Cell-line specific chromatin acetylation at the Sox10–Pax3 enhancer site modulates the *RET* proto-oncogene expression

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Abstract **The *RET* gene is expressed with high tissue and stage specificity during development. Understanding its transcriptional regulation might provide new clues to clarify developmental mechanisms. Here we show that the histone deacetylase inhibitor sodium butyrate (NaB) increases *RET* transcription in cells displaying low levels of its mRNA, while it has no effect in cells expressing at high levels. Chromatin immunoprecipitation (ChIP) experiments showed increased histone acetylation within the same region, in particular the Sox10–Pax3 enhancer site, due to NaB. Accordingly, ChIP showed different acetylation levels at the Sox10–Pax3 site associated with cell-line specific *RET* transcription rates. Concluding, chromatin acetylation targeted to functional sequences in the *RET* regulatory region may control its transcription.**

Key words: *RET* proto-oncogene; Histone acetylation; Nuclear run-on; Chromatin immunoprecipitation

1. Introduction

Acetylation of core histone tails is now recognized as a conserved mechanism modulating gene expression. It is able to relax higher order chromatin, to promote factor binding to DNA and to recruit unique biological complexes that mediate downstream functions [1]. Switch from inactive to active chromatin is often accompanied by histone hyperacetylation of critical sites in gene regulatory regions [2,3]. The antagonistic activities of histone acetyltransferases and histone deacetylases (HDACs) control the nuclear steady-state balance of this covalent modification [1]. Histone hyperacetylation can be accomplished, in vitro, by treating cells with inhibitors of cellular HDACs [4] such as sodium butyrate (NaB). These drugs selectively affect the transcription of specific genes [4]. Chromatin immunoprecipitation (ChIP) experiments have shown a direct effect of these molecules on the acetylation status of their target genes' regulatory regions [5].

The *RET* proto-oncogene encodes a tyrosine kinase receptor predominantly expressed in the developing embryo [6]. Germline *RET* mutations are associated with both Hirschsprung disease (HSCR) and inherited cancer syndromes: multiple endocrine neoplasia types 2A and 2B (MEN2A, MEN2B) and familial medullary thyroid carcinoma (MTC) [7]. While MEN2 syndromes are due to constitutive activation of the Ret receptor, HSCR phenotype is caused by haploinsufficiency or loss of function of the *RET* gene. However, a proportion of such genetic disorders is not associated with mutations in the *RET* coding sequence, suggesting the possible involvement of expression defects caused by alteration of regulatory sequences. Accordingly, *RET* expression or overexpression has been reported in sporadic pheochromocytomas [8] and in head and neck carcinomas [9], in the absence of mutations in the coding region. Finally, in HSCR disease, defective *RET* expression has also been suggested to contribute in some cases to the disease phenotype [10,11]. Thus, understanding the regulation of *RET* expression will provide new clues to the study of gene-related diseases.

Previously, we analyzed *RET* mRNA expression, transcription rate and promoter activity in different cell lines [12–14]. We showed that this gene transcription is cell-line specific. The functional domains of *RET* minimal promoter were identified and Sp1 was shown to be the main positive regulator of the promoter function. However, transfection experiments in cell lines expressing different levels of *RET* mRNA did not show promoter activities consistent with endogenous gene expression, thus raising the question of how the regulated pattern of *RET* transcription is achieved at the molecular level.

In this report, we analyzed the effect of histone acetylation on *RET* expression in different, *RET*-positive, human cell lines. We show that NaB increases *RET* transcription and promotes hyperacetylation of the gene upstream region. In addition, chromatin acetylation targeted to regulatory sequences in the same region contributes to control cell-line specific *RET* transcription.

2. Materials and methods

2.1. Plasmids

Mae3PCAT has been described previously [13]. Bas+6.5λpCAT

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Abbreviations: HDAC, histone deacetylase; NaB, sodium butyrate; HSCR, Hirschsprung disease; MEN2A, MEN2B, multiple endocrine neoplasia types 2A and 2B; MTC, medullary thyroid carcinoma; ChIP, chromatin immunoprecipitation

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contains the 6557 bp fragment of the commercially available λDNA *Hin*dIII digest (New England BioLabs, Beverly, MA, USA), cloned into acceptor *Hin*dIII sites of the pCATBasic empty vector (Promega Italia, Milan, Italy). The RET promoter sequence referred to in this report corresponds to GenBank accession number AF03214.

