0026-895X/97/061019-08$3.00/0

MOLECULAR PHARMACOLOGY, **52:**1019–1026 (1997).

Both the Cyclic AMP Response Element and the Activator Protein 2 Binding Site Mediate Basal and Cyclic AMP-Induced Transcription from the Dominant Promoter of the Rat α_{1B}-Adrenergic Receptor Gene in DDT$_1$MF-2 Cells

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Received April 21, 1997; Accepted September 4, 1997

SUMMARY

cAMP markedly increases α_{1B} adrenergic receptor (α_{1B}-AR) expression in FRTL-5 and PC C13 rat thyroid cells, DDT$_1$MF-2 smooth muscle cells, primary rat hepatocytes, and K9 rat liver cells. Here, we used DDT$_1$MF-2 cells to evaluate further the mechanisms by which cAMP stimulates α_{1B}-AR expression. Receptor binding assays, Northern blotting, and nuclear run-on analyses demonstrated that forskolin (1 μM) in the presence of isobutylmethylxanthine (0.25 mM) increased α_{1B}-AR numbers, mRNA level, and gene transcription rate by 2.3 ± 0.2-, 2.5 ± 0.3-, and 3.5 ± 0.2-fold over control, respectively. Dibutyryl cAMP (1 mM) plus isobutylmethylxanthine (0.25 mM) also enhanced α_{1B}-AR density by 2.7 ± 0.1-fold over control. Further experiments demonstrated that the induction of α_{1B}-AR by forskolin requires new protein synthesis and is protein kinase A dependent. In DDT$_1$MF-2 cells transfected with α_{1B}-AR gene P2 promoter/CAT constructs, both forskolin and dibutyryl cAMP significantly increased P2 promoter activity. The P2 promoter region of the rat α_{1B}-AR gene (−813 to −432) contains a cAMP response element (CRE) (−444 to −437) and an AP2 binding site (−647 to −638). Mutations in either one of these elements alone led to a decrease in both basal and cAMP-induced P2 promoter activity. Mutations in both elements caused a further inhibition of basal transcription and a complete block of cAMP-induced P2 promoter activity. Direct binding of purified activator protein 2 (AP2) to the AP2 element in the P2 promoter was reported previously. Gel mobility shift and supershift assays using liver nuclear extracts from either rat liver or DDT$_1$MF-2 cells demonstrated that the CRE in the α_{1B}-AR gene bound CRE binding protein. These data indicate that both the CRE and the AP2 element in the P2 promoter contribute to basal as well as cAMP-induced transcription of the α_{1B}-AR gene in DDT$_1$MF-2 cells.

The α_{1B}-AR is a G-protein-coupled receptor that plays a key role in a variety of physiological processes, such as cardiac and smooth muscle contractility, contraction of the spleen, liver glycogenolysis, melatonin secretion in the pineal gland, and cell proliferation (1–3). The expression of the α_{1B}-AR gene is regulated by hormonal and developmental factors in a complex tissue-specific manner (4). To understand the molecular mechanism for such complex regulation, we cloned and characterized the rat α_{1B}-AR gene (5–8). The gene is composed of two exons and a single large intron of ≥16 kb. Primer extension and reverse transcriptase-PCR studies using poly(A)$^+$ RNA prepared from rat liver identified three *tsp*, located between −54 and −57 bp (*tsp1*) and at −443 bp (*tsp2*) and a cluster between −1035 and −1340 bp (*tsp3*) upstream from the translation start codon. Northern blot analyses of α_{1B}-AR mRNA have documented three mRNA species that are 3.3, 2.7, and 2.3 kb. The 3.3-kb species is preferentially expressed in rat liver (9, 10), whereas the 2.7-kb species is dominant and widely expressed in many tissues and cells, including rat liver (9, 10). The low-abundance 2.3-kb species is difficult to detect and has been reported only in rat liver (6) and rat medullary thyroid carcinoma 623 cells (11). A similar pattern of three α_{1B}-AR mRNA species was detected in DDT$_1$MF-2 cells (12). The 3.3-, 2.7-, and 2.3-kb mRNAs of the α_{1B}-AR gene in rat liver are likely transcribed from *tsp3*, *tsp2*, and *tsp1*, directed by three distinct promoters: the distal promoter (P3, −1363 to −1107), middle promoter (P2, −813 to −432), and proximal promoter (P1, −127 to −49), respectively (6). However, Ka-

This work was supported in part by Grant IN-105U from the American Cancer Society and Grant J-379 from the Thomas F. Jeffress and Kate Miller Jeffress Memorial Trust.

