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Activation of the mouse cytokeratin A (*endoA*) gene in teratocarcinoma F9 cells by the histone deacetylase inhibitor Trichostatin A

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Abstract Treatment of cultured cells with sodium butyrate, that is the histone deacetylase inhibitor, induces the histone hyperacetylation and the expressions of various mammalian genes without affecting the level of protein synthesis. However, butyrate is a non-specific inhibitor of deacetylase because of its effects on various other enzymes and nuclear proteins other than histones. On the other hand, Trichostatin A (TSA) was recently found to be a potent and specific inhibitor of histone deacetylase. We examined the effect of TSA on the expression of mouse cytokeratin A (*endoA*). TSA increased *endoA* expression in F9 cells, and was efffective at a much lower concentration than sodium butyrate. We also examined the changes of chromatin structure induced by the two drugs by a DNase I-hypersensitivity assay. Both drugs induced the formation of a DNase I-hypersensitive site (DH site) in only the promoter region. The precise mechanism(s) by which the two drugs increase *endoA* gene expression is unknown, but these results suggest that *endoA* expression is induced by inhibition of histone deacetylase and that the effect is at the transcriptional level.

Key words: endoA; Trichostatin A; F9; Histone acetylation; Chromatin; DNase I-hypersensitive site

1. Introduction

The mouse *endoA* gene is a useful model for studies on developmental regulation [1,2], and particularly the features of chromatin structure that differ in active and inactive genes [3].

Transcriptionally competent chromatin differs from the bulk of inactive nuclear chromatin in various characteristics. The active gene appears to be packaged in forms of nucleosomes that are more sensitive than the bulk of nucleosomes to digestion by DNase I [4]. Moreover, transcriptionally active chromatin is more soluble than inactive chromatin in various buffers, thus allowing fractionation of chromatin into active and inactive components [5,6]. The increased solubility of transcriptionally active chromatin suggests partial unwinding or loosening of nucleosome-nucleosome interactions, reflecting a more open conformation. The loosening of the chromatin structure in transcribed regions is thought to be due to rapid, transient acetylation and deacetylation of histones, and results by various experimental approaches have shown that histone acetylation is correlated with transcription [7–9].

Sodium butyrate, an inhibitor of histone deacetylase, enhances the extent of histone acetylation, which has been correlated with modifications of the chromatin structure and increased susceptibility to digestion by DNase I or micrococcal nuclease [10,11]. Sodium butyrate has been used to induce differentiated functions in a variety of cell culture systems [12,13] and induces selective transcriptional activation of some genes [13,14]. These activations seem to be due to its effect on histone deacetylase. However, experiments using sodium butyrate at a high concentration in vivo must be interpreted with caution because this compound not only blocks histone deacetylase but also inhibits the phosphorylation of histones and influences the phosphorylation of nuclear proteins [15]. Recently, the fungistatic antibiotic trichostatin A (TSA) has been shown to be a potent, specific inhibitor of histone deacetylase, inhibiting the enzyme activity at a nanomolar concentration [16] and activating a plaminogen activator gene in F9 cells [17].

To elucidate the role of histone hyperacetylation in the regulation of gene expression, we examined the correlation between expression of the mouse *endoA* gene and chromatin structure in cultured F9 cells in the presence and absence of the histone deacetylase inhibitor TSA.

2. Materials and methods

2.1. Cell culture

F9 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum on gelatin-coated culture dishes. All cultures were supplemented with 5 mM glutamine/10 units penicillin/0.5 μg streptomycin per ml. For treatment with trichostatin A (TSA) or sodium butyrate, F9 cells were plated at a density of 2×10^6 cells/100 mm dish and 24 h later, TSA and sodium butyrate, respectively, were added at final concentrations of 30 nM and 5 mM. Cells were harvested after 1 day for DNase I-hypersensitivity assay.

2.2. RNA isolation and analysis

Cells were plated at 8×10^5 cells/100 mm dish, and after incubation for 24 h, were exposed to 30 nM TSA or 5 mM sodium butyrate for various times. Total cellular RNA was extracted with guanidinium isothiocyanate followed by centrifugation in cesium chloride solution according to a standard method [18]. Samples of 20 μg of RNA per lane were separate by electrophoresis in 1.2% agarose gel containing 2.2 M formaldehyde [18] and then transferred to nylon membranes in 20 × SSC. The blots were hybridized to ^{32}P-labeled *endoA* cDNA [19].

