGluIleSerProProProProAsnProVal 90

GTTAAAGGTAGGAGGCGACGAGGTGCTATCAGCGCTGAGGTCTACACGGAGGAAGATGCGGCATCCTATGTTAGAAAGGTTATACCAAAA 369
ValLysGlyArgArgArgArgGlyAlaIleSerAlaGluValTyrThrGluGluAspAlaAlaSerTyrValArgLysValIleProLys 120

GATTACAAGACAATGGCCGCTTTAGCCAAAGCCATTGAAAAGAATGTGCTGTTTTCACATCTTGATGATAATGAGAGAAGTGATATTTTT 459
AspTyrLysThrMetAlaAlaLeuAlaLysAlaIleGluLysAsnValLeuPheSerHisLeuAspAspAsnGluArgSerAspIlePhe 150

GATGCCATGTTTTCGGTCTCCTTTATCGCAGGAGAGACTGTGATTCAGCAAGGTGATGAAGGGGATAACTTCTATGTGATTGATCAAGGA 549
AspAlaMetPheSerValSerPheIleAlaGlyGluThrValIleGlnGlnGlyAspGluGlyAspAsnPheTyrValIleAspGlnGly 180

GAGACGGATGTCTATGTTAACAATGAATGGGCAACCAGTGTTGGGGAAGGAGGGAGCTTTGGAGAACTTGCTTTGATTTATGGAACACCG 639
GluThrAspValTyrValAsnAsnGluTrpAlaThrSerValGlyGluGlyGlySerPheGlyGluLeuAlaLeuIleTyrGlyThrPro 210

▼
AGAGCAGCCACTGTCAAAGCAAAGACAAATGTGAAATTGTGGGGCATCGACCGAGACAGCTATAGAAGAATCCTCATGGAGGATCCAAAG 729
ArgAlaAlaThrValLysAlaLysThrAsnValLysLeuTrpGlyIleAspArgAspSerTyrArgArgIleLeuMetGluAspProLys 240
┌—>TK RIα <——┴——> ret
TGGGAATTCCCTCGGAAGAACTTGGTTCTTGGAAAAACTCTAGGAGAAGGCGAATTTGGAAAAGTGGTCAAGGCAACGGCCTTCCATCTG 819
TrpGluPheProArgLysAsnLeuValLeuGlyLysThrLeuGlyGluGlyGluPheGlyLysValValLysAlaThrAlaPheHisLeu 270

AAAGGCAGAGCAGGGTACACCACGGTGGCCGTGAAGATGCTGAAAGAGAACGCCTCCCCGAGTGAGCTTCGAGACCTGCTGTCAGAGTTC 909
LysGlyArgAlaGlyTyrThrThrValAlaValLysMetLeuLysGluAsnAlaSerProSerGluLeuArgAspLeuLeuSerGluPhe 300

AACGTCCTGAAGCAGGTCAACCACCCACATGTCATCAAATTGTATGGGGCCTGCAGCCAGGATGGCCCGCTCCTCCTCATCGTGGAGTAC 999
AsnValLeuLysGlnValAsnHisProHisValIleLysLeuTyrGlyAlaCysSerGlnAspGlyProLeuLeuLeuIleValGluTyr 330

GCCAAATACGGCTCCCTGCGGGGCTTCCTCCGCGAGAGCCGCAAAGTGGGGCCTGGCTACCTGGGCAGTGGAGGCAGCCGCAACTCCAGC 1089
AlaLysTyrGlySerLeuArgGlyPheLeuArgGluSerArgLysValGlyProGlyTyrLeuGlySerGlyGlySerArgAsnSerSer 360

TCCCTGGACCACCCGGATGAGCGGGCCCTCACCATGGGCGACCTCATCTCATTTGCCTGGCAGATCTCACAGGGGATGCAGTATCTGGCC 1179
SerLeuAspHisProAspGluArgAlaLeuThrMetGlyAspLeuIleSerPheAlaTrpGlnIleSerGlnGlyMetGlnTyrLeuAla 390

GAGATGAAGCTCGTTCATCGGGACTTGGCAGCCAGAAACATCCTGGTAGCTGAGGGGCGGAAGATGAAGATTTCGGATTTCGGCTTGTCC 1269
GluMetLysLeuValHisArgAspLeuAlaAlaArgAsnIleLeuValAlaGluGlyArgLysMetLysIleSerAspPheGlyLeuSer 420

CGAGATGTTTATGAAGAGGATCCCTACGTGAAGAGGAGCCAGGGTCGGATTCCAGTTAAATGGATGGCAATTGAATCCCTTTTTGATCAT 1359
ArgAspValTyrGluGluAspProTyrValLysArgSerGlnGlyArgIleProValLysTrpMetAlaIleGluSerLeuPheAspHis 450

ATCTACACCACGCAAAGTGATGTATGGTCTTTTGGTGTCCTGCTGTGGGAGATCGTGACCCTAGGGGGAAACCCCTATCCTGGGATTCCT 1449
IleTyrThrThrGlnSerAspValTrpSerPheGlyValLeuLeuTrpGluIleValThrLeuGlyGlyAsnProTyrProGlyIlePro 480