ABBREVIATIONS: AR, adrenergic receptor; CRE, cAMP-response element; AP2, activator protein 2; *tsp*, transcription start point(s); PCR, polymerase chain reaction; DMSA, DNA mobility shift assay; CAT, chloramphenicol acetyltransferase; CREB, cAMP-response element binding protein; ATF-1, activating transcription factor 1; IBMX, isobutylmethylxanthine; Bt2cAMP, dibutyryl cAMP; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; bp, base pair(s); kb, kilobase pair(s).

nasaki *et al.* (13) identified only a single *tsp* located −173 bp upstream from the translation start codon in the FRTL-5 rat thyroid cell line. This *tsp* was directed by a promoter fragment between −451 and + 95, which encompasses the P1 promoter that we identified in rat liver. Our subsequent experiments have established that the dominant P2 promoter interacts with multiple transcription factors, including NF1, CP1, AP2, and Sp1 (8). The results indicated that the transcription of the α_{1B}-AR gene is controlled by combinatorial mechanisms via three promoters, including a tissue-specific promoter, and multiple transcription factors, which may account for the complex changes in α_{1B}-AR expression and function in a variety of physiological and pathological conditions.

Signals generating increased cellular levels of cAMP markedly increase α_{1B}-AR expression in FRTL-5 (13) and PC C13 rat thyroid cells (14), DDT_1MF-2 smooth muscle cells (15), primary rat hepatocytes,[1] and K9 rat liver clonal cells.[1] This mechanism may be physiologically important in tissues such as the thyroid and pineal glands. In thyroid cells, iodide efflux and the iodination of thyroglobulin are concerted actions of TSH and norepinephrine acting on α_{1B}-AR (16), and regulation of α_{1B}-AR gene expression by TSH via a cAMP-dependent mechanism (13) may serve to amplify this function. In the pineal, the circadian rhythm of melatonin secretion is controlled by norepinephrine via a complex mechanism involving both β_1-AR and α_{1B}-AR, and a similar diurnal rhythm in α_{1B}-AR gene transcription is mediated by cAMP generated via β_1-AR (17). The molecular mechanisms involved in the cAMP-stimulated gene transcription are complex and still not fully understood. Two classes of *cis*-acting elements, the CRE and the AP2 element, have been identified in cAMP-regulated genes (18). Sequence analyses revealed that the P2 promoter region of the rat α_{1B}-AR gene contains both CRE and AP2 elements (5–7), with the latter being able to bind purified AP2 protein (7). Recently, Kanasaki *et al.* (13) reported that this CRE mediated cAMP-induced transcription but was not involved in basal transcription from the P1 promoter of the rat α_{1B}-AR gene in FRTL-5 cells. Here, we report that forskolin stimulates the transcription of the α_{1B}-AR gene and the expression of α_{1B}-AR in DDT_1MF-2 cells. The results of mutational analyses indicate that both the CRE and AP2 element contribute to basal as well as cAMP-induced P2 promoter activity. In addition, we demonstrate by using DNA mobility competition and supershift assays that the CRE in the α_{1B}-AR gene P2 promoter binds CREB.

Experimental Procedures

Materials. The DDT_1MF-2 hamster smooth muscle cell line was obtained from American Type Culture Collection (Rockville, MD), and cultured under conditions specified by the supplier. Forskolin, IBMX, and Bt2cAMP were purchased from Sigma Chemical (St. Louis, MO).