2.3. Preparation of nuclei

Nuclei from F9 cells were purified by a modification of the method of Oshima et al. [20]. Monolayers were washed with cold phosphate-buffered saline (PBS), scraped into cold PBS, and collected by centrifugation for 10 min at 1,000 rpm. Subsequent steps were carried out at 0–4°C. The pellet was resuspended in lysis buffer (0.25 M sucrose, 0.45% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 5 mM MgCl2, 0.1 mM EGTA, and 0.1 mM phenyl-methylsulfonyl fluoride (PMSF)). After vigorous vortexing for 10 s, the mixture was stood at

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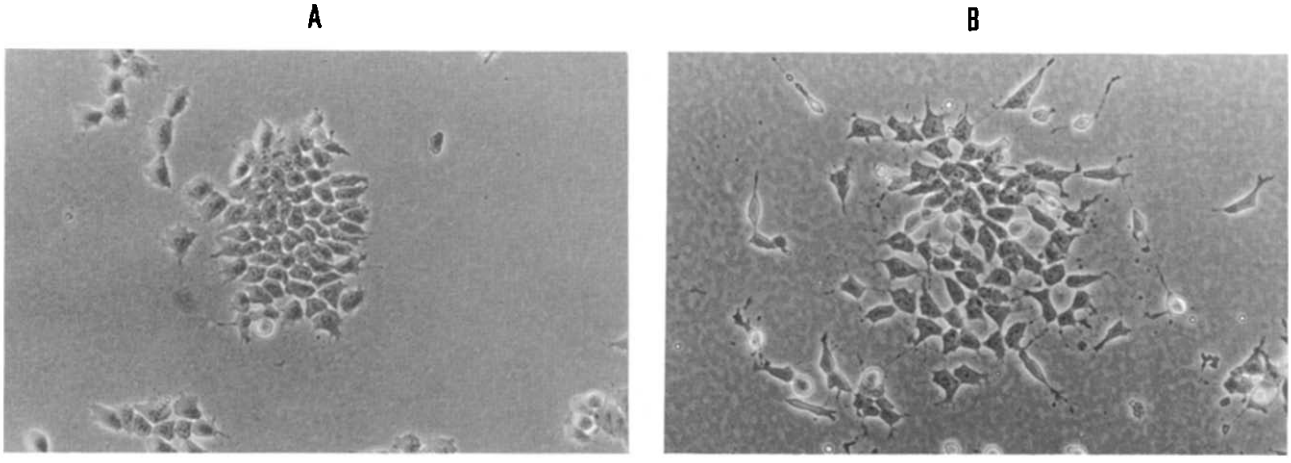

Fig. 1. Effect of TSA on the morphology of F9 cells. Cells were inoculated at 5×10^5 cells per 100-mm culture dish. One day later, TSA was added at 30 nM, and the morphology of the cells was observed 24 h later. (A) control; (B) with 30 nM TSA.

0°C for 15 min, and then, nuclei were pelleted by centrifugation at 1,500 rpm for 5 min, and resuspended in lysis buffer. The nuclear suspension was centrifuged at $1,300 \times g$ for 10 min through a cushion of 30% sucrose in 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 3 mM $MgCl_2$. The nuclear fraction was washed once with suspension buffer (10 mM Tris-HCl (pH7.5), 10 mM NaCl, 3 mM $MgCl_2$, and 0.1 mM $CaCl_2$), precipitated by low-speed centrifugation and resuspended in the same buffer at a concentration of 2×10^8 nuclei per ml.

