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Sequence and characterisation of the *RET* proto-oncogene 5′ flanking region: analysis of retinoic acid responsiveness at the transcriptional level

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Abstract **The *RET* proto-oncogene encodes a receptor tyrosine kinase expressed during neural crest development. *RET* expression is enhanced in vitro by several differentiating agents, including retinoic acid (RA), which up-regulates *RET* expression in neuroblastoma cell lines. In the present work we sequenced and analysed a 5 kbp genomic fragment 5′ to *RET*. Three deletion fragments of this region were tested for their RA inducibility in transient transfection assays and failed to support the hypothesis of a direct transcriptional activation. Finally, our functional analysis of a candidate RA response element present in the *RET* promoter provides new hints for the understanding of the interaction between nuclear receptors and their specific recognition sites.**

Key words: *RET* proto-oncogene; Retinoic acid response element; Neuroblastoma; Hirschsprung; Multiple endocrine neoplasia type 2; Medullary thyroid carcinoma

1. Introduction

The *RET* proto-oncogene encodes a receptor tyrosine kinase [1,2] predominantly expressed during neural crest development and involved in different human neurocristopathies [3].

Somatic rearrangements of *RET* are responsible for a variable proportion of papillary thyroid carcinomas (PTC) [4–6], while germline *RET* mutations are associated with the three variants of the inherited cancer syndrome known as multiple endocrine neoplasia type 2: MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC) [7–9]. Finally, germline *RET* mutations have been repeatedly associated with an autosomal dominant form of the Hirschsprung disease (HSCR) [10–13], a congenital disorder of the enteric nervous system characterised by a marked genetic heterogeneity [14–17].

Recently, the identification of Ret ligand proteins, the glial cell line-derived neurotrophic factor (GDNF) [18–20], as well as neurturin (NTN) [21,22], and of Ret co-receptors (GDNF receptor α and β [23,24] and NTN receptor [22]) has provided new insights into the molecular events leading to Ret signal transduction and therefore into the understanding of the physiological role of the *RET* proto-oncogene.

As suggested by the complex phenotypes which arise from mutations in *RET*, this gene plays a crucial role in neural crest development. Its action is specifically required at certain stages of embryogenesis and for limited periods of time depending on the cell type [25,26]. This implies a fine transcriptional regulation, with the involvement of different specific transcription factors.

To date, only a few data have been reported with regard to the *RET* promoter region: a short genomic fragment spanning 453 bp 5′ of the human gene has been analysed, where the consensus sequences recognised by AP-2, ETF, GCF and Sp-1 transcription factors have been identified [27]. The *RET* transcription start site has been localised and the CAT (chloramphenicol acetyltransferase [28]) functional assay, performed with a fragment spanning from −167 to +98, has shown that this region contains the basal promoter of the gene [27]. This initial identification of the *RET* regulatory region, far from being exhaustive, can be regarded as a starting point for further studies aimed at the identification of the factors which regulate *RET* expression.

Recent evidence has been collected on the in vitro activation of *RET* expression by a variety of differentiating agents. Increased *RET* mRNA levels are observed in TT cells derived from medullary thyroid carcinoma following dibutyryl-cAMP treatment [29], and in several neuroblastoma (NB) cell lines upon treatment with retinoic acid (RA) [30], as well as with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), α-, γ-interferon and glial cell conditioned medium (GCM), all of them inducing a neuronal phenotype [31]. NB is an embryonic tumour that arises in tissues of the peripheral nervous system [32]; NB cell lines, which retain many phenotypic features of the tissue from which they originate, provide a natural model system for the study of *RET* expression. In NB cells, RA has been shown by Bunone et al. [31] to exert its action by enhancing the level of *RET* transcription rather than by increasing the stability of *RET* mRNA; moreover, this effect does not require de novo protein synthesis [31]. These findings suggest that the regulation of *RET* expression might be mediated by a direct effect of RA on the gene regulatory region.