α_1-AR binding assay. α_{1B}-ARs were identified by binding assays using [^{3}H]prazosin as the radioligand. Briefly, DDT_1MF-2 cells were suspended in 50 mM Tris·HCl buffer containing 10 mM $MgSO_4$ at pH 7.5, and subjected to one cycle of freeze-thawing to reduce nonspecific binding. The whole cells (10^6 cells) were then incubated with a saturating concentration of [^{3}H]prazosin (2 nM) in the absence (total binding) or presence of 10 μM phentolamine (nonspecific binding) for 50 min at 30°. Incubations were terminated by rapid vacuum filtration over Whatman GF/B filters presoaked in assay buffer, and the radioactivity retained by the filters was measured by liquid scintillation counting.

Northern blotting analyses. Northern blotting analyses were done as described previously (6). Briefly, total cellular RNA was extracted and purified according to the guanidinium isothiocyanate method, using the RNAzol B kit (Cinna/Biotech Laboratory, Friendswood, TX). The RNA was size-fractionated by electrophoresis on a 1.0% agarose gel containing 6.5% formaldehyde and transferred onto a nylon membrane. RNA sizes were estimated by comparison with the migration of a 0.24–9.5-kb RNA ladder (GIBCO, Grand Island, NY). Blots were prehybridized for 2 hr in a buffer containing 50% formamide, 5× Denhardt's solution, 100 μg/ml sheared salmon sperm DNA, and 0.5% SDS. Hybridization was carried out at 42° overnight in the above solution using ^{32}P-labeled α_{1B}-AR or β-actin probes. Blots were washed in 1× SSC/1% SDS for 20 min at room temperature and twice in 0.2× SSC/0.1% SDS for 20 min at 65° and were analyzed using a PhosphorImager ImageQuant program (Molecular Dynamics, Sunnyvale, CA) (1× SSC = 15 mM sodium citrate, 0.15 M NaCl, pH 7.0).

Nuclear run-on transcription assay. Nuclear run-on assays were carried out as described previously (19). Briefly, nuclei (1×10^7) in 100 μl of nuclear storage buffer were mixed with 100 μl of reaction buffer (10 mM Tris·HCl, pH 8.0; 5 mM $MgCl_2$; 300 mM KCl; 0.5 mM concentration each of ATP, CTP, and GTP; 100 units of RNasin; 200 μCi of [^{32}P]UTP), and incubated at 30° for 30 min with gentle shaking. The nuclei were digested with 10 μl of RNase-free DNase (10 mg/ml; Promega, Madison, WI) and 10 μl of $CaCl_2$ (20 mM), and incubated at 37° for 30 min. They were treated further with 2 μl of proteinase K (10 mg/ml) and 25 μl of 10× SET buffer (5% SDS, 100 mM Tris, pH 8.0, 50 mM EDTA) at 30° for 30 min. Nuclear ^{32}P-labeled RNA was extracted by the addition of 550 μl of RNAzol B and 500 μl of phenol/chloroform (1:1), precipitated with ethanol, and dissolved in Northern hybridization buffer. For binding onto the nitrocellulose membrane, 1–10 μg of linearized plasmid DNA was denatured by boiling in 0.1 M NaOH for 5 min, neutralized with 10 μl of 20× SSC, and then spotted onto the membrane using a slot-blot apparatus, washed with 6× SSC, and UV cross-linked. The membranes containing α_{1B}-AR or β actin cDNA were prehybridized for 6 hr, hybridized for 72 hr at 42°, and washed the same way as for Northern analysis.

Oligonucleotide synthesis. The synthetic oligonucleotides were prepared on a Cyclone Plus DNA synthesizer (Milligen, Marlborough, MA). After ammonium hydroxide deprotection, oligonucleotides were evaporated to dryness by vacuum centrifugation (SpeedVac; Savant, Marietta, OH) and purified by electrophoresis on a 10% polyacrylamide-8 M urea gel.