CCTGAGCGGCTCTTCAACCTTCTGAAGACCGGCCACCGGATGGAGAGGCCAGACAACTGCAGCGAGGAGATGTACCGCCTGATGCTGCAA 1539
ProGluArgLeuPheAsnLeuLeuLysThrGlyHisArgMetGluArgProAspAsnCysSerGluGluMetTyrArgLeuMetLeuGln 510
TK<——┐
TGCTGGAAGCAGGAGCCGGACAAAAGGCCGGTGTTTGCGGACATCAGCAAAGACCTGGAGAAGATGATGGTTAAGAGGAGAGACTACTTG 1629
CysTrpLysGlnGluProAspLysArgProValPheAlaAspIleSerLysAspLeuGluLysMetMetValLysArgArgAspTyrLeu 540

GACCTTGCGGCGTCCACTCCATCTGACTCCCTGATTTATGACGACGGCCTCTCAGAGGAGGAGACACCGCTGGTGGACTGTAATAATGCC 1724
AspLeuAlaAlaSerThrProSerAspSerLeuIleTyrAspAspGlyLeuSerGluGluGluThrProLeuValAspCysAsnAsnAla 570

CCCCTCCCTCGAGCCCTCCCTTCCACATGGATTGAAAACAAACTCTATGGTAGAATTTCCCATGCATTTACTAGATTCTAGCACCGCTGT 1819
ProLeuProArgAlaLeuProSerThrTrpIleGluAsnLysLeuTyrGlyArgIleSerHisAlaPheThrArgPhe***

CCCCTCTGCACTATCCTTCCTCTCTGTGATGCTTTTTAAAAATGTTTCTGGTCTGAAC 1867

4°C. The supernatants were incubated with protein A-Sepharose (Pharmacia), precoated with various antibodies, for 2 h at 4°C. After three washes with lysis buffer, the immunocomplexes were eluted by boiling in 50 μl of Laemmli buffer (14) for 5 min. For Western blotting under nonreducing conditions, 2-mercaptoethanol was omitted.

Immunoprecipitates were resolved by electrophoresis on SDS-polyacrylamide gels (6.5 to 8.5%). Proteins were transferred to nitrocellulose filters and immunoblotted with various antisera essentially as described by Towbin et al. (28). Immunoreactive bands were visualized by use of ^{125}I-labeled protein A and then autoradiographed.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article have been entered in the EMBL nucleotide sequence data library under accession number L03357.

RESULTS

Detection of novel *ret*/PTC transforming rearrangements in human papillary thyroid carcinomas. A Northern blot analysis was performed to investigate the sizes of *ret* mRNAs expressed in the NIH 3T3 cell lines transformed by the different oncogenic versions of the *ret* proto-oncogene. The probe used consisted of an *Eco*RI-*Bgl*II cDNA fragment from the 5′ region of the tyrosine kinase domain of the gene (11). The results reported in Fig. 1A demonstrate that, compared with the SK-N-SH human control cell line (lane 8), all six NIH 3T3 transformants, including that transformed by previously characterized *ret*/PTC1 (lane 1), expressed different patterns of truncated *ret*-related transcripts (lanes 2 to 6).