2.4. DNase I-hypersensitivity assay

Determination of DNase I-hypersensitive site was carried out as described [3]. Nuclei (10^7/200 μl) were treated with increasing concentrations of DNase I (Worthington Biochemicals, DPFF grade) for 5 min at 25°C, and the reaction was terminated by adding final concentrations of 12.5 mM EDTA and 0.25% sodium dodecyl sulfate. The mixtures were then treated overnight with 100 μg proteinase K per ml at 37°C, followed by phenol/chloroform extraction and ethanol precipitation of the DNA. DNA samples were dissolved in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA] and digested with appropriate restriction enzymes, and RNA was removed by treatment with 25 μg/ml of RNase A. The enzymes were removed by phenol/chloroform extraction. The DNA was precipitated with ethanol, dissolved in TE buffer, and separated by electrophoresis in 1.2% agarose gel in TAE buffer (40 mM Tris-acetate (pH 8.0), 1 mM EDTA). It was then transferred to nitrocellulose filters (Schleicher&Schuell) and hybridized to specific probes labeled with [α-^{32}P]dCTP (Amersham) by the random primer procedure.

3. Results

3.1. Effect of TSA on the morphology of F9 cells

Previous studies showed that sodium butyrate induced a slightly dispersed arrangement of F9 cells, which had a flat, polygonal shape [13]. We examined the effect of TSA on the morphology of F9 cells. For change in cell morphology, the minimum concentration of TSA required was as low as 20 nM (data not shown). The morphology of F9 cells after treatment with 30 nM TSA for 24 h is shown in Fig. 1. The morphological change, that is the same as that of previous report [17], became apparent within 6 h after TSA addition.

3.2. Effect of TSA on expression of the mouse endoA gene

The effects of exposure of F9 cells to 30 nM TSA or 5 mM sodium butyrate for various times on the expression of *endoA* mRNA were examined by Northern blot analysis (Fig. 2). Treatment with TSA markedly enhanced the mRNA level of *endoA* by 8 h after its addition and the increased level persisted until at least 24 h after treatment. Sodium butyrate also induced endo A expression, as reported previously [13].

3.3. Association of induction of the endoA gene by inhibitors of deacetylase with an active chromatin configuration

The mouse *endoA* gene is 12-kb long and consists of 9 exons and 8 introns [21]. We have previously identified an enhancer located in the 3′-flanking region and shown that in parietal yolk-sac-like cells, PYS-2, the promoter is activated by a 3′-downstream enhancer [22]. We first examined whether DH sites are present within a 2.6-kb *Eco*RI fragment consisting of the 5′-flanking region, the first exon, and about half the first intron. For this, nuclei from mouse teratocarcinoma F9 stem cells were

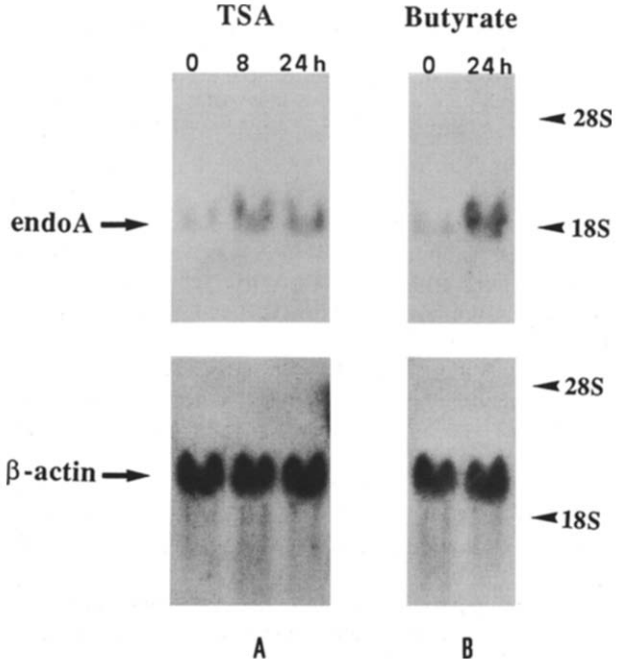

Fig. 2. Effects of TSA and sodium butyrate on *endoA* expression. Cells were treated with 30 nM TSA (A) or 5 mM sodium butyrate (B) for the indicated times. Cytoplasmic RNAs were isolated and analyzed as described in section 2.