Construction of plasmids. The P2/CAT, $P2_{CREm}$/CAT, $P2_{AP2m}$/CAT, and $P2_{CREm+AP2m}$/CAT constructs were prepared by subcloning P2, $P2_{CREm}$, $P2_{AP2m}$, and $P2_{CREm+AP2m}$ promoter regions into pCAT enhancer reporter vectors, respectively. The P2 promoter region was amplified by PCR using the rat α_{1B}-AR gene 5′ flanking region as template, antisense primer 1 (5′-CTGCTGCAGGGTGACATCAGG-3′) containing a *Pst*I site as 3′ primer, and sense primer 2 (5′-GATGTGACTCAAGCTTCTGCCACTG-3′), containing a *Hin*dIII site, as 5′′ primers. The $P2_{CREm}$ region was amplified by PCR using the rat α_{1B}-AR gene 5′ flanking region as a template and primer 1m (5′-CTGCTGCAGGGTGCTATCAGG-3′) and primer 2 as 3′ and 5′ primers, respectively. The $P2_{AP2m}$ region was amplified by sequential PCR (8). Briefly, partially overlapping sense primer (−650 to −626) (5′-GGGCTAAATTGGAGTATGAACCGG-3′) and antisense primer (−633 to −661) (5′-ATACTCCAATTTAGCCCCGCTGGATTAT-3′) containing the point mutations (underlined) were synthesized and used in the sequential PCR amplification steps. The primer pairs used in the sequential steps were sense primer plus primer 1 and antisense primer plus primer 2. The template used in the sequential PCR was the rat α_{1B}-AR gene 5′

[1] B. Gao, J. Chen, C. Johnson, and G. Kunos, unpublished observations.

flanking region. PCR was carried out as described previously (6). The two PCR products were combined and amplified by primers 1 and 2. The final PCR product was purified and subcloned into the pCAT enhancer vector. The mutations in the AP2 site were verified by sequencing. The $P2_{CREmplusAP2m}$ region was amplified by PCR using $P2_{AP2m}$ as a template, primer 1m and primer 2 as 3′ and 5′ primers, respectively.

Transient transfections and CAT assays. Transient transfections and CAT assays were performed as described previously (6).

DMSA and DNA mobility supershift assay. DMSA was carried out and nuclear extracts for DMSA were prepared as described previously (6). Briefly, 1 ng of ^{32}P-labeled probe was incubated with 10 μg of nuclear extract in 20 mM Tris·HCl, pH 7.9, 1.5% glycerol, 50 μg/ml of bovine serum albumin, 1 mM dithiothreitol, 0.5 mM PMSF, and 2 μg of poly(dI/dC) in a volume of 20 μl. In competition experiments, 1 ng of radioactive probe and 100 ng of competitor oligonucleotides were mixed before the addition of nuclear extract. Reactions were incubated at 25° for 20 min and subsequently analyzed by electrophoresis through nondenaturing 10% polyacrylamide gels in 0.5× TBE buffer containing 44.5 mM Tris·HCl, pH 8.2, 44.5 mM boric acid, and 1 mM EDTA. After prerunning of the gels at 100 V for 2 hr, electrophoresis was performed at 270 V for 2 hr at 4°. The gels were analyzed using a PhosphorImager and ImageQuant software (Molecular Dynamics). The following double-stranded oligonucleotides (sense strands only are shown) were used in gel shift assays: oligonucleotide I, 5′-CCGCCTGATGTCACCGCCG-3′ (−431 to −449 in the 5′ flanking region of the rat α_{1B}-AR gene); CREm, 5′-CCGCCTGATAGCACCGCCG-3′ (mutated oligonucleotide I, mutated nucleotides are underlined); AP1, 5′-CGCTTGATGAGTCAGCCGGAA-3′; AP2, 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′; CRE, 5′-AGAGATTGCCTGACGTCAGAG AGCTAG-3′; and Sp1, 5′-ATTCGATCGGGGCGG GGCGAGC-3′. The antibodies against ATF-1, CREB, and NF1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analysis. For comparing values obtained in three or more groups (see Figs. 1–4), one-factor analysis of variance was used, followed by Tukey's *post hoc* test, and $p < 0.05$ was taken to imply statistical significance.

