**Sp1 up-regulates cAMP-response-element-binding protein expression during retinoic acid-induced mucous differentiation of normal human bronchial epithelial cells**

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**INTRODUCTION**

RA (retinoic acid) exerts a profound effect on vertebrate development, cellular differentiation, and homeostasis [1–4]. In addition, RA plays a pivotal role in the induction and maintenance of mucociliary differentiation of airway epithelial cells [5–10]. In the respiratory system, RA deficiency causes squamous metaplasia, and RA supplementation restores the normal mucous cell phenotype in cultured primary bronchial epithelial cells [7]. It has been well documented that the physiological effects of RA is mediated through two families of NRs (nuclear receptors), the RAR (RA receptor) and the RXR (retinoid X receptor), which control transcription initiation by binding to RAREs (RA-response elements) [1,11,12]. However, we recently demonstrated that RA rapidly activates CREB [CRE (cAMP-response element)]-binding protein] without using RARs (RA receptors) or RXRs in NHTBE cells (normal human tracheobronchial epithelial cells) [13,14].

CREB is a 43 kDa protein that belongs to a family of bZip (basic domain-leucine zipper) transcription factors that includes ATF1 (activating transcription factor 1) and CREM (CRE modulator) [15,16]. CREB activates gene transcription by binding to regulatory DNA elements in the promoter regions of genes with the consensus motif: binding has been found to occur at an 8-bp segment of the canonical CRE motif, 5′-TGACGTCA-3′, and at other sequence variants (called CREs) [17–19]. Phosphorylation of CREB at Ser133 promotes recruitment of the transcriptional co-activator CBP (CREB-binding protein)/p300 and/or the TBP (TATA-box-binding protein)-associated proteins (e.g. TAFII110) of the polymerase II complex to stimulate gene transcription [20–22].

CREB is widely distributed in most tissues and has an essential role in controlling cell growth, differentiation, survival and cell cycle progression and in determining the fate of many cell types [23–27], as well as being a proto-oncogene in myeloid transformation [28].

The only mechanism reported to be involved in CREB expression is CREB phosphorylation, because CREB expression has been considered constitutive [29,30]. However, there is increasing evidence that CREB gene expression is subject to dynamic regulation that varies depending on the cell type. In primary rat Sertoli cells, activation of the cAMP pathway up-regulates CREB expression via CREs present in the 5′ promoter region of the CREB gene [31]. Regulation of CREB expression has also been demonstrated in neuronal cells in vivo and in vitro [32–35]. However, these findings in Sertoli and neuronal cells are in marked contrast with findings in certain other systems (e.g. CATH.a cells). Widnell et al. [35] reported that mRNA of CREB was down-regulated in CATH.a cells (a neural-derived cell line) of endogenous Sp1 using siRNA (small interfering RNA) decreased RA-induced CREB gene expression. However, the converse was not true: knockdown of CREB using CREB siRNA did not affect RA-induced Sp1 gene expression. We conclude that RA up-regulates CREB gene expression during the early stage of NHTBE cell differentiation and that RA-inducible Sp1 plays a major role in up-regulating human CREB gene expression. This result implies that co-operation of these two transcription factors plays a crucial role in mediating early events of normal mucous cell differentiation of bronchial epithelial cells.

**Key words:** airway epithelial cell, cAMP-response-element-binding protein (CREB), normal human tracheobronchial epithelial cell (NHTBE cell), retinoic acid (RA), retinoic acid receptor (RAR)/retinoid X receptor (RXR), specificity protein 1 (Sp1).
by activation of the cAMP pathway. Thus it would appear that perturbation of the cAMP pathway could exert opposite effects on CREB expression depending on the type of cells involved.

Our previous studies showed that RA-activated CREB plays a critical role in the expression of mucin genes, which are typical indicators of bronchial mucous cell differentiation [13,14]. We also observed that CREB expression is very low in fully differentiated metaplastic squamous bronchial epithelial cells. As it has been shown that CREB can be positively autoregulated [31], we hypothesized that RA-activated CREB induces CREB gene expression in bronchial epithelial cells. To test the hypothesis, we determined the level of CREB expression after RA treatment in NHTBE cells and analysed the transcriptional regulation mechanism using the upstream region of the human CREB gene. Interestingly, we found that RA up-regulates Sp1 (specificity protein 1) expression and RA-inducible Sp1 contributes to the up-regulation of CREB, whereas the basal level of CREB is autoregulated by CREB itself. These results suggest a co-operative role of these two transcription factors in regulation of CREB expression during mucous cell differentiation of normal bronchial epithelial cells.

**MATERIALS AND METHODS**

**Cell cultures and chemicals**

NHTBE cells (Clonetics) were cultured by the air/liquid interface method as described previously [5,7,13,36]. Briefly, second-passage NHTBE cells were seeded at a density of 1 × 10^5 cells on to 24 mm semipermeable membrane inserts (Transwell–Clear; Corning) and grown in a serum-free culture medium supplemented with growth factor and hormones. The air/liquid interface was initiated when the cultures were confluent. CHX (cycloheximide), all-trans-RA and ActD (actinomycin D; Sigma–Aldrich) were dissolved in DMSO. Ro 61-8431 (pan-RAR antagonist) and Ro 26-5405 (pan-RXR antagonist) were provided by Roche Bioscience. All cells were maintained at 37°C in an incubator containing 5% CO₂.