RA, a natural derivative of vitamin A, has profound effects in a variety of biological processes and plays a crucial role during embryogenesis [33–37], especially in neural crest development. RA acts at the transcriptional level through nuclear receptors which are members of the steroid/thyroid hormone receptor superfamily. Retinoid receptors include RAR (all-*trans* retinoic acid receptor) α, β and γ isoforms, and RXR (9-*cis* retinoic acid receptor) α, β and γ isoforms [38–43]. RARs and RXRs form functionally active heterodimers [44] which recognise retinoic acid response elements (RAREs) within the regulatory regions of their target genes. Typical RAREs are composed of direct repeats of a core motif 5′-PuG(G/T)TCA-3′ or closely related sequences [40,45–47] spaced 1–5 bp apart (DR1-DR5). Nonetheless, several natural

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response elements contain half-site arrangements which are more complex and/or more widely spaced than prototypic DR1-DR5 elements [48–50]. Moreover, a systematic study of widely spaced DR elements, separated by up to 150 bp, has been reported, showing that these sequences can act as functional RAREs [51].

In the present work, we completed our previous characterisation of the genomic region encompassing the entire human *RET* gene [52] by analysing the regulatory 5′ region. Given the crucial role of RA during neural crest development, we investigated the mechanism of the response of *RET* to RA treatment. To this purpose, we coupled different fragments of the *RET* promoter to the prokaryotic CAT gene and studied their inducibility in transient transfection assays in HeLa and neuroblastoma CHP134 cells.

2. Materials and methods

2.1. Plasmid construction, sequence determination and analysis

A 6.3 kbp *Bam*HI-*Xho*I fragment isolated from cosmid cret1 [27,52] was cloned in pBluescript SK$^-$ (Stratagene) and sub-cloned to allow for sequencing (PrBX clone, see Fig. 1). The sequence was determined by the dideoxynucleotide chain termination method either manually, using a USB sequenase 2.0 DNA sequencing kit (Amersham), or automatically, using an ABI-PRISM cycle sequencing kit for the ABI373 DNA sequencer (Applied Biosystems, Perkin-Elmer). The complete sequence obtained was submitted to GenBank under accession number GenBank AF032124. A collection of transcription factor recognition sites described by Faisst and Meyer [53] and by Leonard [54], and a representative group of RARE motifs [50,55–62] were used to search the available sequence for relevant sites (Fig. 2).

For transient transfection assays, three CAT gene reporter constructs were prepared in pCATBasic (Promega): Mae3PCAT, dAccI-CAT and dStuICAT (see Fig. 1). Mae3PCAT was prepared as follows: the 6.3 kbp *Xho*I-*Bam*HI fragment was partially digested with *Mae*II restriction endonuclease; a 5109 bp band (from −5056 to +53) was isolated, cloned into the *Cla*I sites of the pBluescript vector (Stratagene) and subsequently transferred into the *Xba*I-*Hin*dIII sites of the pCATBasic vector (Promega). Two deletion clones were derived from Mae3PCAT by taking advantage of the *Acc*I and *Stu*I restriction sites, giving respectively the dAccICAT (from −3528 to +53) and dStuICAT (from −1296 to +53) constructs.

The positive control clone for RA responsiveness (a DR5 element cloned in pBLCAT2 vector) and the negative control clone (pBLCAT2 vector alone) were a gift of Prof. P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). The RETDR16 and WildDR16 reporter gene constructs were produced by inserting synthetic oligonucleotides carrying the *Hin*dIII-*Sal*I sites into the corresponding sites of pBLCAT2; the insert sequence of RETDR16 was AAGCTTGGATCCCTTGGAGGTCATTCATTGCTTCTGAGCAGGTGAAGATTGTCGAC, while the insert sequence of WildDR16 was AAGCTTGGATCCCTTGGAGGTCATTCATTGCTTCTGAGCAGGTCAAGATTGTCGAC, displaying a single mismatch (C/G) from the RETDR16 insert sequence in the second RARE motif.

2.2. Cell culture and RA treatment

Neuroblastoma CHP134 cells were a gift of Prof. Della Valle (Istituto di Genetica Biochimica ed Evoluzionistica, Università di Pavia, Italy). CHP134 and HeLa cells were grown in Dulbecco's modified Eagle medium (Hyclone) supplemented with 10% foetal calf serum (FCS) (Gibco BRL), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. RA treatment was performed as follows: all-*trans* RA (Sigma-Aldrich) was dissolved in 100% ethanol and added to the cell medium containing 10% charcoal-treated FCS, to a final concentration of 1 μM RA and 0.1% ethanol. In control samples, ethanol alone was added to medium containing 10% charcoal-treated FCS.