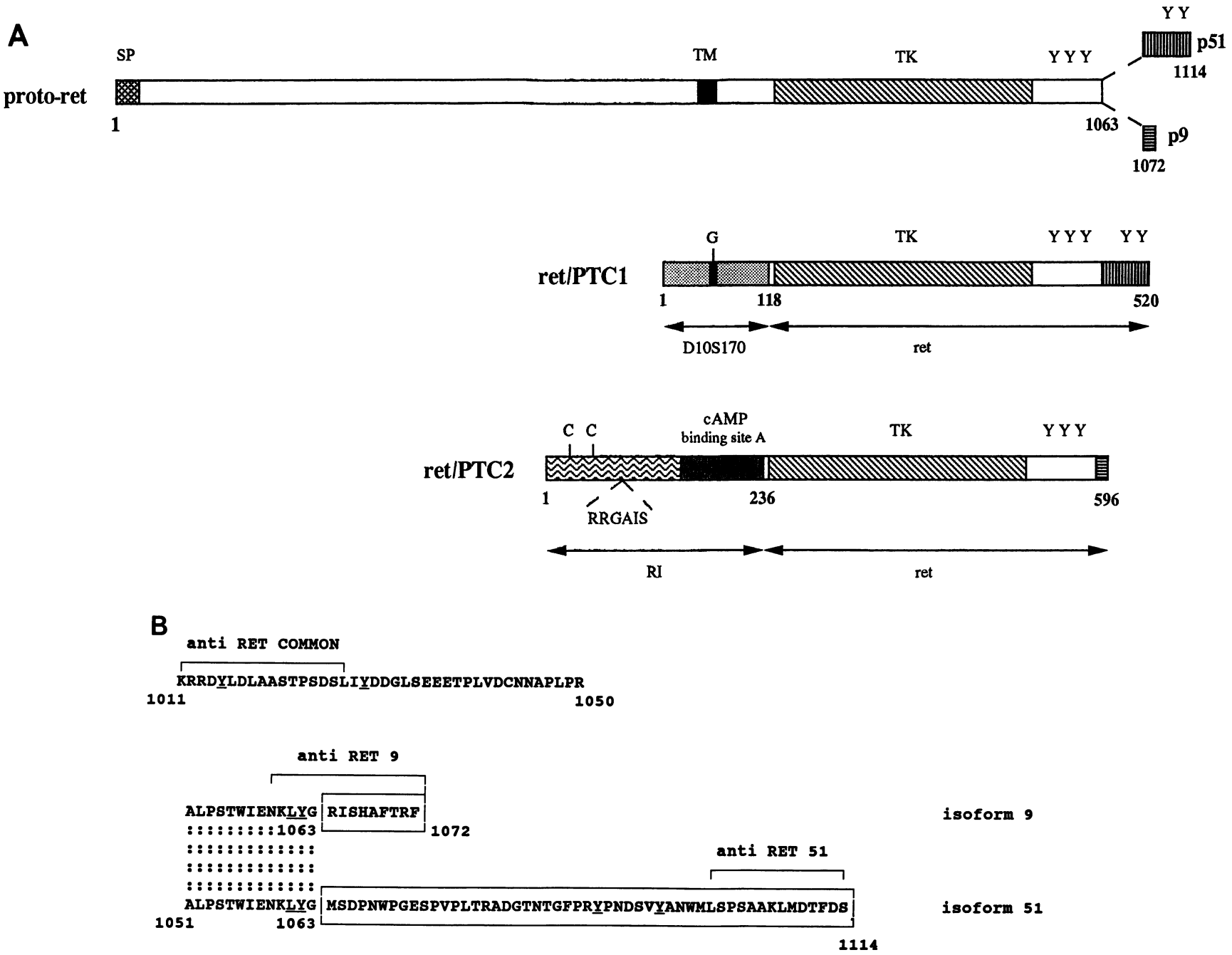
Following this observation, we decided to further characterize these putative new *ret*-transforming sequences. We started with case 65 (Fig. 1A, lane 2), which represented the first case of *ret* activation without involvement of the D10S170 locus. The transforming sequence present in this case was designated *ret*/PTC2 (16). Figure 1B demonstrates the presence of a *ret*-related rearranged band of 2.3 kb in *Eco*RI-restricted DNA from primary tumor 65 (lane 1), a metastasis sample from the same patient (lane 2), and the derived NIH 3T3 transformant cell lines (lanes 4 and 5) when a 1-kb *Bgl*II-*Bam*HI genomic fragment of the *ret* proto-oncogene (probe 2 described in Fig. 2 of reference 19) was used as a probe. In normal *Eco*RI-restricted human DNA, this probe detects a *ret* germ line band of 6.3 kb (Fig. 1B). The tumor specificity of the 2.3-kb band was demonstrated by its absence in the normal DNA obtained from lymphocytes of the same patient (Fig. 1B, lane 3) and in that derived from NIH 3T3 cells (lane 6). These results indicate that the rearrangement of *ret* occurred somatically in the original tumor cells of case 65 and that the 2.3-kb *ret*-related rearranged band was cosegregated with the transforming activity of that tumor DNA.

Molecular cloning of the new *ret*/PTC2 transforming sequence, isolation of cDNA, and sequencing. To elucidate the structure of the *ret*/PTC2 transforming sequence, we constructed an oligo(dT)-primed cDNA library from NIH 3T3 cells transformed by DNA from the primary tumor of patient 65 by using the λ-gt10 phage as a vector. Part of the library (5×10^5 PFU) was screened with a *ret*-related cDNA probe (11); eight positive clones were isolated and characterized by restriction mapping (data not shown). The longest (clone F2) was about 1.85 kb long. Clone F2 was cloned into the *Eco*RI site of the Bluescript (Stratagene) plasmid vector, and its sequence was determined by the dideoxy chain termination method. The analysis of the obtained sequence of 1,867 nucleotides (Fig. 2) revealed the following features. An open reading frame was identified starting at nucleotide 10 and ending at nucleotide 1788; it encoded a putative polypeptide of 596 amino acids. The sequence appeared to be colinear with the *ret* proto-oncogene cDNA sequence starting from the primer at the 3′ end of the clone for 1,149 nucleotides. From this point, the sequence differed completely from that of the *ret* proto-oncogene. The matching region comprised the entire tyrosine kinase domain of the *ret* proto-oncogene. No mutation was found in the coding sequence of *ret*, suggesting that the activation of *ret* occurred solely by a DNA rearrangement. When the sequence upstream of position 718 was compared with known nucleotide sequences by computer-assisted analysis, it was found that this portion of the transforming sequence was nearly identical to the 5′ end of the human RIα gene, which encodes the regulatory subunit of the type I cAMP-dependent PKA (22). Namely, this sequence coincided with the first 717 nucleotides of the RIα gene, except for one conservative change (G→A) in a nucleotide at position 96 (Fig. 2). Thus, this activated *ret* gene is predicted to encode a fusion protein consisting of 236 amino-terminal amino acid residues of the RIα gene product and 360 carboxy-terminal amino acid residues of the normal *ret* protein (Fig. 2 and 3A).