**QRT-PCR (quantitative real-time PCR) and conventional RT–PCR (reverse transcription–PCR) analyses**

Total RNA was extracted from NHTBE cells cultured in the absence or presence of RA (1 µM) for specific times and concentrated by using a RNeasy Mini kit (Qiagen). The reverse transcription reaction was performed using 1 µg of total RNA that was reverse-transcribed into cDNA using a random hexamer primer (GeneAmp RNA PCR core kit; Applied Biosystems) according to the manufacturer’s instructions. All primer sets produced amplicons of expected sizes and sequences. For the quantitative analysis of mRNA expression, the iCycler thermal cycler (Bio-Rad Laboratories) was used with the DNA-binding dye SYBR Green (Applied Biosystems). The PCR cycling conditions for CREB expression were 95°C for 5 min, followed by 40 cycles at 95°C for 30 s and 60°C for 1 min. Initial PCR conditions for Sp1 expression were 95°C for 5 min, followed by 40 cycles at 95, 55 and 72°C for 30 s each. PCR for human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) labelled with VIC dye (Applied Biosystems) was performed as an endogenous control. Primer sequences were as follows: CREB, 5'-ACTGTAACCGTCCGCAACTCC-3' (sense) and 5'-GAATTGATGATCAGGGGCTGA-3' (antisense). Human Sp1 primer sets were purchased from SuperArray Biosciences. For conventional RT–PCR, total RNA was extracted and then reverse-transcribed into cDNA using a random hexamer primer. The conditions for PCR were 30 cycles at 95, 55 and 68°C for 30 s each. PCR for β-actin (Ambion) was performed as an internal control. Sp1 primer sequences were used in QRT-PCR. Other primer sequences were as follows: CREB, 5'-ACCATGGAATCTCTGGACGCAGAAC-3' (sense) and 5'-CTGTAGGAAAGGCCTCTTGAAGA-3' (antisense); and RARβ, 5'-AGCTTACGTGCCAAAAAAGG-3' (sense) and 5'-TCTAGGTGGGAGCCTAATTG-3' (antisense). PCR products were then separated in a 2% agarose DNA gel and stained with ethidium bromide.

**Cloning of the human CREB promoter and construction of chimaeric luciferase reporter plasmid**

The BAC (bacterial artificial chromosome) clone (RPCI-11.C 17I6; Invitrogen) was used to clone the human CREB promoter region. PCR was performed to amplify the 5'-flanking regions of the CREB gene using the human BAC DNA as the template. The primers used to generate the promoter deletion plasmids were as follows: forward, 5'-GAGGA-GGTCAGCGGTTGAGTAAATGC-3', 5'-GAGGACTTCGATGCTCCGTCG-3'; 5'-GAGGAAGTCCCGCCTCGGCTT-3'; 5'-GAGGACCTTACAGGATCTCCG-3'; 5'-GAGGAGTCATCGATCGTTTGTGCTTGC-3'; 5'-GAGGAAGTCGTGGGTACG-3'; 5'-GAGGAGCCTGCTCGCTTGCAGT-3' and 5'-GAGGAGTCGGTGGTGGTGTG-3'; and reverse, 5'-GGCTCTAGTCCTCCTCCACGTAAAC-3'. Primers were designed to contain SacI or NheI restriction sites so that the resulting PCR-amplified fragments could be cloned into the multicloning sites upstream of the luciferase reporter gene in the pGL3-basic vector (Promega).

Site-directed mutations of the human CREB promoter were made in the context of −321/−70 CREB-LUC, using the QuikChange® site-directed mutagenesis kit (Stratagene). The oligomers used to introduce multiple point mutations were: CRE1, 5'-GGAGGTGTTTCTGAGGTTGTTGTA-3'; CRE2, 5'-TGGTGGTAGCTGCAGCCGCGTGGTTG-3'; CRE1 + 2, 5'-TGGTGGTAGCTGAGGCGGCA-3'; CRE1 + 5, 5'-TGGTGGTAGCTGAGGCGGCA-3'; CRE1 + 6, 5'-TGGTGGTAGCTGAGGCGGCA-3'; CRE1 + 7, 5'-TGGTGGTAGCTGAGGCGGCA-3'; and Sp1, 5'-CTCGCAGGACCCGTCGTCG-3' where bold, underlined type indicates a mutation [37]. To generate the Sp1-1 and Sp1-2 mutant, the Sp1-1 and Sp1-2 primers were used in combination. All constructs were confirmed by DNA sequencing.