2.3. Transient transfection and CAT assay

The day before transfection, cells were seeded in 100 mm dishes at 80% confluence. Transfection in HeLa cells was performed with 20 μg DNA using a modified calcium-phosphate co-precipitation method [63]; transfection in neuroblastoma CHP134 cells was performed using a modified protocol of the recently described [64,65] PEI (polyethyleneimine) technique. 10 μg DNA was diluted into 300 μl medium devoid of serum and antibiotics and mixed to 30 μl of 10 mM PEI (25 kDa, Sigma-Aldrich) (300 mmol PEI/mg DNA). The DNA/PEI mixture was vortexed, incubated for 10 min at RT and diluted into 3 ml complete medium. Cells were depleted of medium prior to addition of the transfection mixture. After 2 h the transfection mixture was removed, cells washed with phosphate-buffered saline (PBS) and fresh medium added. The DNA transfection mixture consisted of 7 μg of promoter construct and 3 μg of pCMV-βgal (Clontech) containing the β-galactosidase coding sequence under the control of the CMV (cytomegalovirus) promoter; in HeLa cells 250 ng of either RARα or RXRα expression vectors (kindly provided by Prof. P. Chambon) were added to the transfection mixture. Total DNA in the transfection mixture was kept constant by adding pBluescript plasmid DNA.

Two days after transfection, cells were collected by scraping in 300 μl of 250 mM Tris-HCl (pH 7.8) and lysed by freezing and thawing. Transfection efficiency was determined by spectrophotometric titration of the β-gal activity in cell lysates. Amounts of cell extracts with equivalent β-gal activity were used in CAT reactions. CAT activity was measured using acetyl-CoA and [^{14}C]chloramphenicol as described by Gorman [28]; CAT reactions were run on thin layer chromatography silica gels and scintillation counting of the spots was performed.

Each transfection presented in this report was performed with duplicate samples and was repeated at least three times.

2.4. Northern blot analysis

Cytoplasmic RNA was extracted as described by Buckler et al. [66]. 20 μg RNA was fractionated by 1% agarose/2.2 M formaldehyde gel electrophoresis, blotted onto nylon membranes (Hybond), and hybridised to ^{32}P-labelled cDNA probes according to standard procedures [63]. A full-length *RET* cDNA fragment (4.5 kbp) was used as a probe. To normalise for housekeeping gene expression, we used a 983 bp human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe obtained by RT-PCR using commercially available primers (Clontech).

3. Results

3.1. Physical characterisation and sequence analysis of the 5′ genomic region of the RET gene

An *Xho*I-*Bam*HI 6.3 kbp genomic fragment was isolated from a cosmid clone (cret1) [27,52] containing the 5′ portion of the *RET* gene and cloned in pBluescript (indicated in Fig. 1 as PrBX). PrBX contains 5075 bp upstream of the transcription start site, the first exon and part of the first intron of the

Table 1
Search for RARE single motifs within the *RET* 5′ flanking region

RARE half-site	No. of sites on the + strand	No. of sites on the − strand
1) AGTTCA	−	1
2) GGTTCA	−	2
3) AGGTCA	1	1
4) AGGGCA	5	5
5) GGGTCA	2	−
6) AGGTGA	−	6
7) AGGTTA	1	−
8) CGGCCA	2	1

Single half-motifs were derived from natural RAREs present in the promoter sequence of mammalian genes encoding: the mouse RAR-α2 isoform (1 [55]), the human and mouse RAR-β2 isoform (1 and 2 [56,57]), the mouse complement factor H (3 and 4 [58]), the human alcohol dehydrogenase (1 and 5 [59]), the rat cardiac myosin heavy chain α subunit (3 and 6 [60]), the mouse laminin B1 (3, 6 and 7 [50]) and the rat phosphoenolpyruvate carboxykinase (8 [61,62]). The table shows the number of sites found in the *RET* promoter on the sense (+) and antisense (−) strands.