2.2. Cells and NaB treatment

Human neuroblastoma IMR32 and MTC-TT cells were grown as already described [14]. NaB (Sigma–Aldrich, Milan, Italy) was dissolved in water (1 M stock solution), and delivered to cells at a final concentration of 5 mM. Treatment was performed overnight.

2.3. Transfection and CAT assay

Transfection was performed with polyethylenimine (800K, Sigma–Aldrich) as previously described [14]. Upon transfection and NaB treatment, CAT assays were performed [14]. Each transfection was performed with duplicate samples and repeated at least three times.

2.4. Nuclear run-on assay

Nuclei were prepared from $30–80\times10^6$ cells [15]. Biotin-16-UTP (Roche Molecular Biochemicals, Milan, Italy) was added to a standard reaction mix and Dynabeads M-280 (Dynal, Oslo, Norway) were used to capture the labeled molecules from purified nuclear RNA, as described [15]. Beads were resuspended in DEPC-treated water (30 μl).

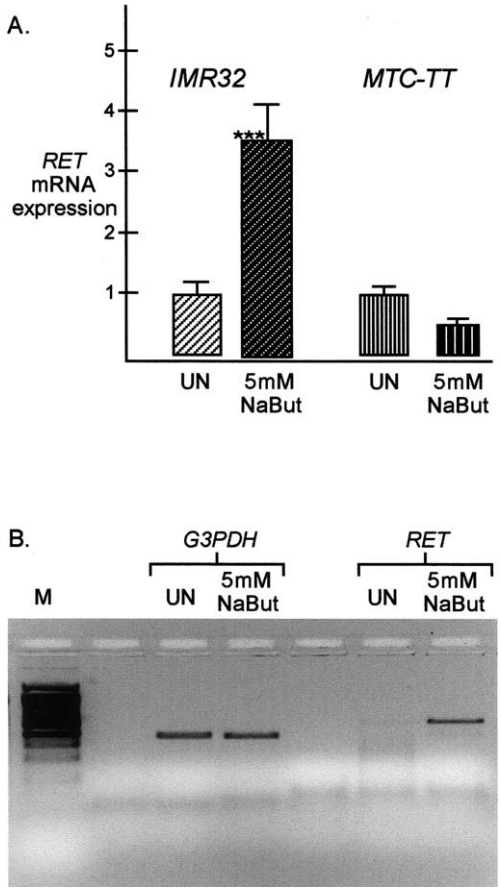

Fig. 1. Up-regulation of *RET* expression and transcription in *RET* mRNA-positive cells. The IMR32 and MTC-TT cells were treated with 5 mM NaB and the *RET* gene expression and transcription rate assessed as described in the text. A: The histogram depicts *RET* expression values of total RNA from treated (NaB) relative to untreated (UN) cells. Mean values and error bars are referred to eight independent calculations. Asterisks indicate significance of results: three asterisks indicate $P<0.01$. B: Single point amplification of both *RET* and the housekeeping *G3PDH* gene, performed using nuclear run-on RNA derived from untreated (UN) and 5 mM NaB-treated (NaB) cells.

2.5. Semi-quantitative RT-PCR

Random hexamer primed cDNA was prepared from 3–10 μl run-on RNA or 500 ng total RNA. The fluorescence-based semi-quantitative PCR method was already described in [15]. In both total RNA and run-on analysis, different *R*-values were determined from independent treatments and, for each treatment, from independent sets of PCR reactions.

2.6. ChIP

IMR32 and MTC-TT cells were labeled for 24 h with 0.5 μCi/ml [^{3}H]thymidine (Amersham Pharmacia Biotech, Milan, Italy), treated with 5 mM NaB, collected and washed in PBS/5 mM NaB to prevent histone deacetylation. Isolation of nuclei from 2.5×10^8 cells and digestion with micrococcal nuclease (Amersham Pharmacia Biotech, Italy) were performed, with minor modifications, as described in [16]. Chromatin was immunoprecipitated with antibodies against hyperacetylated histone H4 (Upstate Biotechnology, Dundee, UK) as reported by Johnson [17]. DNA was isolated from the antibody-bound (i.e. highly acetylated), unbound (i.e. underacetylated) and input chromatin fractions by phenol/chloroform extraction. The [^{3}H]thymidine incorporation was determined by scintillation counting. Equal amounts of DNA from each fraction, based on [^{3}H]thymidine content, were used for semi-quantitative PCR.