**Transfection and luciferase assays**

For the luciferase assay, NHTBE cells were plated on to 12-well culture plates at a density of 1 × 10^4 cells/well. Cells were grown in BEGM (bronchial epithelial growth medium) without RA. When the cells reached 80% confluence, they were subjected to transient transfection with 0.5 µg of plasmid DNA with FuGENE™ 6 transfection reagent (Roche Diagnostics), which was performed according to the manufacturer’s instructions. To normalize the transfection efficiencies of the various luciferase constructs, the phRL-CMV plasmid containing the Renilla luciferase gene was co-transfected into the cells at a molar ratio of 1:50 (ratio of phRL-CMV and pGL3). At 4 h after transfection, the medium was replaced and the cells were treated with RA. The cultures were maintained for an additional 48 h. CREB promoter activity was determined by measuring firefly luciferase activity, and Renilla luciferase activities were measured sequentially with a dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions; a Lumat LB 9507 luminometer (EG & G Berthold) was used to make the measurements.
RA-induced Sp1 increases CREB expression

Figure 1 RA up-regulates CREB expression

(A–E) NHTBE cells grown in RA-deficient medium for 7 days were incubated with or without 1 μM RA for various periods of time or with various concentrations of RA for 24 h, as indicated on the Figures. CREB expression was analysed by Western blotting (A, C) and QRT-PCR (B, D). Vehicle (DMSO) was used as negative control. (E) ActD and CHX effects on RA-induced CREB expression.

EMSA (electrophoretic mobility-shift assay)

To assess the DNA-binding activity of CREB, we performed EMSA as described previously [13,38], with necessary modifications. Briefly, nuclear proteins from RA-treated or untreated cells were prepared and stored at −80°C. For EMSA, oligonucleotide–corresponding sequences were as follows: Sp1-1, 5′-TGTGGCCGGCGTGCTGGAGAGCG-3′; Sp1-2, 5′-CTCGGCACCGGCGGCGTCGCTGGCT-3′; CRE1, 5′-GGAGGTGTAGTTTGACGCGGTGTGTTACGTG-3′; and CRE2, 5′-TGTTGGTGAGTGACGCGGCGGAGGTGTA-3′. These oligonucleotides were annealed and end-labelled with [γ-32P]ATP using T4 polynucleotide kinase. Nuclear extract (10 mg) was incubated at room temperature (25°C) for 30 min with the 32P-labelled CRE probe in binding buffer [20% (v/v) glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT (dithiothreitol), 250 mM NaCl, 50 mM Tris/HCl, pH 7.5, and 0.25 mg/ml poly(dI-dC)·(dI-dC)]. DNA–nuclear-protein complexes were separated from the DNA probe by electrophoresis on 5% non-denaturing polyacrylamide gels in 0.5× TBE (Tris/borate/EDTA; 1× TBE = 25 mM Tris base, 25 mM boric acid and 0.5 mM EDTA) buffer. For supershift analysis, nuclear extracts were incubated with anti-CREB and anti-Sp1 antibodies and then incubated with oligonucleotide probes for 30 min at 37°C. The complex was then analysed by EMSA. Antibodies against pre-immune serum were included as negative controls. The DNA–protein complexes were resolved by electrophoresis on 5% non-denaturing polyacrylamide gels in 0.5× TBE. Gels were dried and autoradiographed at −80°C. All EMSAs were repeated three times, and consistent results were obtained.

ChIP (chromatin immunoprecipitation) analyses

ChIP assays were performed as described elsewhere [39,40]. Briefly, NHTBE cells were activated with RA for 4, 8, 24 or 48 h and then cross-linked by reaction with 1% formaldehyde for 10 min at 37°C. The cells were then washed with cold PBS. The cell pellet was resuspended in lysis buffer (1% SDS, 100 mM NaCl, 50 mM Tris/HCl, pH 8.1, and 5 mM EDTA) and then sonicated to an average DNA length of 500–1000 bp. Antibodies were added to each of the samples, which were then rotated overnight at a temperature of 4°C. After interaction with Protein A
Figure 2  RA-induced CREB regulation is independent of RAR and RXR

(A, B) NHTBE cells grown in RA-deficient medium for 7 days were pre-incubated with or without pan-RAR or pan-RXR antagonist (ant; 10 µM) for 2 h and then further incubated with RA (1 µM) for 48 h. Vehicle (DMSO) was used as a negative control. (A) QRT-PCR analysis of CREB mRNA expression normalized to the GAPDH endogenous control. Results represent the means ± S.E.M. for three independent experiments, each performed in triplicate. *P < 0.05, compared with RA-untreated control. (B) To show the effects of pan-RAR antagonist and pan-RXR antagonist, RARβ mRNA expression was measured by RT–PCR. After treatment, total RNA was extracted and then used to measure the RARβ mRNA level. RT–PCR products were analysed on a 2 % agarose gel using β-actin as a loading control. (C) Effects of siRNA knockdown of RAR/RXR on CREB regulation. NHTBE cells were transiently transfected with siRNAs of RAR and RXR together in combination using siRNA-RARα, -RARβ, -RARγ/RXRα or a non-specific control pool (NS siRNA). At 3 days after transfection, the cells were further incubated with or without RA for 24 h. After treatment, CREB mRNA expression was measured by QRT-PCR and the results were normalized to those for GAPDH endogenous control. To show the effects of RAR/RXR siRNA, RARβ expression was measured by Western blotting. Equal loading was confirmed by stripping the blot and reprobing it for β-actin antibody. Results are representative of those from three independent experiments. *P < 0.01, compared with the untreated control.

beads and incubation overnight at 65°C to reverse the cross-links, the DNA was dissolved in Tris/EDTA buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0) and then analysed by PCR. The anti-CREB, anti-phospo-CREB and anti-Sp1 antibodies (Upstate Biotechnology) or normal rabbit IgG was added separately into the reaction solutions. Primers used for PCR were obtained from CREB promoter sequences: 5′-AAGGTCTTCGGCAAGTTCC-3′ (sense) and 5′-TTCTCTCCACGTAACAC-3′ (antisense).