As shown in Fig. 3A, the fusion point of the RIα-*ret* transforming sequence (*ret*/PTC2) is located at the same position in the *ret* portion as that found for D10S170-*ret* in *ret*/PTC1 (11), i.e., between the transmembrane and tyrosine kinase domains of the *ret* proto-oncogene. On the other hand, Fig. 3 shows that the carboxy terminus of *ret*/PTC2 is different from that of *ret*/PTC1 because of an alternative splicing of the *ret* proto-oncogene, as discussed below.

Biological activity of the chimeric *ret*/PTC2 cDNA. *ret*/PTC2 cDNA clone F2 was inserted into the *Xba*I site of the pMAM-neo plasmid expression vector. For this purpose, F2 cDNA was amplified by PCR with primers (N7 and N8 described in Materials and Methods) located on each side of λ-gt10. The obtained product was digested with *Xba*I and inserted in both sense and antisense orientations with respect to the Rous sarcoma virus promoter and the dexamethasone-inducible mouse mammary tumor virus long terminal repeat present in the vector to produce pGF1 (sense) and pGF2 (antisense) expression plasmids. After transfection into NIH 3T3 cells, selection for resistance to G418 and treatment with dexamethasone were performed. Plasmid pGF1 showed significant transforming capacity (about 3,500 focus-forming units per μg of DNA), whereas the vector alone or the plasmid expressing the antisense *ret*/PTC2 sequence (pGF2) yielded no transformants. These results demonstrate that the pGF1 insert contains the entire sequence essential for the biological activity of transforming

FIG. 2. cDNA sequence and deduced amino acid sequence of the λ-gt10 F2 insert. The position of the RIα-*ret* fusion point is indicated by a solid triangle (nucleotides 717 and 718). The proposed ATG initiation codon and the 3′ sequence that belongs to a cloned shorter form of the *ret*/PTC2 protein (see Fig. 3B) are underlined by thin and thick solid lines, respectively. The only nucleotide difference between the RIα gene (22) and the RIα portion of *ret*/PTC2 is shown above the sequence at nucleotide 96. TK, tyrosine kinase.


A
SP
TM
TK
Y Y Y
Y Y
p51
1114
proto-ret
1
1063
p9
1072
G
TK
Y Y Y
Y Y
ret/PTC1
1
118
520
D10S170
ret
C C
cAMP
binding site A
TK
Y Y Y
ret/PTC2
1
236
596
RRGAIS
RI
ret
B
anti RET COMMON
KRRDYLDLAASTPSDSLIYDDGLSEEETPLVDCNNAPLPR
1011
1050
anti RET 9
ALPSTWIENKLYG
RISHAFTRF
:::::::::1063
1072
isoform 9
anti RET 51
ALPSTWIENKLYG
MSDPNWPGESPVPLTRADGTNTGFPRYPNDSVYANWMLSPSAAKLMDTFDS
1051
1063
1114
isoform 51

sequence *ret*/PTC2 generated by the tumor-specific RIα-*ret* rearrangement.

Characterization of *ret*/PTC2 proteins in NIH 3T3 transformants. As predicted by Ishizaka et al. (12) and Tahira et al. (25), Fig. 3A shows that because of alternative splicing, the *ret* proto-oncogene could generate two isoforms of the *ret* protein differing at the carboxy terminus, one 51 and the other 9 amino acids long. By comparing the carboxy termini of the cloned *ret*/PTC1 and *ret*/PTC2 transforming sequences, we found that they were derived from the longer and shorter isoforms, respectively (Fig. 3A). Moreover, in preliminary experiments with a PCR after reverse transcriptase and isoform-specific primers, we identified both forms of the *ret*/PTC2 cDNA in NIH 3T3 transformants (data not shown). Therefore, to identify the two putative protein isoforms derived from the activation of the *ret* proto-oncogene, we generated three different rabbit polyclonal antisera (Fig. 3B). They were directed against a 17-amino-acid peptide belonging to a portion of the protein shared between the two isoforms and a 14-amino-acid peptide representing a region totally unique to the 51-amino-acid isoform. Since immunization with a peptide of only 9 amino acids was inefficient, antiserum against the 9-amino-acid isoform was generated by use of a 14-amino-acid peptide containing an additional 5 amino acids in common with the other isoform (Fig. 3B).

