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A Novel Type of *RET* Rearrangement (PTC8) in Childhood Papillary Thyroid Carcinomas and Characterization of the Involved Gene (*RFG8*)[1]

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ABSTRACT

As part of ongoing studies on the *RET* rearrangement frequency in children with papillary thyroid carcinoma (PTC) after their exposure to radioactive iodine after the Chernobyl reactor accident, new methods for the detection of novel types of *RET* rearrangements are being developed. In this study, an improved reverse transcription-PCR strategy is used successfully to identify a new type of *RET* rearrangement. This rearrangement is designated PTC8 and the involved *RET*-fused gene (*RFG*) as *RFG8*. The identification of two reciprocal transcripts coding for the *RFG8/RET* and *RET/RFG8* fusions suggests that the PTC8 rearrangement results from a balanced chromosomal translocation. With a view to clarify its role in tumor induction, we compared the fusion products with those of previously described *RET* rearrangements. We therefore sequenced and characterized the *RFG8* cDNA, which showed no significant similarity to any functional protein described as yet. *RFG8* is located on chromosome 18q21–22 and is expressed ubiquitously. Bioinformatic analysis predicts with a high probability that the corresponding rfg8 protein is located in the cytoplasm and is involved putatively in intracellular transport processes. Furthermore, we identified coiled-coil structures upstream of the breakpoint with one of the coiled-coils showing dimerization capability. Thus the rfg8/ret fusion protein exhibits structures for oncogenic activation that are similar to those observed in previously described *RET* fusions.

INTRODUCTION

Proto *RET* codes for a membrane-associated receptor TK[3] that is expressed in a developmental stage-specific manner in subsets of neural crest-derived cells (1). Oncogenic *RET* rearrangements are detected frequently in PTCs of children from Belarus who had been exposed to radioactive iodine after the Chernobyl power plant accident (2–4). These *RET* activations are caused by balanced chromosomal translocations that lead to the expression of at least one aberrant fusion product. Typically, these fusion proteins have a deletion of the *RET* NH_2-terminal and fusion of its remaining TK domain to the expressed 5′end of RFGs. The described RFGs are expressed ubiquitously and contain dimerization domains. Each *RET* rearrangement produces chimeric mRNAs and proteins in the thyroid epithelial cells, which is a cell type that does not originate from the neural crest (for review, Refs. 5, 6). Moreover, the fusion proteins show an intrinsic and constitutive TK activity that is sufficient to induce PTC in transgenic mice (7, 8).

Previously, we have published a RT PCR-based strategy to rapidly identify hitherto unknown *RET* rearrangements in a large series of tumor samples (2, 9). Using RT PCR-based strategies, we have investigated thus far 191 PTCs of children and adolescents who were up to 18.3 years of age at the time of the reactor accident (10). As a result, we have identified three new types of *RET* rearrangement (PTC5, PTC6, and PTC7; Refs. 6, 11) and two variants of the PTC3 rearrangement (12). Thus, six different types of *RET* rearrangements have been reported until now for childhood thyroid carcinomas. The involved RFGs are *H4* (PTC1), *RIα* (PTC2), *ELE1/ARA70* (PTC3 and PTC4; for review, Ref. 13), *RFG5/GOLGIN84* (PTC5), *HTIF1* (PTC6), and *RFG7* (PTC7). We have now improved the identification of novel *RET* rearrangements by the establishment of the MPI PCR. In this study, we report the discovery of the PTC8 rearrangement in two tumor samples of our series and discuss its role in tumor induction.

MATERIALS AND METHODS

Thyroid Tissues. Tumor material was obtained from patients who underwent thyroidectomy at the Department of Surgery, Medical High School of Minsk, Belarus. The tumor specimens of interest were from a female (M119) and a male (M163) patient exposed to radioactive fallout at the age of 2 years 3 months and 2 years 10 months, respectively. Both tumors were follicular variants of PTC (14) with Tumor-Node-Metastasis classifications $T_{4a}N_0M_0$ (M119) and $T_{2a}N_{1a}M_0$ (M163).