RNA interference analyses

For knockdown by RNA interference, we used SMARTpool siRNA (small interfering RNA) of CREB, RARα, RARβ, RARγ and RXRα (Upstate Biotechnology). Sp1 siRNA was purchased from Santa Cruz Biotechnology. Briefly, NHTBE cells at 60–70 % confluence were transfected with a final concentration of 100 nM SMARTpool siRNA or non-specific control pool (siRNA negative control) using the siIMPORTER siRNA transfection reagent (Upstate Biotechnology) according to the manufacturer’s instructions. Sp1 siRNA was also transfected at 100 nM. For luciferase reporter assay, −321−/−70 CREB-LUC was co-transfected with siRNA of CREB or Sp1. After 72 h of transfection, when the target protein levels had been reduced by 70–80 % as assessed by Western-blot analysis, the cells were treated with or without RA for another 24 or 48 h and then assessed by the luciferase reporter assay. Then, whole-cell lysate was prepared for Western blot, QRT-PCR, RT–PCR and luciferase assays.

Western-blot analyses

Western-blot analysis was performed as previously described [13]. Briefly, whole-cell extracts were prepared using 2 × SDS Laemmli lysis buffer. Equal amounts of total protein (20 µg) were resolved by SDS/10 % PAGE. We used mouse monoclonal antibodies against β-actin (clone AC-15; Sigma–Aldrich), rabbit polyclonal antibodies against Sp1 (Upstate Biotechnology) and total CREB and phospho-CREB (Upstate Biotechnology). Proteins reactive with primary antibody were visualized with a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents (Amersham Biosciences).

Statistical analyses

For all transfection assays and QRT-PCR analysis, results are shown as means ± S.E.M. for triplicate assays in three
RA-induced Sp1 increases CRE expression

**Figure 3 Functional deletion analysis of the human CREB promoter region**

The diagram represents the locations of the Sp1 and CRE motifs, and the numbers on the left-hand side represent the upstream start site of the CREB promoter region. NHTBE cells were transiently transfected with serially deleted CREB promoters containing luciferase reporter constructs and then treated with RA. After 48 h of transfection, cell extracts were assayed for luciferase activity, which was normalized to Renilla luciferase activity, as described in the Materials and methods section. The results are expressed relative to the basal activity of the CREB promoter (indicated as −2313/−70 CREB-LUC = 100%) and represent the means ± S.E.M. for three independent experiments, each performed in triplicate. The asterisk indicates the difference from the RA-stimulated −2313/−70 CREB-LUC construct (∗P < 0.01). The numbers on the left-hand side of the diagram represent the start position of the 5′ location on the CREB promoter region.

independent experiments. Statistical analysis was performed using a Student’s t test (Prism; GraphPad Software), and statistical significance is expressed as P < 0.05 or P < 0.01.

**RESULTS**

**RA up-regulates CREB expression in a time- and dose-dependent manner**

To determine whether RA regulates the CREB gene, we determined the level of CREB gene expression before and after RA treatment. CREB mRNA and protein expression was significantly increased by RA treatment for 1, 3 and 5 days compared with vehicle-treated control (Figures 1A and 1B). CREB protein and mRNA levels also increased in a dose-dependent manner after 24 h of RA treatment levels in this analysis (Figures 1C and 1D).

To further investigate the effects of RA on CREB gene regulation due to an increase in transcription and translation, we used ActD, a general inhibitor of mRNA transcriptional synthesis, and CHX, a general inhibitor of protein synthesis, to inhibit de novo synthesis of mRNA and protein; NHTBE cells were then treated with RA. Pretreatment with either ActD or CHX alone did not affect the endogenous level of CREB; however, RA-induced CREB mRNA expression was completely blocked (Figure 1E). Taken together, these findings demonstrate that RA increases CREB gene expression, and that this up-regulation occurs at the transcription level, where de novo synthesis of protein is required.

**RA-induced up-regulation of CREB is independent of RAR**

Next, we examined whether RA-induced CREB gene expression is mediated by the conventional RA receptors (RAR and RXR). In cultured NHTBE cells, pretreatment with 10 μM Ro 61-8431 (pan-RAR antagonist) or Ro 26-5405 (pan-RXR antagonist) alone did not affect the endogenous level of CREB. In addition, neither the pan-RAR antagonist nor the pan-RXR antagonist could block RA-induced CREB gene expression (Figure 2A). The activities of pan-RAR and RXR antagonists were confirmed by measuring the mRNA level of the RAR/RXR-mediated RA target gene RARβ (Figure 2B). RA-induced RARβ mRNA expression was almost completely blocked by either antagonist.