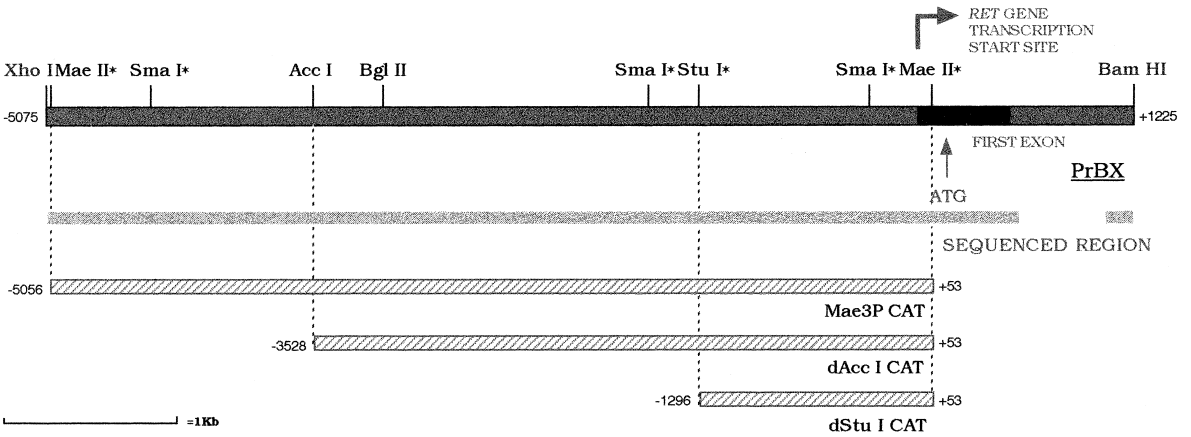

Fig. 1. Physical map of the *RET* gene 5′ flanking region. The 6.3 kbp clone is indicated as PrBx, and the transcription start site and the first exon of the gene are located within it. A restriction map of the clone is shown, where only relevant sites recognised by *Sma*I, *Stu*I and *Mae*II (indicated by asterisks) are shown. A dotted box underlines the sequenced region. CAT gene reporter constructs are represented by thick striped boxes.

gene. PrBx was then sub-cloned and almost fully sequenced (GenBank accession number GenBank AF032124).

A computer analysis of the available sequence, summarised in Fig. 2, revealed the presence of putative recognition sites for several transcription factors [53]. Among them there are widely expressed transcriptional regulators (AP-2, E2A, GCF, PEA3 and Sp1), inducible nuclear factors (ISGF1, 2, 3, STAT factors and MEP1), as well as developmentally regulated, tissue-specific transcription factors (krox-20, krox-24, TTF1 and WT1). Our preliminary sequence analysis also included the search for candidate RAREs, which could provide a useful tool for any subsequent functional analysis. As shown in Table 1, a selected group of RARE half-motifs from mammalian gene promoters [50,55–62] was used to search for single