RT PCR, 5′ RACE, Marathon RACE, and Sequence Analysis. Poly(A)$^+$ mRNA isolation and RT PCR were performed as described in detail elsewhere (2) with the following modification. The identification PCR (2) was replaced by the MPI PCR that was carried out using specific primer pairs for all of the known types of *RET* rearrangements in one PCR reaction under the conditions described for the identification PCR. Unknown fusion partners of *RET* were identified by the 5′ RACE system (Roche Diagnostics, Boehringer Mannheim, Germany). To obtain the cDNA sequence of the *RET* fusion partner, the Thyroid Marathon Ready cDNA Kit (Clontech, Palo Alto, CA; marathon RACE) was used. The marathon RACE products were cloned using the TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, NM). Cycle sequencing of vector DNA and direct sequencing of the RT PCR fragments were performed according to protocols for the ABI PRISM 310 genetic analyzer (Applied Biosystems-Perkin Elmer, Weiterstadt, Germany). The strategy used in this study, including data of primers tm1 and retc5, has been described in detail (2, 6, 9). Additional details concerning primer sequences and positions are shown in Figs. 1 and 3 or are available on request.

Northern Blot Hybridization. Northern blot analysis was carried out as described earlier (11). We used the RT PCR fragment rfg8.1V/R (780 bp; Fig. 3) as a hybridization probe, which is specific for *RFG8* and a commercial β-actin probe (Clontech).

FISH. We used the services of the RZPD to obtain a hybridization probe for the FISH analysis. The PCR fragment *PTC8*-M119 (primer pair: rfg8.1V/rfg8int1R, GCCCATCAAAGGATATGGTC) of ~2.3 kb in length was sent to RZPD as a probe to screen the human PAC library No. 704. Potentially positive PAC clones were handled as advised by the RZPD and rescreened by PCR using the primer pair: rfg8.1V/rfg8int1R (see above). We isolated at least 2 μg of PAC DNA using the QIAfilter Plasmid Midi Kit (Qiagen, Hilden, Germany) from those clones that had been positive after rescreening. One μg of DNA from PACs RPCIP704B06764Q2 and RPCIP704H1212Q25 was biotin labeled (La Roche Diagnostics, Mannheim, Germany) by a standard nick-translation reaction. Before hybridization, metaphase spreads prepared from phytohemagglutinin-stimulated blood lymphocytes were treated with

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[3] The abbreviations used are: TK, tyrosine kinase; MPI PCR, multiple primer identification PCR; RACE, rapid amplification of cDNA ends; 5′ RACE, rapid amplification of 5′-cDNA ends; FISH, fluorescence *in situ* hybridization; PTC, papillary thyroid carcinoma; RT PCR, reverse transcription-PCR; RFG, *RET*-fused gene; RZPD, Resource Center of the German Human Genome Project.