To further investigate the effects of RA on CREB gene expression, we used RAR/RXR siRNA to suppress the endogenous expression of RAR/RXR in NHTBE cells. Co-transfection of the NHTBE cells was done using SMARTpool-sequenced siRNA targeting the human RAR and RXR receptors with a combination of RARα, RARβ and RARγ/RXRα (Figure 2C). We silenced only the α isotype of RXR because it is known that RXRα plays a critical role in RAR/RXR heterodimers [41,42]. We observed maximal reduction of endogenous RAR/RXR protein 72 h after transfection. Successful suppression of RAR/RXR has been demonstrated previously, and residual RAR/RXR was not sufficient to transmit RA signalling [13,14]. Likewise, the RA-induced expressions of CREB mRNA and protein were not significantly reduced after co-transfection with a combination of RAR and RXR siRNAs (Figures 2C and 2D), strongly indicating that RAR and RXR receptors are not involved in RA-induced CREB regulation.

**Sp1-binding sites are required for CREB promoter transactivation**

To determine the key transcription factor involved in RA-induced CREB regulation, we first generated a series of 5′ deletion constructs of the human CREB promoter by subcloning appropriate restriction fragments upstream of the luciferase gene in the pGL3-Basic luciferase reporter vector. Then, we transiently transfected them into NHTBE cells to determine their RA-induced
Figure 4 Analysis of mutated Sp1 and CRE motifs in the CREB promoter

(A) The schematic diagram represents the locations and sequences of the wild-type (wt) and mutated (mt) Sp1 and CRE motifs. (B) NHTBE cells were transiently transfected with various mutants of Sp1 and CRE in the CREB promoter-luciferase reporter constructs. The diagrams show the relative locations of the Sp1 and CRE motifs in the −321 to −70 CREB-LUC construct. An X in a motif designates a mutation. After transfection, the cells were further incubated with 1 µM RA or vehicle control for 48 h. The results are expressed relative to the fold increase in luciferase activity over untreated control and represent the means ± S.E.M. for three independent experiments, each performed in triplicate. The asterisk indicates the difference from the RA-stimulated wild-type −321/−70 CREB-LUC construct (*P < 0.05, **P < 0.01).

Luciferase activities. In the current analysis (Figure 3), we found that RA caused a 7.84-fold increase in the luciferase activity of the reporter construct containing the CREB promoter (nt −2313 to −70), relative to untreated control. In addition, RA increased luciferase activity even further, to 8.07-fold over the control, in tests using a deletion mutant of the CREB promoter that contained nt −260 to −70. However, the deletion mutants that contained nucleotides from the −196 to −70 and −134 to −70 regions of the CREB promoter significantly decreased RA-induced luciferase activity compared with the 2.2 kb CREB promoter (nt −2313 to −70); the −196/−70 and −134/−70 CREB-LUC mutants yielded increases of only 2.07- and 1.35-fold over the control, whereas that yielded by the −2313/−70 CREB-LUC mutant was 7.84-fold over the control (P < 0.01; Figure 3). These results show that the region between nt −260 and −134 upstream of the CREB coding sequence, which includes two Sp1 motifs, is critical for transactivation of the CREB promoter by RA.

By analysing the CREB promoter sequences in the TFSEARCH database (http://mbs.cbrc.jp/research/db/TFSEARCH.html) at a threshold score of 74.0, we identified two putative Sp1-binding sites (Sp1 motifs) and two putative CREB-binding sites (CRE motifs) in the −260 to −70 CREB promoter region, as shown in Figure 4(A). To further determine the role of Sp1 and CRE motifs in CREB expression, we next generated point-mutated Sp1 and CRE motifs in the CREB promoter luciferase reporter vector and tested their transactivation by RA (Figure 4A). We found that Sp1 mutants, both as single mutants (Sp1-1 at −150 or Sp1-2 at −217) and as a double mutant (Sp1-1 + Sp1-2), significantly reduced promoter activity when compared with the RA-stimulated CREB promoter (nt −321 to −70)/luciferase reporter construct. Using this as a template to make a construct with site-specific mutations, Sp1-1 alone increased activity 2.72-fold over the control, Sp1-2 alone increased it 2.52-fold, and Sp1-1 + Sp1-2 increased it 1.67-fold, whereas the RA-stimulated CREB promoter (nt −321 to −70)/luciferase reporter construct increased it 8.04-fold. Whereas neither CRE motif mutant alone (CRE1 mt nor CRE2 mt) affected promoter activity, the double CRE mutant (CRE1 +2 mt) slightly decreased the promoter activity compared with the RA-stimulated CREB promoter (nt −321 to −70)/luciferase reporter construct. CRE1 alone increased promoter activity 7.14-fold over the control, CRE2
RA-induced Sp1 increases CREB expression

Figure 5  Alignment of the nucleotide sequences around the transcription initiation sites of CREB genes from four different species

The respective transcription initiation sites are indicated by the boldface dot (●). Gaps in the sequence are indicated by dashes, and nucleotide numbers are shown for the human sequence. The positions of sequence identity are also indicated (*). The previously known sequence elements, including Sp1 and CRE sequences, are boxed.

alone increased it 6.82-fold, and CRE1 + 2 increased it 5.92-fold. These results clearly demonstrate that the Sp1 motifs, which surround the transcription initiation start site, are required for RA-induced CREB promoter transactivation.