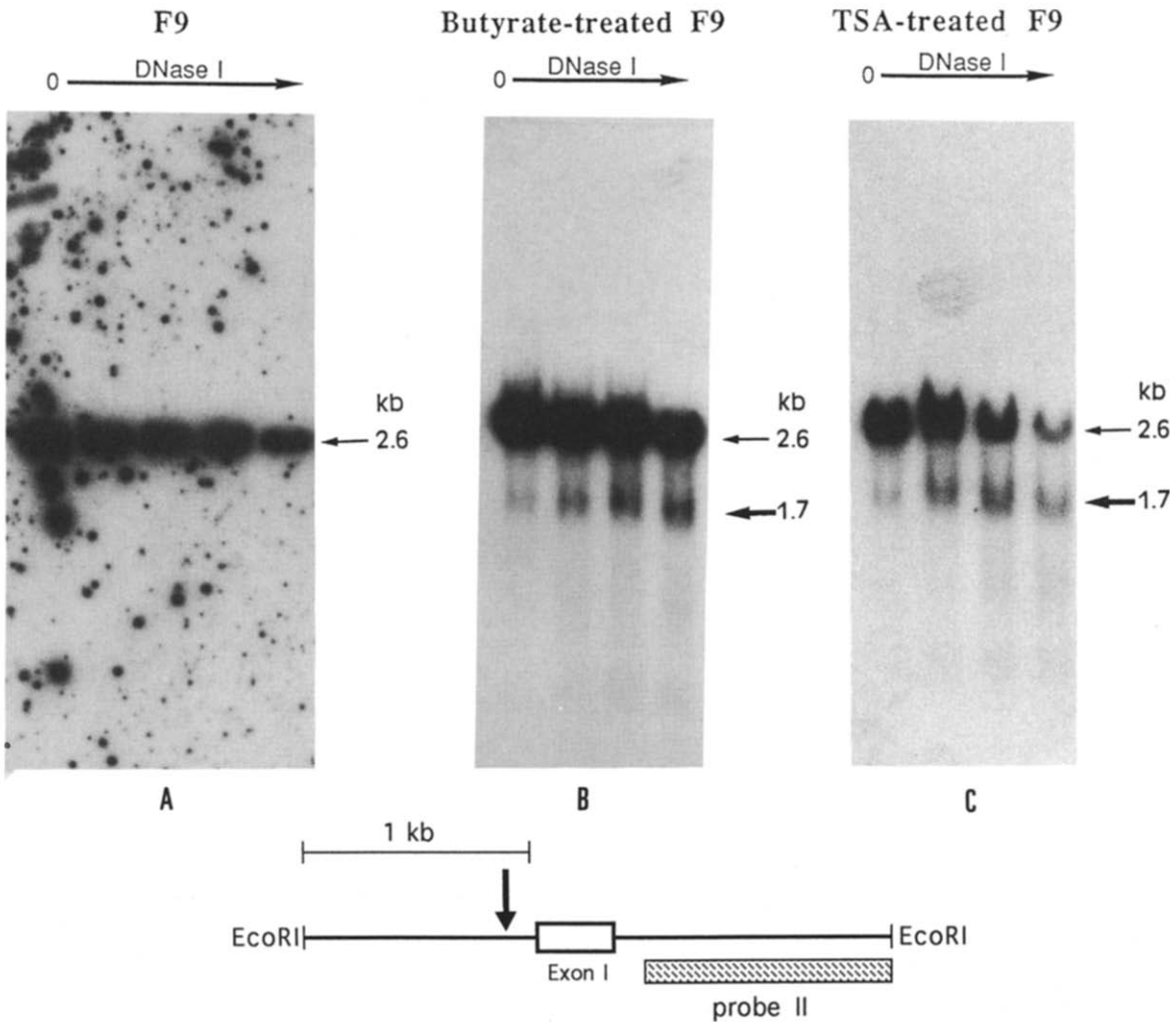

Fig. 3. DNase I-hypersensitivity in the 5'-end 2.6-kb *Eco*RI fragment of the mouse *endoA* gene in stem (A), sodium butyrate-treated (B) and TSA-treated (C) F9 cells. Nuclei were treated with DNase I at increasing concentrations indicated by the rightward arrow (0, 0.02, 0.04, 0.08, and 0.15 units/ml (panel A); 0, 0.04, 0.08, and 0.15 units/ml (panels B, C)) The *Eco*RI digest of DNA was separated in 1.2% agarose gel followed by hybridized to probe II. Sizes in kb are those of the parental fragment (2.6-kb; thin arrow) and the subfragment obtained by DNase I digestion (1.7-kb; thick arrow). The positions of the DH site (downward arrow) and of the probe (stippled box) are shown at the bottom.

incubated with increasing concentrations of DNase I, and after *Eco*RI digestion, Southern blotting was performed followed by hybridization to probe II. No positive subfragment was detected, suggesting that no DH site is present in the 2.6-kb *Eco*RI fragment (Fig. 3A).