Results

Effects of forskolin on α_{1B}-AR density, mRNA levels, and gene transcription rate in DDT_1MF-2 cells. The effects of elevated intracellular cAMP concentrations on α_{1B}-AR density, mRNA level, and gene transcription rate in DDT_1MF-2 cells were studied using forskolin in the presence of the phosphodiesterase inhibitor IBMX. As shown in Fig. 1, α_{1B}-AR density in DDT_1MF-2 cells was significantly increased 30 min after the addition of forskolin and IBMX, reached a maximum at 6 hr (2.3 ± 0.2-fold of control), and remained at this level for ≥48 hr. To confirm that the effects of forskolin on α_{1B}-AR gene expression are cAMP mediated, DDT_1MF-2 cells were incubated with the cAMP-dependent protein kinase A inhibitor U89 (20) and then stimulated with forskolin for 2 or 6 hr. As shown in Fig. 1C, pretreatment with U89 blocked the forskolin-stimulated α_{1B}-AR gene expression. This strongly suggests that activation of α_{1B}-AR gene expression by forskolin is cAMP mediated. Fig. 1B shows that the cAMP analog Bt2cAMP in the presence of IBMX also increased the α_{1B}-AR numbers significantly in DDT_1MF-2 cells.

To determine whether the forskolin-induced increase in α_{1B}-AR gene expression required new protein synthesis or was due to some change in receptor distribution or recovery, DDT_1MF-2 cells were incubated with puromycin, a protein