Phylogenetic analysis of CREB promoter shows conservation of CRE and Sp1 motifs in several species

To determine the evolutionarily conserved transcription factor that may play a critical role in the regulation of the CREB gene, we analysed the human CREB gene promoter around the transcription start site and compared it with mouse, Xenopus and zebrafish CREB gene promoters (Figure 5). Genomic DNA sequences of the CREB promoter region from four different species were obtained (Ensembl genome browser, http://www.ensembl.org/) and aligned using the NCBI BLAST two-sequence alignment program (bl2seq). The human CREB promoter is enriched in the nucleotides guanosine and cytosine (65 % G + C) and contains no CAAT- or TATA-box motifs [43]; however, it does contain the 5'-TCTCTCAGTCGG-3' sequence, which resembles the initiator sequence 5'-PyCCTAPyTCTG-3' (where Py is any pyrimidine) that has been shown in TATA-less promoters to initiate gene transcription at the conserved A nucleotide [44]. The numbering was based on the translation start site, defined as +1. Because of the lack of a TATA-box, the CREB promoter is numbered relative to the translation start site, placing the major transcription initiation start site at position −181. As Figure 5 shows, two Sp1 motifs and CRE motifs are present within the 191-bp promoter fragment; they are located between nt −260 and −70. Sp1 sites surround the major transcription initiation site (at positions −217 and −150), and the two CREs are located −119 and −98 bp downstream of the transcription start site. The two CRE motifs are highly conserved among the four species, but Sp1 motifs are conserved only in human and mouse.

Transcription factors Sp1 and CREB bind specifically to the CREB promoter

To determine whether Sp1 and CREB specifically bind to their binding motifs (boxes in Figure 5) in the CREB promoter, we performed competition EMSA and ChIP assays with anti-CREB, anti-phospho-CREB and anti-Sp1 antibodies. EMSA results showed that the radiolabelled oligonucleotides of Sp1-1, Sp1-2, CRE1 and CRE2 formed DNA–protein complexes. In addition, pretreatment with unlabelled oligonucleotides abolished nuclear protein binding to radiolabelled oligonucleotides. To further confirm Sp1 and CREB binding to putative Sp1 and CRE motifs after RA treatment, supershift assays were performed in the presence of anti-Sp1 or anti-CREB antibodies. After incubation of either of these antibodies, attenuation and band shift were seen in the DNA–protein complexes (Figures 6A and 6B, lanes with Ab). However, normal rabbit IgG did not induce attenuation or band...
The DNA-binding reaction was performed in the absence or presence of a 100-fold excess of each oligonucleotide unlabelled (i.e. an unlabelled competitor). Supershift assays were performed using 2 µg of CREB promoter that corresponded to the position −321/−70 region; 1% of the chromatin was assayed to verify equal loading (Input), and non-specific IgG was assayed under otherwise identical conditions as a negative control. Results are representative of those from three independent experiments.

Figure 6 The effect of RA on CREB promoter binding of Sp1 and CREB

(A, B) EMSA was performed using NHTBE cells that were cultured for 7 days with or without RA and then treated with RA for 24 h. Nuclear extract (10 µg) was prepared, and DNA–protein binding activity was measured by EMSA using various [γ-32P]ATP-labelled oligonucleotides corresponding to Sp1-1, Sp1-2, CRE1 and CRE2, as described in the Materials and methods section. The DNA-binding reaction was performed in the absence or presence of a 100-fold excess of each oligonucleotide unlabelled (i.e. an unlabelled competitor). Supershift assays were performed using 2 µg of the anti-CREB and anti-Sp1 antibodies, as indicated above each lane in (A) and (B). The labelled nucleotide and nuclear protein complexes were separated by electrophoresis on 5% polyacrylamide gels, and the gels were dried and autoradiographed at −80°C. The asterisks (*) indicate shifted bands. (C) ChIP analysis was performed using NHTBE cells grown in RA-deficient medium for 7 days. The cells were then incubated with or without 1 µM RA for the indicated times. ChIP assays were performed using anti-phospho-CREB (pCREB), anti-CREB or anti-Sp1 antibodies to precipitate CREB promoter that corresponded to the position −321/−70 region; 1% of the chromatin was assayed to verify equal loading (Input), and non-specific IgG was assayed under otherwise identical conditions as a negative control. Results are representative of those from three independent experiments.

RA induces Sp1 expression

Because ChIP analysis (Figure 6C) showed that RA increases Sp1 binding to the CREB promoter, we examined the possibility that RA is also involved in the regulation of endogenous Sp1 in NHTBE cells. As shown in Figure 7A, Sp1 protein expression levels remained unchanged until after 4 h of RA treatment and then gradually increased thereafter, with a significant increase after 24 h of treatment. In addition, QRT-PCR revealed that Sp1 mRNA was up-regulated 3.91-fold within 1 day of RA treatment and remained elevated thereafter (Figure 7C). These results indicate that RA increased Sp1 and phosphorylated CREB binding to the CREB promoter in vivo.