We next examined the chromatin structure of the same region of F9 cells treated with TSA or sodium butyrate. Probe II hybridized to only a 1.7-kb subfragment of preparations after both treatments (Fig. 3B and C). This DH site was located in the promoter region. We have reported that probe II hybridizes to two subfragments of 1.9-kb and 1.7-kb within this region in differentiated F9 cells induced by retinoic acid (RA) and dibutyryl cAMP [3].

Subsequently, the 4-kb *Bam*HI fragment, containing an enhancer located 1.2-kb downstream from the last exon, was examined. This region has no DH site in undifferentiated F9 cells, but one DH site has been identified in the enhancer region in differentiated F9 cells (Fig. 4A; see [3]). Nuclei from F9 cells treated with TSA or sodium butyrate were incubated with increasing concentrations of DNase I, and after *Bam*HI digestion, Southern blotting was performed to probe V. Unexpectedly, we did not detect any subfragments (Fig. 4B and C). These results suggest that induction of *endoA* gene expression by inhibitors of histone deacetylase does not cause change of chromatin structure in the enhancer region.

4. Discussion

Histone acetylation plays an important role in chromatin assembly. There is a close correlation between histone acetylation and transcription, and several studies have shown that actively transcribed genes are selectively enriched in acetylated histones [7–9]. Butyrate has been suggested to be rather unspecific inhibitor of histone deacetylase, also affecting some other enzymes [14]. Recently TSA, which differs in chemical structure from sodium butyrate, was shown to enhance accumulation of highly acetylated histones in vivo, and its effect was shown to be due to its specific inhibition of histone deace-