To characterize the *ret*/PTC2 products, we immunoprecipitated cell extracts from normal or *ret*/PTC2 transformant NIH 3T3 cell lines first with anti-common peptide antibodies. The immunoprecipitates were divided into three aliquots and blotted onto nitrocellulose filters. The filters were developed either with anti-common peptide antibodies or with anti-isoform 51 or anti-isoform 9 peptide antibodies (Fig. 4A). The antibodies recognizing an amino acid sequence shared between the two *ret* isoforms did not show any reactivity with normal NIH 3T3 cells (lane 1) but detected in *ret*/PTC2 NIH 3T3 transformants two bands with relative mobilities of 76 and 81 Da, in agreement with the expected different molecular masses of the two predicted isoforms (lane 2). In particular, a mass of 76 kDa is in agreement with that predicted for the protein encoded by the *ret*/PTC2 cDNA clone, whose sequence is reported in Fig. 2 and which was expected to code for the shorter isoform (Fig. 3A). Although in Fig. 4A, lane 2, the relative amounts of the two isoforms appeared unbalanced, in Fig. 5, lane 2, the two isoforms were equally expressed, so the observed variability was merely due to the experimental conditions. The specificity of the anti-51 antibodies was further confirmed by their ability to identify only the 81-Da protein whereas, because of the presence of the 5 common amino acids in the immunizing peptide used to generate the anti-9 antibodies, the latter detected a weak 81-Da band in addition to the expected 76-Da band. In this experiment, the common band of about 50 Da was most likely due to the reactivity of ^{125}I-labeled protein A with the reduced immunoglobulin G heavy chain. When the experiment using the anti-common peptide antibodies in both immunoprecipitation and Western blotting was run under nonreducing conditions, the cell extract from *ret*/PTC2 NIH 3T3 transformants yielded two bands, of 175 and 190 Da (Fig. 4B, lane 1). No specific bands were noted in the same region in the extract from normal NIH 3T3 cells (Fig. 4b, lane 2), whereas the detected common band of 150 Da was compatible with the unreduced immunoglobulin G used in the immunoprecipitation. Therefore, the two *ret*/PTC2-related proteins, p175 and p190, could represent dimers, stabilized by disulfide bonding, of the *ret* isoforms. To verify whether heterodimers could be formed between the different *ret*/PTC2 isoforms, we first immunoprecipitated the cell extract from *ret*/PTC2 NIH 3T3 transformants with the antibodies against the peptide 51-containing isoform and developed the resulting Western blot with the anti-common peptide antibodies. Figure 4C, lane 2, shows that the two isoforms were present in the immunoprecipitate, demonstrating that p81-p76 heterodimers were formed. The excess p81 isoform could have been due both to homodimers and to the presence of free p81 in the cell extract.

These results demonstrate that the *ret*/PTC2 oncogene encodes two isoforms of the transforming protein able to form both homo- and heterodimers. To demonstrate the involvement of RIα sequences in the two *ret*/PTC2 protein isoforms, we performed another immunoprecipitation with the anti-common peptide antibodies using normal NIH 3T3 cells and *ret*/PTC1 and *ret*/PTC2 NIH 3T3 transformants. In two cases, the immunoprecipitates were divided into three aliquots, and in one case, they were divided into two aliquots, and the aliquots were blotted. Figure 5 shows the results obtained when one filter was developed with the same antibodies as those used for the immunoprecipitation (anti-common peptide). Again, no bands were detected in the normal NIH 3T3 cell extract (lane 1), whereas the 76- and 81-Da *ret*/PTC2 protein isoforms were readily identified (lane 2). Moreover, the same analysis of a *ret*/cell extract from PTC1 transformants identified the *ret*/PTC1 protein isoforms of 64 and 59 Da (lane 3). When the second filter was developed with anti-RIα antibodies, as shown in Fig. 5, bands of 76 and 81 Da were detected in the cell extract from *ret*/PTC2 transformants (lane 5). The specificity of the reaction was confirmed by the negative result for the extract derived from the NIH 3T3 cells transformed by the *ret*/PTC1 oncogene sequence, which did not contain the RIα-related sequences (lane 6).

Finally, the last blot was reacted with anti-phosphotyrosine antibodies. Figure 5 shows that in the *ret*/PTC2 NIH 3T3 transformant cell extract, the antibodies decorated the isoforms of 76 and 81 Da (lane 8), and no bands were detected in the normal NIH 3T3 cell extract. This result confirms a previously reported preliminary analysis of *ret*/PTC2 NIH 3T3 products, which showed that the oncogenic proteins display constitutive tyrosine phosphorylation and an in vitro tyrosine kinase enzymatic activity (16).

DISCUSSION

For papillary carcinomas of the thyroid gland, we previously described the generation of chimeric transforming sequences by rearrangement of the tyrosine kinase domains

FIG. 3. (A) Schematic structure of the products of the *ret* proto-oncogene and of its oncogenic versions *ret*/PTC1 and *ret*/PTC2. Shown are the signal peptide (SP), transmembrane (TM) and tyrosine kinase (TK) domains, a glycine stretch in D10S170 (G), cysteine (C) and tyrosine (Y) residues, an autoinhibitor (RRGAIS), and cAMP binding sites in RIα. p51 and p9 indicate the different COOH termini of the two *ret* isoforms. (B) Amino acid sequences of the different carboxy termini of *ret* gene products due to alternative splicing. The peptides used to produce the different antisera are indicated.