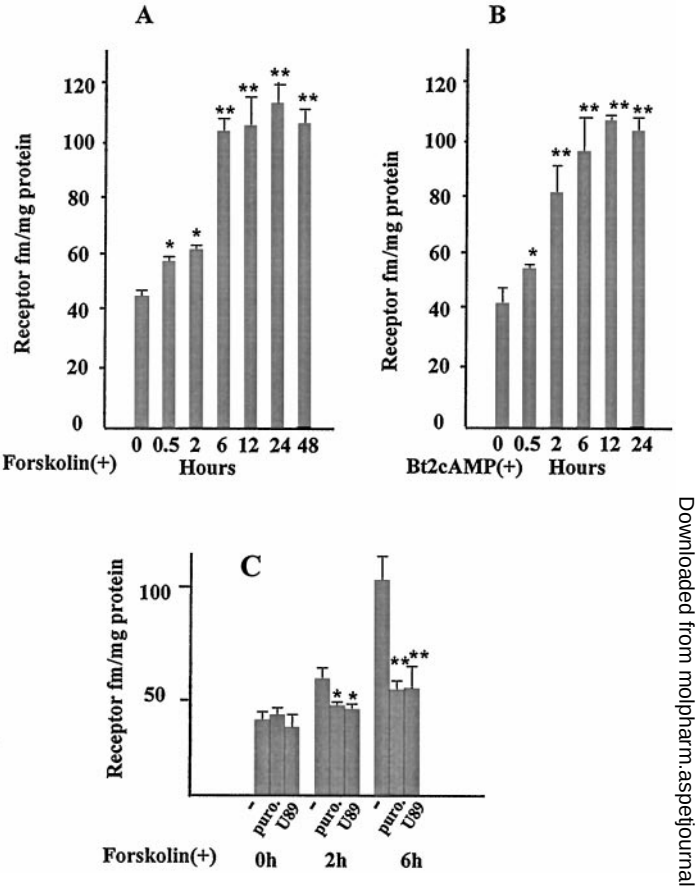

Fig. 1. Effects of cAMP on α_{1B}-AR densities in DDT_1MF-2 cells. A and B, DDT_1MF-2 cells were incubated with forskolin (1 μM) plus IBMX (0.25 mM) (A) or Bt2cAMP (1 mM) plus IBMX (0.25 mM) (B) for the indicated times. Cells were used for α_{1B}-AR binding assay as described in Experimental Procedures. The receptor density values were derived from Scatchard plots. Values are mean ± standard error for four experiments. *, $p < 0.05$; **, $p < 0.01$, significant difference from corresponding 0-hr values. C, DDT_1MF-2 cells were pretreated with puromycin (*Puro*. 20 μg/ml) or U89 (0.1 μM) for 60 min and then stimulated with forskolin (1 μM) plus IBMX (0.25 mM) for 2 or 6 hr. Cells were used for α_{1B}-AR binding assay. *, $p < 0.05$; **, $p < 0.01$, significant difference compared with corresponding forskolin-treated group.

synthesis inhibitor that was shown to have no effect on basal α_{1B}-AR gene expression (21), and then stimulated with forskolin for 2 or 6 hr. As shown in Fig. 1C, pretreatment with puromycin blocked forskolin-stimulated α_{1B}-AR gene expression. This suggests that activation of α_{1B}-AR gene expression by forskolin requires new protein synthesis.

To define the level at which forskolin regulates α_{1B}-AR gene expression, we quantified the steady state levels of α_{1B}-AR mRNA as well as the rate of transcription of this receptor gene. Total RNA extracted from forskolin-treated DDT_1MF-2 cells at various time points was analyzed by Northern blotting. As shown in Fig. 2A, the α_{1B}-AR cDNA probe hybridized with two major mRNA species of 2.7 and 3.3 kb. The pattern of these two bands is similar to that in a previous report, in which 2.3- and 2.0-kb α_{1B}-AR mRNAs were detected in DDT_1MF-2 cells (12). The discrepancy of the apparent size of the α_{1B}-AR mRNA species between the two studies may be due to the use of different RNA ladders as standard. Forskolin significantly increased the amounts of both α_{1B}-AR mRNA species, which peaked at 6 hr and remained at this level for ≥48 hr. Quantification by phosphor-

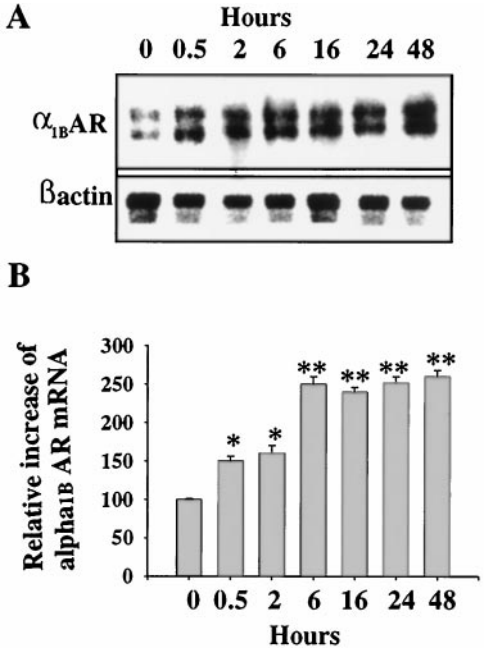

Fig. 2. Time course of changes in α_{1B}-AR mRNA induced by forskolin and IBMX. DDT_1MF-2 cells were incubated with forskolin (1 μM) plus IBMX (0.25 mM) for 0.5–48 hr. Total RNA was then isolated and analyzed by Northern blotting, using cDNAs for the rat α_{1B}-AR and β-actin genes as probes. The amount of radioactivity in the bands was quantified with a PhosphorImager (B). Values are mean ± standard error of three independent experiments. *, $p < 0.05$; **, $p < 0.01$, significant difference from corresponding 0-hr values.