Both Sp1 and CREB are required for RA-induced CREB gene expression

To further investigate the roles of Sp1 and CREB in CREB gene regulation and transactivation, we used an siRNA approach to suppress the endogenous expression of Sp1 and CREB in NHTBE cells and determined the level of RA-inducible Sp1 and CREB protein expressions. We found that RA-induced CREB protein expression was reduced after transfection with Sp1 siRNA, whereas CREB siRNA did not affect RA-induced Sp1 expression (Figure 8A). Furthermore, RT–PCR analysis demonstrated that the decrease in endogenous CREB mRNA was dependent on Sp1 silencing, whereas CREB siRNA did not affect RA-induced Sp1 mRNA expression (Figure 8B). This result shows that Sp1 is important for CREB gene expression.

Next, to further determine the role of Sp1 and CREB in RA-induced transactivation of CREB promoter activity, we co-transfected pGL3-Basic luciferase reporter vector containing CREB promoter (nt −321 to −70) region and Sp1 siRNA or CREB siRNA. Using a luciferase activity assay, we found that knockdown of either endogenous Sp1 or endogenous CREB significantly decreased RA-inducible CREB gene transactivation (Figure 8C). Sp1 knockdown decreased the CREB promoter activity significantly more than CREB knockdown did (82.9% versus 69.1%), compared with RA-treated non-transfected control. These results strongly support that both Sp1 and CREB are required for RA-induced transactivation of CREB promoter activity.

Moreover, these results suggest that Sp1 has a more important role than CREB in RA-inducible CREB gene regulation and that
Sp1 and CREB may interact and/or that these protein interactions dependently or independently support binding to the imperfect CRE or Sp1 motifs to facilitate transcription from the CREB promoter.

**DISCUSSION**

Earlier studies have shown that RA deficiency induced metaplastic squamous differentiation in airway mucosa and normal mucociliary phenotype can be restored by RA treatment [5,9]. Our recent study [13,14] showed that one of the earliest events that occurred during this restoration of normal mucous phenotype from squamous metaplasia was CREB activation by RA. In the present study, we found that RA not only activated CREB but also induced expression of the CREB gene in bronchial epithelial cell and that Sp1 played an important role in the up-regulation of the CREB gene expression during mucous differentiation of normal human bronchial epithelial cells. By conducting a detailed analysis of the promoter of CREB in order to gain an understanding of the molecular mechanisms associated with the up-regulation of CREB transcription in response to RA, we found that Sp1 and CREB motifs in the essential promoter region of the CREB were required for full transcriptional activity in the RA-induced transcription of the human CREB gene. These RA-inducible CREB gene up-regulation mechanisms appeared to be mainly controlled by the binding of two Sp1-binding motifs in
overexpression of Sp1 significantly induces CREB promoter activity in Drosophila SL2 cells, suggesting that Sp1 is a potent activator of CREB gene expression [45]. Mutation of the downstream Sp1 motif (Sp1-1 mt) in the CREB promoter resulted in a 50% decrease in basal activity in Sertoli cells. However, mutation of the Sp1 site, which is located upstream of the transcription initiation site (Sp1-2 mt), did not affect CREB promoter activity [37]. However, Coven et al. [46] reported that only one of the Sp1 sites (Sp1-2) was required for the normal up-regulation of CREB promoter activity in C6 cells. Thus both Sp1- and CRE-binding motifs might have important roles in CREB gene regulation in many organisms. As shown in Figure 3, the transcription start sites are located in different regions of human and mouse CREB promoters. The Sp1-1 and Sp1-2 motifs alone and combined (Sp1-1 mt + Sp1-2 mt) significantly reduced RA-inducible CREB promoter activity compared with wild-type CREB promoter (Figure 4). Our results strongly suggest that two CRE motifs bind CREB, which contributes to basal promoter activity, and that two Sp1-binding motifs bind Sp1, which is essential for RA up-regulation of CREB gene transcription.

In contrast with our findings, Walker et al. [37] reported that the presence of three CRE-like sequences in the first 1240 bp upstream of the translation start site in the CREB promoter region suggested regulation of the cAMP-inducible CREB gene expression pathway. They showed that cAMP-inducible CREB gene expression required the two CREs located downstream of the major transcription start site in the CREB promoter gene. They also showed that only Sp1-1-binding sites contributed to basal promoter activity. In neuronal cells, up- or down-regulation of CREB expression by activation of the cAMP pathway has been reported both in vivo and in vitro [32,34,35]. Activation of the cAMP pathway resulted in down-regulation of CREB mRNA and protein in CATHa cells [47], whereas the same treatments did not alter CREB expression in PC12 cells (pheochromocytoma cells) [34]. These findings imply that regulation of CREB expression by the cAMP pathway depends on specific cell type.