Semi-quantitative PCR analysis was performed from serially diluted DNA samples [15]. PCR reactions were run on a 1.5% agarose gel with SYBR Gold Nucleic Acid Gel Stain (Molecular Probes, Eugene, OR, USA) and analyzed on Storm 860 (Amersham Pharmacia Biotech, Italy) with the ImageQuant software. A 700 bp fragment, containing both Sox10 and Pax3 binding sites, was obtained with primers: SPX1F (5′-TGTGGGGACATGGAAAACTG-3′) and SPX1R (5′-TGGCATTCCTGGAACTGCTG-3′). A 402 bp fragment, including the minimal promoter sequence, was amplified with Prom1F (5′-CCCGCACTGAGCTCCTAC-3′) and Prom1R (5′-GGACGT-CGCCTTCGCCAT-3′). Primers SSCP12F (5′-TCTTCTCCCCCT-TCCCTCAT-3′) and SSCP12R (5′-GCCCCGGAGACTCCCCCA-G-3′) were used to amplify a 225 bp fragment including *RET* exon 12 [18]. The human housekeeping β-actin gene was amplified with the forward primer 1F (5′-TCACCCACACTGTGCCCATCTACGA-3′) and the reverse primer 1C (5′-CAGCGGAACCGCTCATTGC-CAATGG-3′) to yield a 297 bp fragment.

2.7. Statistical analysis

In all experiments presented in this report, significance of results was determined by Student's *t*-test, available on-line (Tools for Science, Statistics site, at www.physics.csbsju.edu/stats).

3. Results

3.1. Activation of RET expression and transcription in two human cell lines

In order to determine whether histone acetylation can modulate *RET* expression, we used the HDACs inhibitor NaB to convey histone hyperacetylation in two human cell lines, showing different endogenous amounts of the gene mRNA, as we previously reported [14]. *RET* was shown to be expressed at high levels in MTC-TT cells, derived from MTC, while only a low level was detected in neuroblastoma IMR32 cells.

Upon treatment, we collected total RNA and quantified *RET* expression using a fluorescence-based semi-quantitative PCR [15]. As depicted in Fig. 1A, inhibiting cellular HDACs resulted in a significant up-regulation of *RET* mRNA in IMR32 cells while it had no effect on MTC-TT cells.

To test if NaB directly affects the gene transcription, we collected intact nuclei from IMR32 cells and performed the nuclear run-on assay [15]. Fig. 1B shows a representative single point amplification of four independent run-on experiments. Statistical analysis indicated a 10-fold activation of *RET* expression in IMR32 treated nuclei ($P<0.01$, after four independent experiments). The observed activation was even more evident as compared to the total RNA.

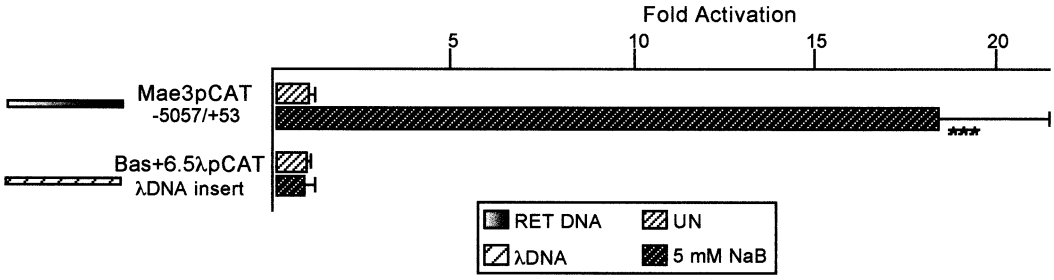

Fig. 2. NaB sensitivity of *RET* promoter region. IMR32 cells were transfected with the indicated constructs. After NaB treatment, cells were collected and CAT reactions performed. CAT activities were normalized to protein content and expressed as activity obtained from NaB-treated cells (dark bars) relative to untreated cells (light bars). Means and mean standard errors are given for at least three separate experiments. Three asterisks indicate $P < 0.01$.

3.2. Sensitivity of RET promoter region to NaB

Chromatin assembly has also been shown to take place on plasmid DNA transfected into cells [19,20]. Therefore, sensitivity of promoters to histone acetylation can be assessed on transfected DNA [5,21]. To test whether NaB treatment was able to relax chromatin of the plasmid containing 5 kbp of the *RET* upstream region, we transfected IMR32 cells with the Mae3PCAT plasmid (−5057/+53) [14]. To verify if the observed effects were sequence specific, the *RET* region was substituted by phage λDNA (a 6.5 kbp *Hin*dIII fragment) in a control plasmid: Bas+6.5λpCAT. CAT activities were measured in untreated and NaB-treated cells. NaB dramatically activated the promoter function of Mae3PCAT (Fig. 2), whereas substitution of the *RET* sequences with phage λDNA was unable to reproduce such an effect. Thus, the *RET* region analyzed is specifically sensitive to NaB.