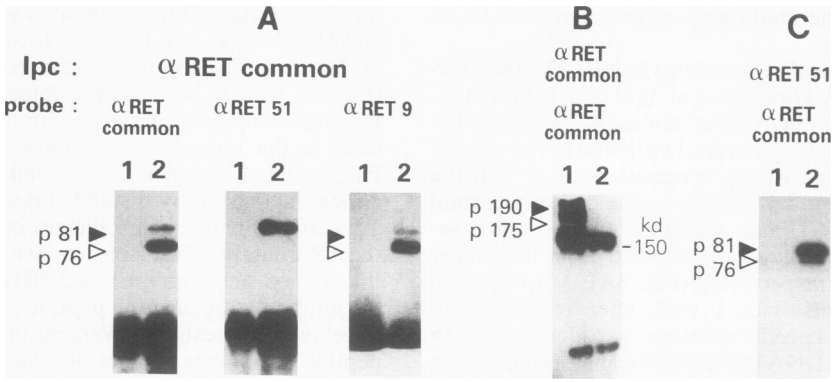


FIG. 4. Western blot analysis of *ret*/PTC2 isoforms. (A) Reducing conditions. Control NIH 3T3 (lanes 1) and *ret*/PTC2 transformant (lanes 2) cell lines were immunoprecipitated (Ipc) with anti-common peptide serum (αRET common) and probed (probe) with the following antisera: anti-RET common, anti-RET 51 (αRET 51) and anti-RET 9 (αRET 9). (B) Nonreducing conditions. Control NIH 3T3 (lane 2) and *ret*/PTC2 transformant (lane 1) cell lines were immunoprecipitated and probed with anti-common peptide serum. (C) Reducing conditions. Control NIH 3T3 (lane 1) and *ret*/PTC2 transformant (lane 2) cell lines were immunoprecipitated with anti-RET 51 serum and probed with anti-common peptide serum. Specific bands of p81 and p76, corresponding to the two *ret*/PTC2 isoforms, and the migration of p190 and p175 are indicated.

of the *trk* and *ret* proto-oncogenes (3). The *trk* tyrosine kinase domain has been found rearranged with portions of two different 5′-end donor genes: the first seven exons of a tropomyosin isoform-encoding gene (1, 18, 24) and *tpr*, a gene encoding a product thought to belong to a kind of cytoskeleton protein (10). In this study, we analyzed a novel rearrangement of *ret* and partially characterized its product. We cloned a biologically active *ret*-related cDNA from NIH 3T3 cells transformed by a tumor DNA that was found to contain a somatically rearranged *ret* allele. The analysis of its sequence revealed that it contained 717 nucleotides of the RIα gene followed by 1,150 nucleotides of the *ret* proto-oncogene, including its entire tk domain. The RIα gene encodes one isoform of the regulatory subunit of cAMP-dependent PKA. The latter is a holoenzyme complex consisting of a dimeric regulatory subunit and two catalytic subunits (27). In higher eukaryotes, most of the physiological responses to cAMP are mediated by the activation of cAMP-dependent PKA (8). Recently, the RIα gene was mapped to human chromosome 17q23q24 (4, 13).

This is the first report of the involvement of a PKA-related gene in the process of oncogenic activation. We recently detected a second instance of a papillary thyroid carcinoma with an oncogenic rearrangement between *ret* and the RIα gene.

Our data indicate that the product of *ret*/PTC2 is a chimeric enzyme with a portion of the regulatory subunit of the PKA serine-threonine kinase and the entire *ret* tyrosine kinase effector domain. The known properties of RIα would match those expected for a 5′-end gene capable of activating *ret* enzymatic activity. RIα is ubiquitously expressed (20) and, as mentioned before, its product exists in native form as a dimer of two subunits whose interaction could be stabilized by interchain disulfide bonding (31). The RIα sequences present in the *ret*/PTC2 oncogene contain the information to code for the two cysteines, at positions 18 and 39, that are involved in the stabilization of that interaction, providing that the environmental redox condition is compatible with the formation of disulfide bonds. This observation could explain the present results, which revealed the presence of high-molecular-weight bands, compatible with homo- and heterodimeric forms of the *ret*/PTC2 gene product, when the analysis was performed under nonreducing conditions. In addition, in accordance with the general model of the acti-