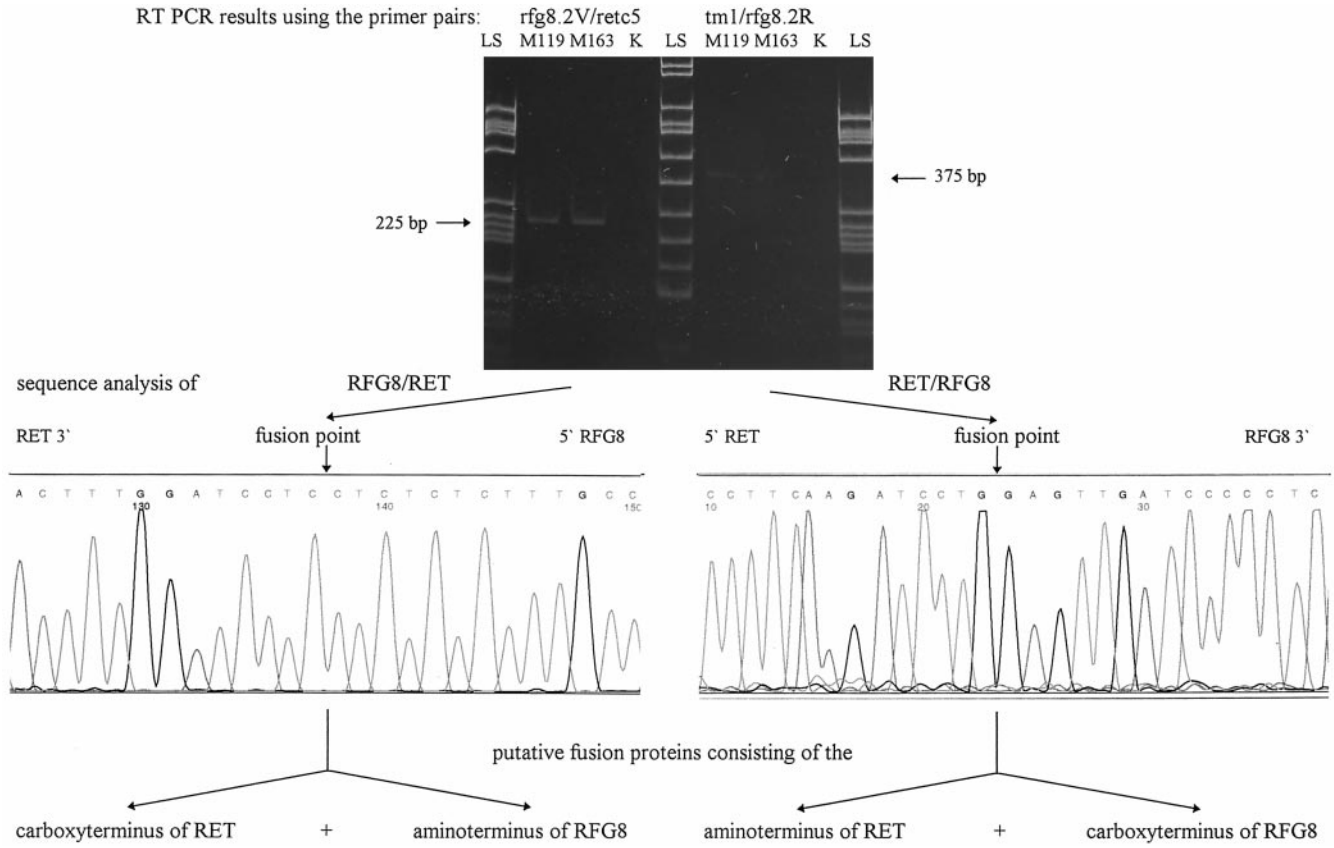

Fig. 1. Results of RT PCR and the corresponding sequencing analysis to identify the PTC8 rearrangement in tumor samples M119 and M163. The PTC8 rearrangement consists of two chimeric transcripts designated RFG8/RET and RET/RFG8. The direction of the sequence analysis is 3′ to 5′ for *RFG8/RET* and 5′ to 3′ for *RET/RFG8*. The corresponding fusion points are indicated by *arrows*. The *RET* part of the *RET/RFG8* sequence ends with nucleotide 1334, whereas the *RET* portion of the *RFG8/RET* sequence starts with nucleotide 1335 of the *RET* cDNA sequence (37).