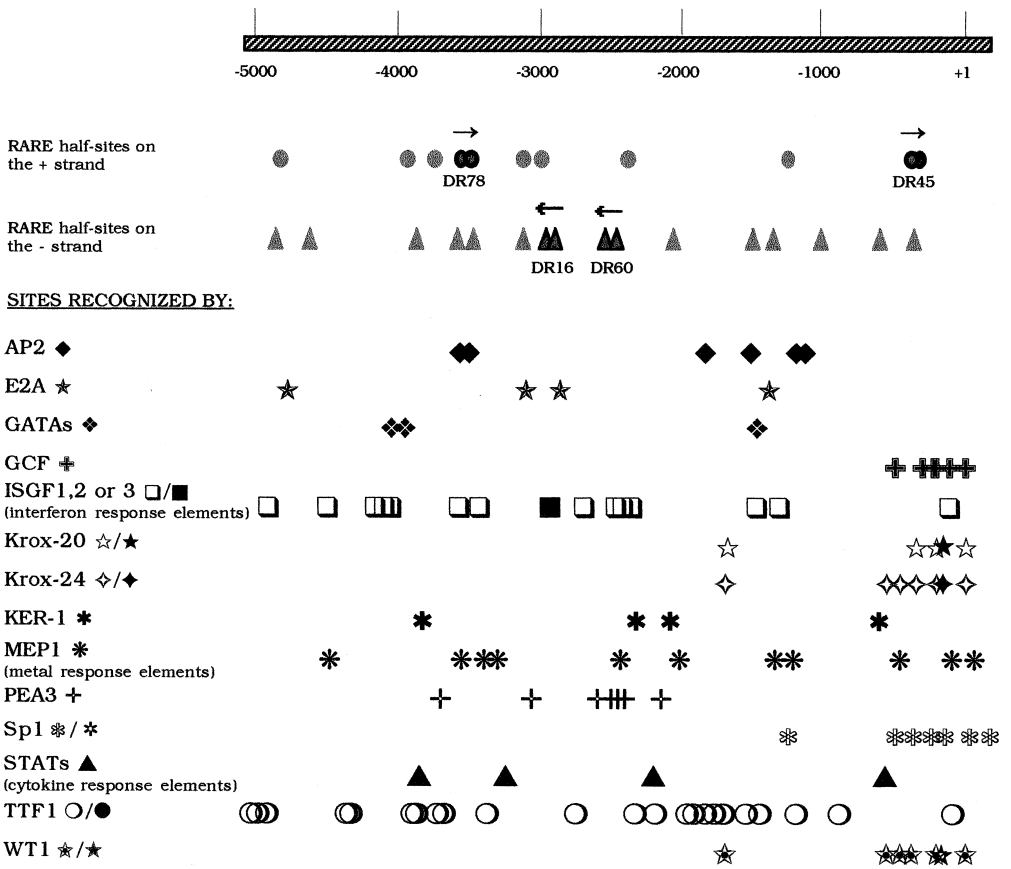

Fig. 2. Sequence analysis. Transcription factor recognition sites and RARE motifs are indicated by symbols and positioned along the *RET* 5′ flanking region (from −5075 to translation start site). Where black and white symbols are present along the same line, the former represent sequences displaying 100% homology to the consensus sequence, while the latter display over 80% homology. Where not indicated, the homology of sites to the consensus sequence is 100%. Candidate RARE direct repeats are outlined by arrows and indicated as DR16, DR45, DR60 and DR78, the number reflecting the length of the spacer sequence.

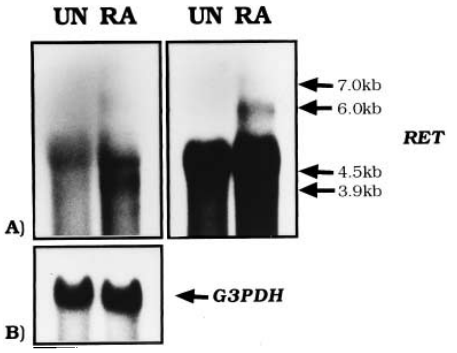

Fig. 3. Northern analysis of *RET* RA responsiveness in CHP134 cells. Cytoplasmic RNA was extracted from untreated cells (UN) and cells treated with 1 μM RA (RA) for 1 day and analysed by Northern blot. A: Hybridisation with a *RET* cDNA full-length probe. The four major *RET* transcripts are indicated by arrows; the 4.5 and 3.9 kbp mRNAs are evident on the left-hand side, while to detect the longer 6.0 an 7.0 kbp mRNAs a prolonged film exposure was necessary (right-hand side). B: Hybridisation with a human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) 983 bp RT-PCR product.

RARE sites: a total of 27 such sequences were identified. Taking into account that even widely spaced directly repeated RARE half-motifs have been shown to behave as functional RAREs, with the maximum transactivating power displayed by DR15 [51], only four direct repeats, in which the spacer sequence was shorter than 100 bp, were considered as candidate RAREs and are outlined in Fig. 2.

3.2. *Northern analysis of RA responsiveness in NB CHP134 cells*

The neuroblastoma CHP134 cell line was chosen as a model system for our study since it showed a relevant RA-induced activation of *RET* expression among several NB cell lines [31]. In order to confirm this activation, we performed Northern blot analysis on cytoplasmic RNA extracted from CHP134 cells untreated and treated with RA for 1 day. As shown in Fig. 3, in RA-treated cells all four *RET* major transcripts (7.0, 6.0, 4.5 and 3.9 kbp) are expressed, although at different intensities, while only the 4.5 kbp transcript is detectable in untreated cells. This finding, together with the evidence already presented by others [30,31], prompted us to study the RA responsiveness of the *RET* gene at the transcriptional level.