3.3. NaB determines histone hyperacetylation at the Sox10–Pax3 enhancer site

We performed the ChIP assay on NaB-treated and untreated IMR32 cells to test whether NaB had a direct effect on the acetylation level of histones within *RET* regulatory sequences. Antibodies against tetra-acetylated histone H4 were used to collect hyperacetylated chromatin and the associated genomic DNA. Antibody-bound (i.e. highly acetylated) and unbound (i.e. underacetylated) fractions were analyzed for relevant sequences by semi-quantitative PCR. Quantification was performed for selected sequences located inside the 5 kbp 5′-*RET* flanking region previously shown to be crucial for expression. The minimal promoter (−147/+53), containing four overlapping Sp1 sites [14,22], and an enhancer element consisting of the Sox10 and Pax3 binding sites (−3996/−3390) [23] were tested. Finally, we analyzed *RET* exon 12, as a control sequence within the gene genomic region, likely to be unaffected by NaB.

Due to its GC-rich sequences, the *RET* minimal promoter signal, quantified by PCR, was also confirmed by filter hybridization (data not shown). In each case, normalization of ChIP sample values was performed quantifying the β-actin housekeeping gene, taken as the reference assay for association between histones and DNA.

NaB treatment significantly enhances the acetylation level

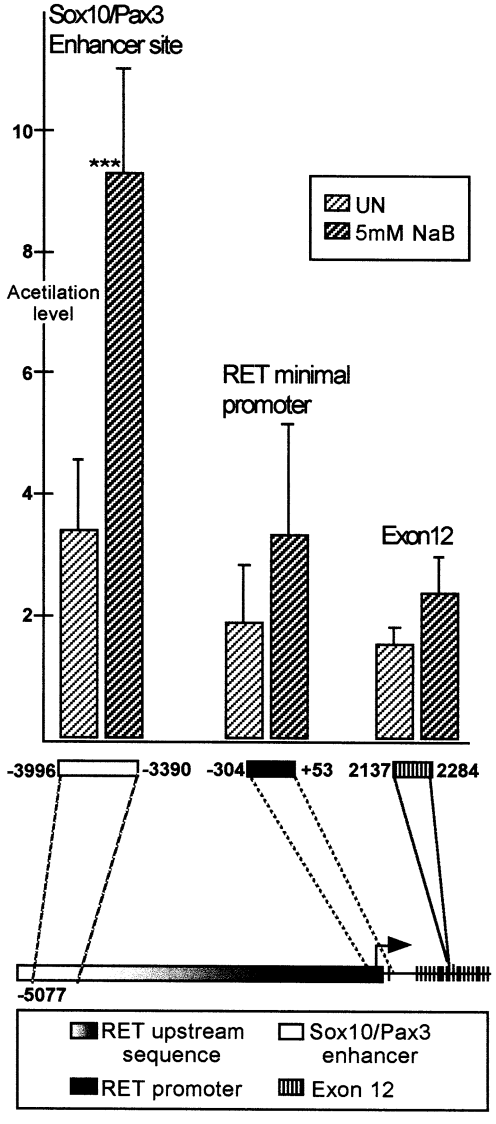

Fig. 3. Quantification of amplification products from ChIP samples: NaB-treated IMR32 cells. The *RET* genomic region (not to scale) is represented in the lower panel. Horizontal bars represent the sequences analyzed in ChIP assays. Bars in the upper panel indicate the relative histone H4 acetylation level within the sequences analyzed by semi-quantitative PCR. Calculation was performed as follows: the ratio of antibody-bound/unbound signals was determined and normalized to the β-actin ratio and total [^{3}H]thymidine content. Dark bars represent values obtained from NaB-treated cells while light bars those from untreated cells. Means and mean standard errors are given for at least three separate experiments. Three asterisks indicate $P < 0.01$.