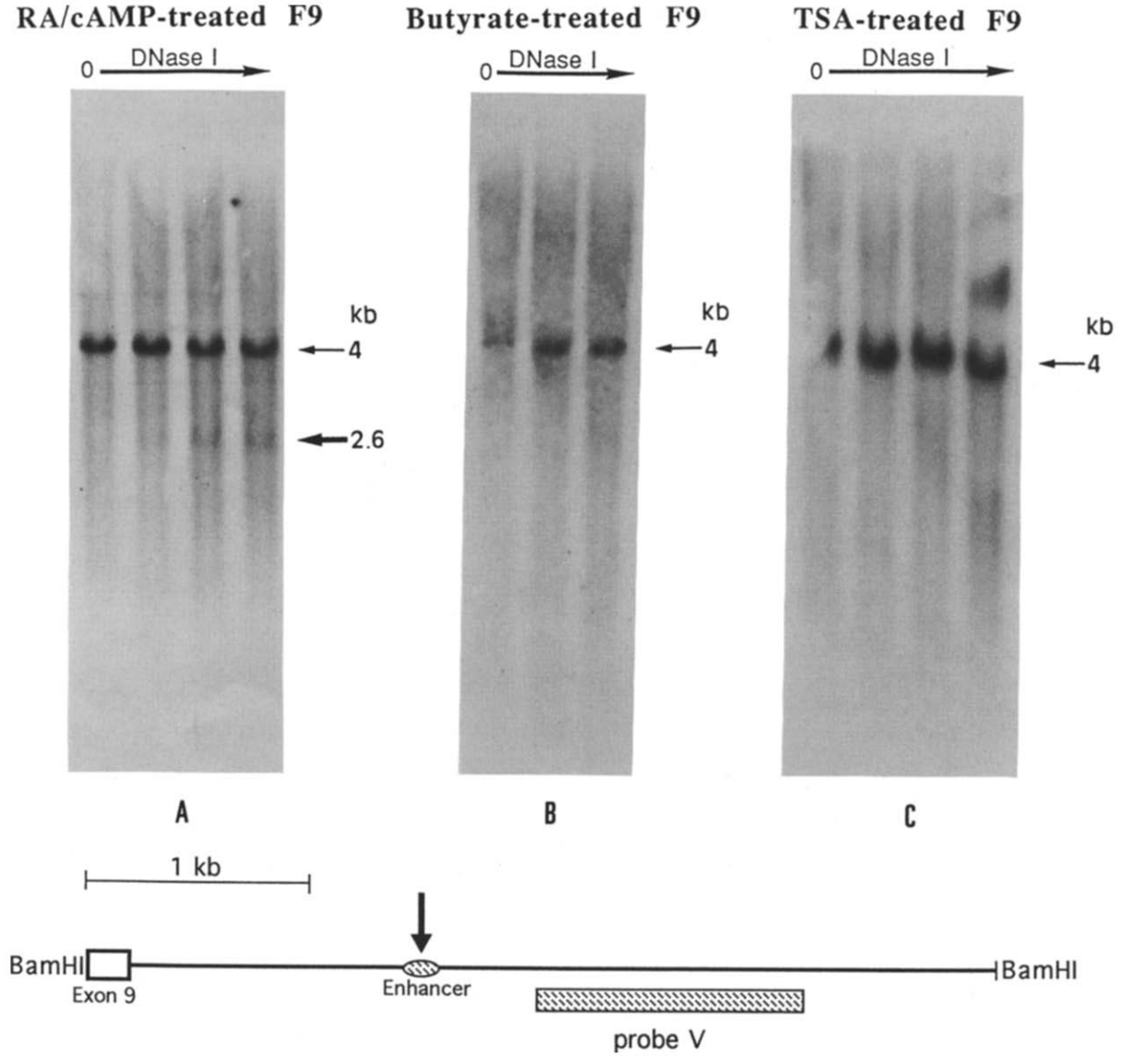

Fig. 4. DNase I-hypersensitivity in the 3′-end 4-kb *Bam*HI fragment of the mouse *endoA* gene in retinoic acid (RA) and dibutyryl cAMP-treated (A), sodium butyrate-treated (B) and TSA-treated (C) F9 cells. Nuclei were treated with DNase I at increasing concentrations indicated by the rightward arrow (0, 0.02, 0.04, and 0.08 units/ml (panel A); 0, 0.08, and 0.15 units/ml (panel B); 0, 0.04, 0.08, and 0.15 units/ml (panel C)). The *Bam*HI digest of DNA was separated in 1% agarose gel and hybridized to probe V. The positions of the DH site (downward arrow) in the enhancer region of the *endo A* gene in differentiatd F9 cells and of the probe (stippled box) are shown at the bottom.

tylase [16]. We examined the effect of TSA on expression of the mouse *endoA* gene in F9 cells. TSA at 30 nM induced a slightly dispersed arrangement of F9 cells similar to that observed on their treatment with 5 mM sodium butyrate. This differentiation-like morphological change of the cells was associated with induction of *endoA* mRNA synthesis. Thus we assume that inhibition of histone deacetylase activity caused both morphological change of F9 cells and induction of *endoA* mRNA.

We also examined the changes in chromatin structure associated with activation of this gene. In a 12-kb genomic fragment carrying the mouse *endoA* gene, a DNase I-hypersensitive site was found only in the promoter region in cells treated with TSA or butyrate. This result is consistent with the general observation that regions around the promoter of genes that are active are DNase I-hypersensitive [23]. The precise mechanism(s) by which the two drugs increase *endoA* expression is still unknown, but these results suggest that *endoA* expression is caused by inhibition of histone deacetylase and that the effect(s) is at the transcriptional level. We could not detect any DH site located upstream of the promoter or in the enhancer region. We do not know why treatment with the histone deacetylase inhibitor does not confer the active chromatin configuration on both regions, as observed in differentiated F9 cells induced by retinoic acid (RA) and dibutyryl cAMP. Tazi and Bird reported that CpG islands, which are involved in the 5′ end of all known housekeeping genes, contain histones H3 and H4 in a highly acetylated form [24]. Since the region of the upstream of the promoter and the enhancer does not contain CpG islands [21], the absence of CpG islands may not guarantee the formation of highly acetylated chromatin. Alternatively, TSA may affect the

formation of a DH site by preventing nucleosome displacement due to histone hyperacetylation [25–27]. Another possibility is that TSA-induced expression of *endoA* gene is excuted through an independent pathway from the retinoic acid- induced expression.

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