3.3. *Transient transfection assays in HeLa and NB CHP134 cells*

In order to elucidate the mechanism of *RET* responsiveness to RA, we produced three CAT reporter gene constructs: Mae3PCAT, dAccICAT and dStuICAT, containing respectively the −5056/+53, −3528/+53 and −1296/+53 regions of the *RET* promoter 5′ to the CAT gene (Fig. 1). These constructs were tested for their retinoic acid sensitivity in transient transfection assays either in neuroblastoma CHP134 cells, which express endogenous retinoid receptors, or in HeLa cells, an ideal neutral recipient for the study of RA-responsive gene promoters. By cotransfecting HeLa cells with retinoid receptor expression vectors (such as RAR-α and RXR-α) it is possible to create a responsive cellular system.

A DR5 natural RARE cloned upstream of the herpes simplex virus thymidine kinase promoter (TK) in the pBLCAT2 vector was used as a positive control of RA responsiveness, while the TK promoter alone present in pBLCAT2 was taken as a negative control. The three *RET* promoter constructs were tested for their inducibility following 24 h RA treatment. As shown in Fig. 4, despite the definite activation displayed by the positive control of responsiveness (over 45-fold in the HeLa system; 7-fold in neuroblastoma cells), the Mae3PCAT construct CAT activity was not enhanced upon RA treatment in either cell line. An identical behaviour was displayed by the dAccICAT and dStuICAT constructs analysed (data not shown).

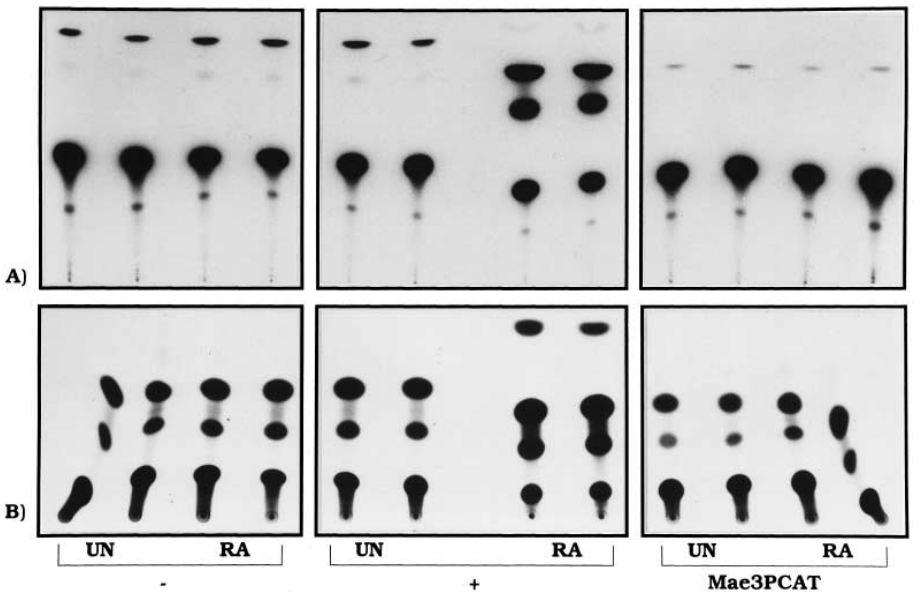

Fig. 4. Transient transfection assays in HeLa (A) and neuroblastoma CHP134 (B) cells. Equal amounts of protein extracts from transfected cells, untreated (UN) and treated with 1 μM RA for 1 day (RA) were used to assay the CAT activity. The positive control of RA responsiveness is indicated as +, while the negative control is indicated as −. The *RET* promoter construct analysed is indicated as Mae3PCAT.

3.4. Functional analysis of the RETDR16 candidate RARE sequence

The data presented above did not rule out the existence of functional RAREs in the gene regulatory region, the activity of which might be overridden by negative transcription regulators acting on the long promoter regions analysed. To test the hypothesis that the negative data obtained so far were due to the use of long promoter constructs, we decided to perform functional analysis on isolated candidate RAREs. Four such sequences were available for our study (see Fig. 2): among them, a AGGTCA$(N)_{16}$AGGTGA direct repeat present at −2972 on the minus strand of the *RET* promoter was the object of our attention. This sequence is in fact closely related to the DR15 motif analysed in the previously mentioned report [51], the directly repeated consensus sequence being the same in the two cases, apart from a single G/C mismatch. The *RET* candidate RARE (RETDR16) and a perfect direct repetition of the consensus motif AGGTCA (WildDR16) were cloned upstream of the heterologous TK promoter in pBLCAT2. In both constructs spacer and flanking sequences surrounding the RARE half-site were the ones present in the *RET* promoter region. RETDR16 and WildDR16 were tested for their RA inducibility as described above. Neither RETDR16 nor WildDR16 displayed any significant RA-mediated activation (data not shown).