RNase and pepsin (15). Hybridization was performed as described by Lichter and Cremer (16) with the following modifications: 100 ng of biotinylated PAC DNA was precipitated together with 30 μg of Cot-1 DNA and 1 μg salmon sperm DNA. The DNA was resuspended in 10 μl of hybridization mixture, containing 50% deionized formamide, 1 × SSC, and 10% dextran sulfate, and hybridized to metaphase spreads of a healthy male donor for at least 12 h. The hybridized metaphase slides were incubated with FITC-conjugated avidin DCS (Vector Laboratories, Burlingam, CA), and signals were amplified once using a biotinylated anti-avidin D affinity purified antibody from goat (Vector Laboratories), followed by a second layer of fluorescein avidin DCS (17). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole and mounted using Vectashield mounting medium (Vector Laboratories). Visualization of FISH signals was accomplished using an epifluorescence microscope (Axiophot, Zeiss) coupled to a cooled charge device camera (Photometrics, Kodak KAF 1400 chip). Image processing and pseudocoloring was performed using the Adobe Photoshop software package.

Homology Search and Computer-assisted Sequence Analysis. Programs provided on the Expert Protein Analysis System proteomics server[4] of the Swiss Institute of Bioinformatics were used for sequence analysis (18).

RESULTS AND DISCUSSION

Detection of PTC8 as a Novel Type of *RET* Rearrangement. A large series of tumor samples are being investigated using a RT PCR-based strategy to identify hitherto unknown *RET* rearrangements (2, 9). We have improved the identification method to screen the samples more efficiently. The resulting combination of multiplex PCR, the new MPI PCR, 5′ RACE, and direct sequencing was used successfully to identify a novel *RET* rearrangement in the thyroid tumor samples M119 and M163. In these two samples, the result of multiplex PCR strongly indicated the presence of a *RET* rearrangement. However, the six published fusion genes *H4/RET*, *RIα/RET*, *ELE1/RET*, *RFG5/GOLG5A/RET*, *HTIF1α/RET*, or *RFG7/RET* had not been detected by MPI PCR. 5′-RACE was carried out, and a PCR product was obtained that included a non-*RET* sequence of ~200 bp upstream of the ret TK domain. The novel type of *RET* rearrangement in tumor samples M119 and M163 was designated PTC8 and the respective gene *RFG8*.

In keeping with our previous experiences with *RET* rearrangements (2, 6, 11, 12), the PTC8 rearrangement also produces two transcripts, namely RFG8/RET and RET/RFG8. We conclude, therefore, that this new type of *RET* rearrangement is, like all of the other *RET* rearrangements, a balanced chromosomal translocation. In Fig. 1, we show the RT PCR results using the original thyroid cDNA of the two tumor samples and primer pairs made using the *RFG8* and *RET* cDNA sequences. The PCR products were sequenced directly using the corresponding PCR primers specific for the *RET* cDNA (Fig. 1).

Consequences of the *RFG8* Gene Expression for the PTC8 Rearrangement and for Identifying the RFG8 cDNA Sequence. To further characterize *RFG8*, we investigated its expression pattern. Northern blot analysis of mRNA from various human tissues was performed. A *RFG8* specific probe detected two transcripts of about 6.0 and 4.4 kb in 6 of 7 tissues tested, including the thyroid gland (Fig. 2). These results demonstrate that the *RFG8* gene is expressed ubiquitously, which is a feature shared with all of the RFGs identified thus far (5, 6, 11). It is assumed that the rearrangement process places the ret TK domain under the control of the ubiquitously expressed *RFG8* promoter. For the reciprocal translocation, the 3′ end of the *RFG8*

[4] http://www.expasy.ch.

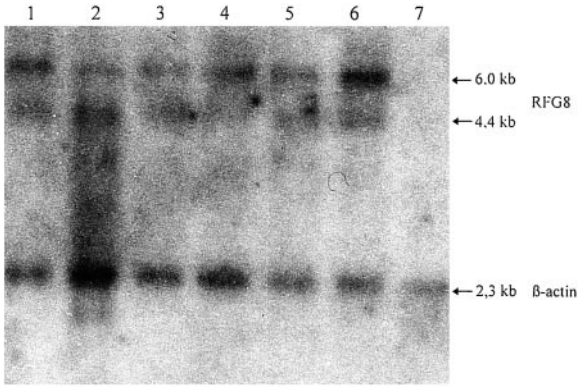

Fig. 2. Expression of RFG8 mRNAs as seen by Northern blot analysis of various tissues. The tissues used are as follows: *1*, stomach; *2*, thyroid; *3*, spinal cord; *4*, lymph node; *5*, trachea; *6*, adrenal gland; and *7*, bone marrow. We detected messages of about 6.0 and 4.4 kb in length in all of the tissues tested except in the bone marrow.