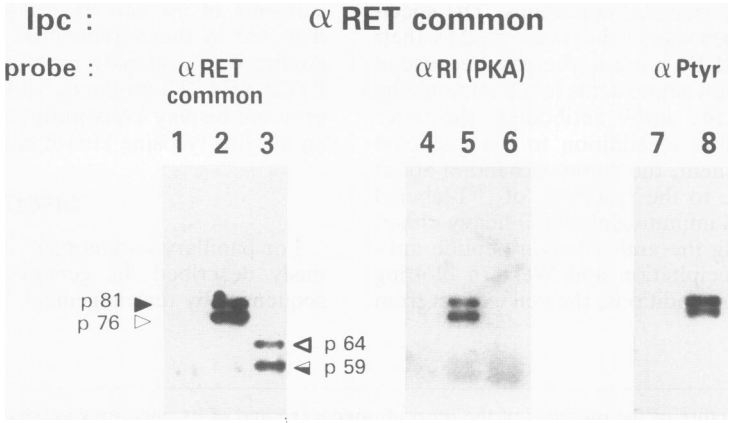


FIG. 5. Western blot analysis of *ret*/PTC1 and *ret*/PTC2 gene products. Control NIH 3T3 (lanes 1, 4, and 7), *ret*/PTC1 transformant (lanes 3 and 6), and *ret*/PTC2 transformant (lanes 2, 5, and 8) cell lines were immunoprecipitated (Ipc) with anti-common peptide serum (αRET common) and probed (probe) with the following antisera: anti-RET common, anti-RIα [αRI (PKA)], and antiphosphotyrosine (αPtyr). The migration of p59 and p64 *ret*/PTC1 and of p76 and p81 *ret*/PTC2 isoforms is indicated. PKA, protein kinase A.

vation of tyrosine kinase membrane receptors (29), *ret*/PTC2 gene product dimerization could be related to the observed constitutive tyrosine phosphorylation of this product.

A still unsolved question concerns the interaction of both cAMP and PKA catalytic subunits with that portion of RIα that is present in the *ret*/PTC2 gene product. In fact, an interaction of the transforming protein with the physiological RIα ligand (cAMP) and its interactive protein (PKA catalytic subunits) is theoretically still possible. For this question to be answered, further experiments are required. However, it is worth pointing out how a single genetic event, while generating the fusion of a portion of RIα with the tyrosine kinase domain of *ret*, not only activates the enzymatic activity of the latter but also alters one allele of RIα, perturbating the normal balance of cAMP receptor proteins. Several reports have indicated that malignancy is correlated with the abnormal signal transduction of cAMP (5, 8).

The analysis of the *ret*-related sequences of the *ret*/PTC2 rearrangement revealed two major points of interest in comparison with the homologous sequences displayed by the previously reported *ret*/PTC1 rearrangement (11). First, although two different genes provide the 5′ end of the *ret* transforming sequences, the breakpoints of the *ret* proto-oncogenes were identical, at the cDNA level, in the two cases, confirming the previously reported finding that tumor-specific *ret* rearrangements all occur within the same intronic region (11). Second, the 3′-end sequences of the two oncogenic rearrangements of *ret* were found, on the contrary, to differ as a consequence of alternative splicing, as originally proposed by Ishizaka et al. (12). Here we demonstrate that two isoforms of *ret*/PTC2 (as well as of *ret*/PTC1) can be detected in NIH 3T3 transformants.

In conclusion, we have provided evidence that in papillary thyroid carcinomas, the *ret* proto-oncogene can be found rearranged with different genes, resulting in constitutive tyrosine phosphorylation of the chimeric *ret* gene product and, most likely, in constitutive activation of its enzymatic activity. This activation could be mediated by the formation of homo- and heterodimers of the two isoforms of the transforming protein that are generated by alternative splicing of *ret* at the 3′ end of the *ret*/PTC2 transforming sequence.

ACKNOWLEDGMENTS

We thank R. A. Gambetta for providing the antiphosphotyrosine antibodies and G. Tortora (University of Naples) for useful discussions and help in obtaining the anti-RIα antibodies. The technical assistance of M. G. Rizzetti and M. Azzini is also acknowledged, as are the competent secretarial work of G. Raineri and the assistance of S. Clegg in the preparation of the manuscript.

This study was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC) and the National Council for Research (CNR), Special Project ACRO.