4. Discussion

In the present work we completed our previous characterisation of the physical map of the *RET* proto-oncogene genomic region by analysing 5075 bp upstream of the transcription start site.

A preliminary analysis of this sequence allowed the identification of putative recognition sites for several transcription factors. In particular, some of them display an expression pattern which is closely related to that of the *RET* proto-oncogene and are likely to play a role in the control of *RET* expression during development: AP-2 and krox-20 are in fact most abundant in the neural crest lineage; krox-24 is involved in nervous system development; while TTF1 is largely responsible for thyroid-specific gene expression and WT1 is thought to regulate transcription during kidney development [53]. In each of these tissues *RET* expression has been documented during development [25,26]. Our CAT reporter gene constructs provide an experimental system to further investigate the role of these factors in the control of *RET* transcription under different experimental conditions.

In order to study the regulation of *RET* expression promoted by RA we used two simple approaches: Northern blot analysis and transient transfection assays. Northern blot analysis has revealed a positive role of RA in the control of *RET* expression in neuroblastoma cell lines, which represent a valuable system for the study of neural crest cells gene expression. This is consistent with the evidence already presented by others [30,31]. The up-regulation of *RET* expression is an early event promoted by RA treatment, which induces a series of complex phenotypic changes that result in cellular differentiation [31]. In order to further investigate the role of *RET* in RA-mediated cellular differentiation it could be of interest to analyse the expression of the newly identified *RET* ligand and coreceptor molecules [18–23] upon RA treatment. The observation of a concerted RA-mediated up-regulation of all or a part of these genes would confirm the physiological role of retinoids in activating the *RET* signalling machinery during cellular differentiation. A preliminary indication in this sense comes from the observation, made in our laboratory, of the activation of GDNFR-α gene expression after RA treatment (unpublished results).

Our CAT reporter gene approach provides a preliminary evidence for the lack of a direct transcriptional effect mediated by RA receptors on the *RET* promoter. It can be suggested that RA might regulate *RET* expression through a more complex mechanism than direct RAR/RXR-mediated transactivation. Among the transcription factors which are likely to regulate *RET* transcription, AP-2 and krox-20 are induced by RA [53] and could be responsible for *RET* indirect response to RA treatment.

Nonetheless, the presence of a DR16 element and other RARE-like sequences within the gene regulatory region suggests another possible explanation for our data. The constructs analysed contain long genomic regions where the recognition sites for negative regulators of *RET* transcription (such as GCF, see Fig. 2) could mask the RA-mediated activation. In fact, an increased level of *RET* expression has been observed in NB cells by inhibiting protein synthesis with cycloheximide [31], suggesting that negative regulators of transcription could be normally associated with the *RET* regulatory region.

Our functional analysis of the *RET* DR16 element seems to rule out the possibility that the negative data presented here were due to the use of long promoter constructs. It could still be argued that RAREs might be present in additional regulatory regions which are not included in our constructs and are still to be identified.

Our comparative analysis of the RETDR16 and WildDR16 indicates that neither sequence behaves as a functional RARE; this is in apparent disagreement with data reported by others, showing that the spacer length between the half-motifs could be as high as 150 bp [51]. Since spacer between the half-sites and flanking sequences are derived from the *RET* promoter region, our results would suggest that such sequences are important to define a functional RARE and may therefore not allow for nuclear receptor-mediated transactivation. Evidence is being collected on this point, indicating a growing complexity in the molecular aspects involved in the recognition between nuclear receptors and their cognate DNA elements [67–70]. In particular it has been suggested that the promoter context in which a specific element is placed can be the determining factor for nuclear receptor selectivity, being capable of overriding the affinity of transcription factors for their cognate binding sites. To further investigate this topic we could envisage developing a functional analysis of DR16 carrying selected and diverse spacer sequences.

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