gene comes under the control of the *RET* promoter. The latter clearly occurs in the tumor cells, generating the reciprocal transcript RET/RFG8 (Fig. 1). The role of this transcript in thyroid carcinogenesis remains unknown.

A BLAST search with the *RFG8* part of the 5′ RACE sequence using all of the available databases revealed no considerable sequence homology to any other sequences. Therefore, we performed the marathon RACE technique to complete the *RFG8* cDNA sequence. This technique is designed to identify unknown 5′ and 3′ cDNA ends, and very little sequence information is required for primer design. We successfully amplified the 3′ end of the *RFG8* message, including a putative stop codon. However, we could not identify a start codon in the 5′ end of the *RFG8* cDNA sequence, although the two detected *RFG8* messages were of 4.4 and 6.0 kb in length (Fig. 2). Several 5′-RACE experiments using different primers showed shorter fragments without potential start site or transcripts of incorrectly spliced mRNA including Alu sequences. This is comparable with our recent published results (6) on the *RFG7* cDNA sequence. *RFG7* was expressed by two messages of 8.2 and 5.2 kb in length, and it was also not possible to obtain the 5′ cDNA end with the start codon using the marathon RACE technique. Therefore, we conclude that only shorter messages can be amplified as complete cDNA sequences by the marathon RACE method. The entire *RFG7* sequence has since been published and is designated *HTIF1*γ (19). Our partial *RFG8* cDNA sequence, which results from the marathon RACE technique, is shown in Fig. 3.

Chromosomal Location of the *RFG8* Gene and the Frequency of the PTC8 Rearrangement in Our Tumor Series. We have detected the PTC8 rearrangement in two samples of 191 PTCs obtained from children from Belarus. This low frequency is in contrast to the high prevalence of PTC1 and PTC3 rearrangements, but comparable with that of the PTC5, PTC6 and PTC7 rearrangements. The PTC2 rearrangement was absent in our tumor series. However, in PTC2 a chromosomal translocation, t(10;17)(q11.2;q23) juxtaposes the TK domain of the *RET* proto-oncogene, which is located on chromosome 10, to the 5′ region of the *RI*α gene on chromosome 17 (20). In contrast, a paracentric inversion of chromosome 10 is responsible for the generation of PTC1 and PTC3 rearrangements (21, 22). This implies that *RET* rearrangements involving genes on chromosome 10 are created more often than those involving genes on different chromosomes. We tested this hypothesis by determining the chromosomal localization of *RFG8* by FISH analysis. Approximately 20 metaphase chromosome spreads from a lymphocyte culture of a healthy male were hybridized with the two PAC probes described in