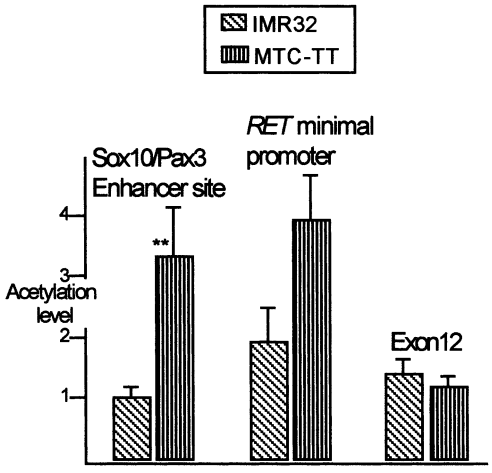

Fig. 4. Quantification of amplification products from ChIP samples: IMR32 and MTC-TT untreated cells. Bars indicate the relative histone acetylation level, calculated as described in the legend to Fig. 3, within the analyzed sequences by semi-quantitative PCR. Different bars represent values obtained from IMR32 and MTC-TT cells. Means and mean standard errors are given for three separate experiments. Two asterisks indicate $P < 0.05$.

of histones associated with the Sox10–Pax3 sequence (Fig. 3). The acetylation level of the minimal promoter fragment was not significantly affected by NaB. As expected, the exon 12 signal detected from treated cells was comparable to control.

3.4. Endogenous association of hyperacetylated histones with RET upstream region

ChIP was then performed on untreated IMR32 and MTC-TT cells to determine whether endogenous levels of acetylation were related to physiological modulation of *RET* expression. We tested the above-described sequences for enrichment in the antibody-bound DNA fraction.

Our results indicate that Sox10–Pax3 enhancer was significantly enriched in histone acetylation in MTC-TT cells as compared to IMR32 cells (Fig. 4). The minimal promoter also appeared to be differentially acetylated. The difference between the two cell lines (Fig. 4), although detectable, did not reach statistical significance in our PCR experiments but it was confirmed by filter hybridization (data not shown). No differences were seen both in the β-actin gene and *RET* exon 12 sequences.

4. Discussion

Chromatin structure is a major determinant of transcription, activation of gene expression being dependent on the interplay of several nucleosome remodeling activities [1,2]. The present work was undertaken to establish a model for studying the *RET* gene transcription and the possible role of histone acetylation on RNA production.

NaB treatment, used to convey histone hyperacetylation, was able to activate *RET* expression and increase the transcription in IMR32 cells displaying low levels of its mRNA, supporting a model in which NaB directly promotes *RET* transcription. Transient transfections confirmed that *RET* upstream sequences are specifically sensitive to NaB. As further confirmation, we have recently shown that NaB also stimulates a detectable level of transcription in *RET*-negative lymphoblastoid cells (P. Griseri et al., submitted). We did not observe an increase of *RET* transcription mediated by NaB in MTC-TT cells. These data can be explained by their high levels of *RET* mRNA, reflecting that histone acetylation at crucial sites might already have reached a plateau level.

ChIP with antibodies against acetylated histone H4 was performed to test whether NaB had a direct effect on the acetylation status of the *RET* upstream region. This was particularly evident at the enhancer element consisting of the Sox10 and Pax3 binding sites [23], indicating that this site is prone to enrichment in histone acetylation level resulting from treatment, and it is important for chromatin acetylation-dependent transcriptional regulation.

ChIP was also performed on untreated IMR32 and MTC-TT cells to determine the endogenous levels of acetylation in the *RET* upstream region. In particular, we found that transcription may be controlled by targeted chromatin acetylation in the Sox10–Pax3 enhancer, suggesting a fine regulation of these two transcription factors. One hypothesis for the role of these proteins is that they might take part in an activating multimeric complex, including histone-acetylating activities. These data further strengthen the indication that the acetylation level within the *RET* 5′ flanking region correlates with endogenous gene expression.

The minimal promoter also appears to display different levels of histone acetylation in different cell lines, thus indicating that Sp1 may contribute to cell-line specific acetylation. However, the minimal promoter is not affected by NaB, thus suggesting that the Sp1 sites are not involved in the up-regulation of *RET* expression induced by the treatment. Specific transcription factors can confer distinct patterns of histone acetylation on target promoters or promoter regions [24]. In this line, we can envisage a differential responsiveness or an insensitivity of the Sp1 DNA sequences to a particular deacetylase inhibitor, such as NaB, as recently reported [25].

RET is one of the first transcribed genes in the pericentromeric region of chromosome 10 [26]. This region is particularly prone to rearrangements, as deduced from changes that have occurred during evolution [26]. Therefore, rearrangements in the pericentromeric region, involving for example insulator sequences or *cis*-acting regulatory elements, might cause position effects resulting in the impairment of *RET* expression. This mechanism would explain in part the proportion of genetic disorders that are not associated with any coding sequence mutations. Present results support the idea that chromatin conformation and therefore the acetylation profile of this genomic domain exert a relevant control on *RET* expression.

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