REFERENCES

1. **Bongarzone, I., et al.** Unpublished data.
2. **Bongarzone, I., et al.** Unpublished data.
3. **Bongarzone, I., M. A. Pierotti, N. Monzini, P. Mondellini, G. Manenti, R. Donghi, S. Pilotti, M. Grieco, M. Santoro, A. Fusco, G. Vecchio, and G. Della Porta.** 1989. High frequency of activation of tyrosine kinase oncogenes in human papillary thyroid carcinoma. Oncogene **4**:1457–1462.
4. **Boshart, M., F. Weih, M. Nichols, and G. Schutz.** 1991. The tissue-specific extinguisher locus TSE1 encodes a regulatory subunit of cAMP-dependent protein kinase. Cell **66**:849–859.
5. **Cho-Chung, Y. S.** 1990. Role of cyclic AMP receptor proteins in growth, differentiation, and suppression of malignancy: new approaches to therapy. Cancer Res. **50**:7093–7100.
6. **Chomczynski, P., and N. Sacchi.** 1987. A single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
7. **Cirillo, D. M., G. Gaudino, L. Naldini, and P. M. Comoglio.** 1986. Receptor for bombesin-associated tyrosine kinase activity. Mol. Cell. Biol. **6**:4641–4649.
8. **Dumont, J. E., J.-C. Jauniaux, and P. P. Roger.** 1989. The cyclic AMP-mediated stimulation of cell proliferation. Trends Biochem. Sci. **14**:67–71.
9. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
10. **Greco, A., M. A. Pierotti, I. Bongarzone, S. Pagliardini, C. Lanzi, and G. Della Porta.** 1992. *TRK*-T1 is a novel oncogene formed by the fusion of *TPR* and *TRK* genes in human papillary thyroid carcinomas. Oncogene **7**:237–242.
11. **Grieco, M., M. Santoro, M. T. Berlingieri, R. M. Melillo, R. Donghi, I. Bongarzone, M. A. Pierotti, G. Della Porta, A. Fusco, and G. Vecchio.** 1990. PTC is a novel rearranged form of the *ret* proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell **60**:557–563.
12. **Ishizaka, Y., M. Ochiai, T. Tahira, T. Sugimura, and M. Nagao.** 1989. Activation of the *ret-II* oncogene without a sequence encoding a transmembrane domain and transforming activity of two *ret-II* oncogene products differing in carboxy-termini due to alternative splicing. Oncogene **4**:789–794.
13. **Jones, K. W., M. H. Shapero, M. Chevrette, and R. E. K. Fournier.** 1991. Subtractive hybridization cloning of a tissue-specific extinguisher: TSE1 encodes a regulatory subunit of protein kinase A. Cell **66**:861–872.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Landt, M., S. C. Boltz, and L. G. Butler.** 1978. Alkaline phosphatase: affinity chromatography and inhibition by phosphonic acids. Biochemistry **17**:915–919.
16. **Lanzi, C., M. G. Borrello, I. Bongarzone, A. Migliazza, A. Fusco, M. Grieco, M. Santoro, R. A. Gambetta, F. Zunino, G. Della Porta, and M. A. Pierotti.** 1992. Identification of the product of two oncogenic rearranged forms of the *RET* proto-oncogene in papillary thyroid carcinomas. Oncogene **7**:2189–2194.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Martin-Zanca, D., S. H. Hughes, and M. Barbacid.** 1986. A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature (London) **319**:743–748.
19. **Pierotti, M. A., M. Santoro, R. B. Jenkins, G. Sozzi, I. Bongarzone, M. Grieco, N. Monzini, M. Miozzo, M. A. Herrmann, A. Fusco, I. D. Hay, G. Della Porta, and G. Vecchio.** 1992. Characterization of an inversion on the long arm of chromosome 10 juxtaposing *D10S170* and *RET* and creating the oncogenic sequence *RET/PTC*. Proc. Natl. Acad. Sci. USA **89**:1616–1620.
20. **Rios, R. M., C. Celati, S. M. Lohmann, M. Bornens, and G. Keryer.** 1992. Identification of a high affinity binding protein for the regulatory subunit RIIβ of cAMP-dependent protein kinase in Golgi enriched membranes of human lymphoblasts. EMBO J. **11**:1723–1731.
21. **Ross, A. H., D. Baltimore, and H. N. Eisen.** 1981. Phosphotyrosine-containing proteins isolated by affinity chromatography with antibodies to a synthetic hapten. Nature (London) **294**:654–656.
22. **Sandberg, M., B. Skalhegg, and T. Jahnsen.** 1990. The two mRNA forms for the type Iα regulatory subunit of cAMP-dependent protein kinase from human testis are due to the use of different polyadenylation site signals. Biochem. Biophys. Res. Commun. **167**:323–330.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
24. **Sozzi, G., I. Bongarzone, M. Miozzo, C. T. Cariani, P. Mondellini, C. Calderone, S. Pilotti, M. A. Pierotti, and G. Della Porta.** 1992. Cytogenetic and molecular genetic characterization of papillary thyroid carcinomas. Genes Chromosomes Cancer **5**:1–7.

25. **Tahira, T., Y. Ishizaka, F. Itoh, T. Sugimura, and M. Nagao.** 1990. Characterization of *ret* proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. Oncogene **5**:97–102.
26. **Takahashi, M., Y. Buma, T. Iwamoto, Y. Inaguma, H. Ikeda, and H. Hiai.** 1988. Cloning and expression of the *ret* proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. Oncogene **3**:571–578.
27. **Taylor, S. S., J. A. Buechler, and W. Yonemoto.** 1990. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu. Rev. Biochem. **59**:971–1005.
28. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
29. **Ullrich, A., and J. Schlessinger.** 1991. Signal transduction by receptors with tyrosine kinase activity. Cell **51**:203–212.
30. **Vaughan, J. M., J. Rivier, A. Z. Corrigan, R. McClintock, C. A. Campen, D. Jolley, J. K. Voglmayr, C. W. Bardin, C. Rivier, and W. Vale.** 1989. Detection and purification of inhibin using antisera generated against synthetic peptide fragments. Methods Enzymol. **168**:588–589.
31. **Zick, S. K., and S. S. Taylor.** 1982. Interchain disulfide bonding in the regulatory subunit of cAMP-dependent protein kinase I. J. Biol. Chem. **257**:2287–2293.