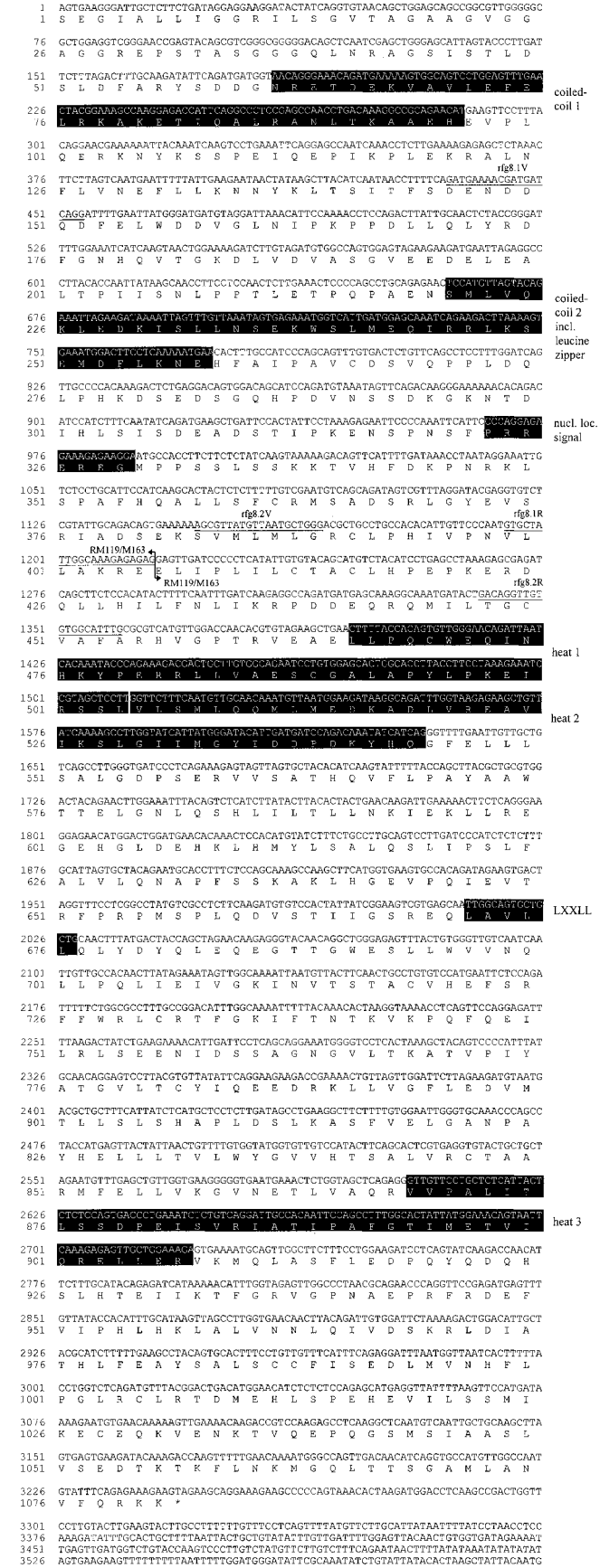

Fig. 3. cDNA sequence of the *RFG8* gene obtained by the marathon RACE, the corresponding amino acid sequence, and the results of protein sequence analysis. The sequence motifs detected are indicated by *rectangles*. The position where the PTC8 rearrangement occurred is marked by *arrows*. Primer sequences are *underlined*.

this study. The PAC probes harbored a considerable portion of the RFG8 genomic sequence or even the complete sequence (this was not investigated in detail). Both PACs revealed specific hybridization signals on chromosome 18q21–22 (Fig. 4) showing that the *RFG8* gene is located on chromosome 18 most proximal to the q22 band.

Computational Characterization of RFG8 and Consequences for the PTC8 Rearrangement. Thus far, the partial *RFG8* cDNA sequence consists of 3673 bp and the corresponding amino acid sequence of 1081 amino acids (Fig. 3). Database searches using both cDNA and protein sequences revealed the following significant similarities: (*a*) the human bacterial artificial chromosome clone RG300C03 (human bacterial artificial chromosome library CITB-HS-A) mapped to chromosome 7q31.2 was detected showing several interrupted sequence similarities between 51 and 78%. We conclude that a *RFG8*-related gene on chromosome 7 may exist; (*b*) several EST clone sequences from mice, rats, and humans have been identified showing very high similarities to the *RFG8* sequence (up to 99%), indicating that the same or a highly related gene is expressed in these species. Most of the human EST sequences cover 3′ *RFG8* sequence regions, and some of them belong to clones that are mapped to chromosome 18 (*e.g.*, cDNA clones NHTBCae15h12 and IMAGE: 36907). Therefore, it cannot be excluded that they have sequences identical to the *RFG8* sequence, but because of sequencing ambiguities the identity is <100%. According to the nucleic and amino acid sequence databases, however, it was not possible to identify the entire *RFG8* sequence and to achieve information on the function of the corresponding protein. Therefore, we performed bioinformatic analyses to characterize the rfg8 amino acid sequence shown in Fig. 3 to postulate the role of the protein of the PTC8 rearrangement.