The results of our EMSA and ChIP analyses further support the belief that Sp1 binds specifically to the Sp1-binding motif and that CREB binds specifically to CRE motifs, in vitro and in vivo (Figure 6). These results also suggest that the Sp1-1, Sp1-2-, CRE1- and CRE2-binding motifs in this promoter region can function in vivo. Our finding that Sp1 and CREB binding on the CREB promoter region was increased after 24 h of RA treatment suggests that both the RA-induced up-regulated Sp1 and phosphorylated CREB may have a role in up-regulating the CREB gene expression during the early stages of epithelial cell differentiation (Figure 6C). It has been reported that the promoter region of human and mouse CREB has three variant CREs spread over nearly 800 bp [31,48]. These CREs are highly conserved in organisms ranging from zebrafish to humans; however, Sp1-binding motifs are conserved only in mice and humans (Figure 5). Thus both Sp1- and CRE-binding motifs might have important roles in CREB gene regulation in many organisms. As shown in Figure 5, the transcription start sites are located in different regions of the promoter region in humans and mice, implying that different transcription regulation mechanisms exist in these two species.

It has also been reported that NF-κB (nuclear factor κB) and TNFα (tumour necrosis factor-α) are regulators of CREB expression. Delfino and Walker [49] showed that overexpressed NF-κB interacts with the NF-κB-binding site in the CREB promoter and increases promoter activity in Sertoli and NIH 3T3 cells. These
RA-induced Sp1 increases CREB expression

Figure 9  A proposed model explaining up-regulation of CREB expression by RA in NHTBE cells

In this model, RA rapidly activates protein kinase C (PKC) and transmits an activation signal to phosphorylate nuclear CREB (CREB–P) via the MAPKinase (Ras/ERK (extracellular-signal-regulated kinase)/RSK (p90 ribosomal S6 kinase)) pathway, thereby increasing its transactivation activity. A basal CREB regulation of the positive feedback loop is constituted by induction of CREB, which is consequently activated by RA binding of CRE motifs. In addition, Sp1 protein, which is increased by RA, binds to Sp1-binding sites in the CREB promoter region to further activate transcription of CREB genes.

results suggest that NF-κB may be an important regulator of the genes required for spermatogenesis and is a general regulator of CREB gene expression in non-testis cells. However, our reporter assay indicates that RA-induced CREB activity was retained by a luciferase vector containing CREB promoter region from nt −447 to −70, from which the putative NF-κB-binding sites had been deleted (Figure 3). We therefore postulate that NF-κB is not required for RA-inducible CREB expression in NHTBE cells.

RA reportedly has a profound effect on the regulation of cell growth and differentiation, primarily through two families of NRs: RARs and RXRs. Several studies have shown that RARs and RXRs, upon binding to ligands, promote transcription through interaction with co-activators [50,51]. However, our results suggest that RA up-regulates CREB independently of RAR and RXR because NHTBE cells depleted of RARs and RXRα by means of siRNAs targeting RARα, RARβ, RARγ and RXRα could still mediate RA-induced transactivation of CREB promoter activity. In addition, in our study, selective pan-RAR and pan-RXR antagonists did not block RA activation of CREB (Figure 2). We previously reported that RA rapidly induced CREB activation via a non-classical RA-signalling mechanism without using its canonical RARs and RXR [13]. All these results strongly suggest that the activation and regulation of the CREB gene by RA are both RAR- and RXR-independent.

We also showed that RA up-regulated Sp1 gene expression in a time- and dose-dependent manner (Figures 7A–7E). Interestingly, our results suggest that RA-inducible Sp1 gene up-regulation is independent of RAR and RXR function: depletion of RARs/RXRα using siRNA did not affect the RA-induced transactivation of Sp1 promoter activity. However, further extensive studies are required to determine how RA up-regulates Sp1 expression without using its conventional receptors, RAR/RXR. Notably, our results showed that endogenous depletion of Sp1 significantly reduced the RA-inducible CREB gene but that CREB depletion did not affect Sp1 gene up-regulation (Figure 8). In addition, Sp1 depletion completely abolished RA-inducible CREB promoter activity (to a level only 1.2-fold higher than that of untreated controls), and CREB depletion significantly abolished the CREB promoter activity. Our results strongly suggest that Sp1 has a more critical role than CREB in RA-inducible CREB gene regulation and that these two proteins facilitate the binding of imperfect CRE and Sp1 motifs to each other. It is plausible that a basal level of CREB is positively autoregulated by RA-activated CREB. In addition, Sp1 protein, whose levels are increased by RA, binds to two Sp1-binding sites in the CREB promoter region to further facilitate transactivation of CREB genes in the early stage of NHTBE cell differentiation.

In summary, our findings further demonstrate that RA up-regulates CREB gene expression during the early stage of NHTBE cell differentiation and that RA-inducible Sp1 plays a major role in up-regulating human CREB gene expression. This finding and our earlier findings suggest that several transcription factors, including CREB, Sp1 and RAR/RXR, may co-ordinately regulate the differentiation programme of bronchial epithelial cells. CREB and Sp1 may play an important role in preparing the environment for mucous cell differentiation when the level of RAR/RXR is not sufficient in the cells, such as in the squamous metaplasia state.

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REFERENCES

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