The partial rfg8 protein contains 145 negatively charged *versus* 112 positively charged amino acids, with leucine being the most abundant (13.6%). The hydrophilicity profile of the protein was plotted by the method of Kyte and Doolittle (23), but no hydrophobic region that could code for a transmembrane domain (data by the EXPASY PROTPARAM tool) was found. These predictions were confirmed by the PSORT II analysis (24, 25) according to which the rfg8 amino acid sequence codes for a protein that is in all probability cytoplasmic. In detail, the predictions are based on two different methods: (*a*) the NNCN score that discriminates the tendency of the protein to be either at the nucleus or in the cytoplasm is calculated based on the amino acid composition (24). NNCN predicted with a probability of 70.6% that rfg8 is a cytoplasmic protein; (*b*) the algorithm k-Nearest Neighbors Classifier (k-NN) was used for assessing the probability of a protein localizing at various candidate sites (25). The prediction is performed using the k-nearest data points, where *k* is a predefined integer parameter. According to the k data points, rfg8 has a probability of 65.2% of being a cytoplasmic protein and of 13% of being localized to the nucleus. The nuclear component of the sequence might be attributable to a putative nuclear localization signal that is shown in Fig. 3 (26). The correct localization of the rfg8 protein may be important for the function of the rfg8/ret fusion protein, because the nuclear localization signal is found upstream of the breakpoint and, therefore, would be transcribed. It could then be possible for the ret TK part of the fusion protein to interact directly with nuclear substrates.

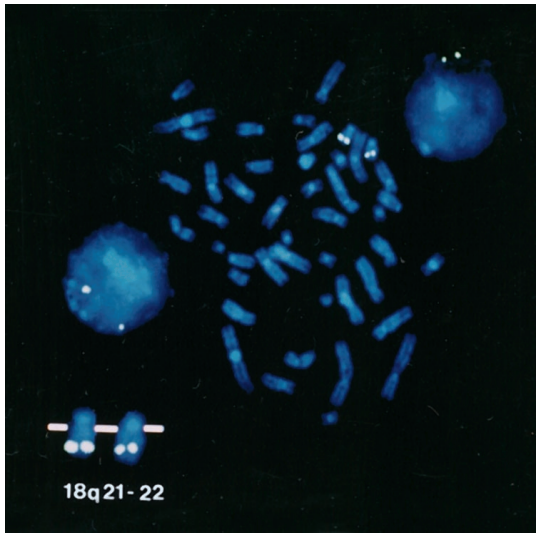

Fig. 4. Digitized pseudocolored images of human metaphase chromosome 18 after FISH of PAC RPCIP704B06764Q2. The similar result obtained using PAC RPCIP704H1212Q25 is not shown. The hybridization signals of both PACs were mapped to chromosome 18q21–22.

We performed a ProfileScan to compare the partial rfg8 amino acid sequence with current PROSITE (27, 28) and Pfam (29) profile libraries. Both databases are collections of protein motifs and families with Pfam generally focusing on classical domains with a high proportion of extracellular modules. In contrast, the PROSITE profile collection emphasizes domains in intracellular proteins and proteins involved in signal transduction, DNA repair, cell cycle regulation, and apoptosis.

The ProfileScan predicted the existence of a leucine zipper in the NH_2-terminal part of the rfg8 amino acid sequence (Fig. 3). To obtain more precise results, we analyzed additionally the rfg8 amino acid sequence using the Paircoil program (30). This program is designed to predict the location of coiled-coil regions. By this method, the rfg8 protein seemed to contain two NH_2-terminal coiled-coil domains (Fig. 3) with the second domain appearing as a typical leucine zipper. Further analysis using the MultiCoil program (31) revealed with a probability of 75% that this second domain forms two-stranded coiled-coils. Coiled-coil domains, which confer the ability to dimerize, have been shown to play a crucial role in the ligand-independent activation of ret TK oncoproteins (32). All of the described *RET* rearranged proteins including rfg8 contain coiled-coils that are located upstream of the breakpoints to become part of the rfg/ret products (5, 6, 11).

Additionally, we found three putative heat repeat motifs in the COOH-terminal part of the rfg8 amino acid sequence (Fig. 3). This motif is very common for helical repeat proteins where 3 to 36 repeat units form a rod-like helical structure that appears to function as a protein-protein interaction surface. Many of the heat repeat-containing proteins seem to be involved in intracellular transport processes (for review, Ref. 33). The heat repeat motifs are located downstream of the breakpoint in the *RFG8* sequence; therefore, the putative interaction function might be important for the ret/rfg8 fusion protein.

Recently, we published the PTC6 and PTC7 types of *RET* rearrangements (6) and identified two transcriptional coactivators for nuclear receptors as being involved in the rearrangements. These are *HTIF1*α (34) and γ (19), which is the same as *RFG7* (6). Furthermore, *ELE1*, the partner of *RET* in the PTC3 rearrangement, has been identified as a transcriptional coactivator of the androgen receptor (35). Therefore, we compared the rfg8 amino acid sequence with those of transcriptional coactivators. We detected a LxxLL motif that is known to be responsible for the interaction of transcriptional coactivators with nuclear receptors (36). This motif is located downstream of the breakpoint in *RFG8* (Fig. 3) and, therefore, is part of the ret/rfg8 fusion product. However, we could not find more sequence similarities that would allow us to conclude that rfg8 is a transcriptional coactivator.

In conclusion, we identified the novel PTC8 type of *RET* rearrangement in two childhood PTCs from Belarus. Like all of the other *RET*

rearrangements described thus far, it is caused by a balanced chromosomal translocation, and reciprocal transcripts are produced. The gene involved was designated *RFG8* and, like the previously detected RFGs, is expressed ubiquitously. The analysis of this novel *RET* rearrangement is reported in this study for the first time. The importance of each new type of *RET* rearrangement is not reflected in a high rearrangement frequency, but rather in the identification of similarities among the involved fusion proteins. This permits us to gain greater insight into their mode of action as rearrangement partners. As bioinformatic tools gradually improve, it becomes easier to obtain characterizations of amino acid sequences. rfg8 is most likely a cytoplasmic protein that might be involved in intracellular transport processes. Like all of the other RFGs, it contains coiled-coiled regions that are important for the activation of the rfg8/ret fusion product and thus for tumor induction.

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Note Added in Proof

After submission of this manuscript, an additional gene fusion involving *RET* has been reported for which the designation PTC8 was chosen [Salassidis, K., Bruch, J., Zitzelsberger, H., Lengfelder, E., Kellerer, A. M., and Bauchinger, M. Translocation t(10;14)(q11.2:q22.1) fusing the kinetin to the *RET* gene creates a novel rearrangement form (PTC8) of the *RET* proto-oncogene in radiation-induced childhood papillary thyroid carcinomas. Cancer Res., *60:* 2786–2789, 2000]. Therefore, we propose PTC9 as final designation of the gene fusion that is described in our present study and the reported *RET*-fused gene as *RFG9*. A consensus about further designations of additional gene fusions that will eventually be found in papillary thyroid carcinomas is required to clarify the nomenclature of *RET*-fused genes.

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