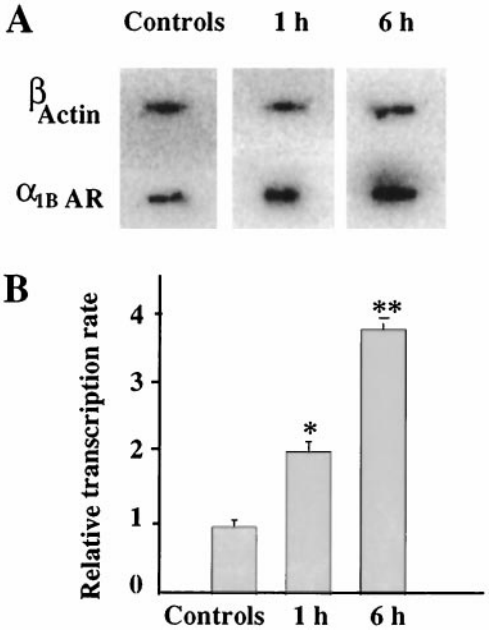

Fig. 3. Effects of forskolin and IBMX on relative transcription rates of the α_{1B}-AR gene in DDT_1MF-2 cells as measured by nuclear run-on assays. DDT_1MF-2 cells were incubated with forskolin (1 μM) plus IBMX (0.25 mM) for 1 and 6 hr. Nuclei were then isolated and used for synthesis of mRNA in the presence of [^{32}P]UTP. Equal amounts of the ^{32}P-labeled RNA were hybridized to the rat α_{1B}-AR or β-actin cDNA probes immobilized on nitrocellulose membranes. This experiment was replicated in three more preparations, and the radioactivity on the blots was quantified with a PhosphorImager (B). *, $p < 0.05$; **, $p < 0.01$, significant difference from corresponding 0-hr values.

imaging revealed that the abundance of both mRNA species at 6 hr was 2.5 ± 0.1 times that in control cells (Fig. 2B). The amount of the 2.2-kb β-actin mRNA remained unchanged during exposure to forskolin, which is in agreement with a previous report (22). To examine whether the increase in α_{1B}-AR mRNA resulted from an increase in the rate of transcription, nuclear run-on assays were performed using nuclei isolated from control and forskolin-treated DDT_1MF-2 cells. As shown in Fig. 3, the rate of transcription of the α_{1B}-AR gene was dramatically increased in forskolin-treated cells, whereas the rate of transcription of the control β-actin gene remained essentially unchanged. This latter finding is in agreement with an earlier report (22), which showed that Bt2cAMP and IBMX had no effect on β-actin gene expression in HT-29 cells. Similar results were obtained in three additional experiments, in which the rate of transcription of the α_{1B}-AR gene was increased 3.5 ± 0.2-fold in the cells treated for 6 hr with forskolin versus control DDT_1MF-2 cells, as quantified by phosphorimaging (Fig. 3B). These results clearly indicate that the induction of α_{1B}-AR and α_{1B}-AR mRNA levels in DDT_1MF-2 cells by forskolin is due to an increase in the rate of transcription of this gene.

Site-directed mutations of both the putative CRE and AP2 elements inhibit basal and cAMP-stimulated P2 promoter activity in DDT_1-MF2 cells. The above findings demonstrated that the rise in intracellular cAMP in response to forskolin significantly increased the rate of transcription of the α_{1B}-AR gene in DDT_1MF-2 cells. To determine whether this induction involves the activation of the dominant P2 promoter, DDT_1MF-2 cells were transfected with a P2/CAT construct. After 60 hr, the cells were harvested and CAT activities were measured. Drugs were added at the time of plating the cells or 6 hr before harvesting of the cells. As shown in Fig. 4, incubation with forskolin for 60 or 6 hr stimulated the P2 promoter activity by 3.3 ± 0.3- and 3.1 ± 0.25-fold, respectively. Incubation with Bt2cAMP for 6 hr also enhanced the P2 promoter activity by 2.5 ± 0.35-fold. This suggests that the induction of the P2 promoter by forskolin or Bt2cAMP contributes to the cAMP-induced transcription of the α_{1B}-AR gene in DDT_1MF-2 cells.

To further explore how the elevated intracellular cAMP stimulated the P2 promoter activity, we examined the sequence of the P2 promoter region and found a one-mismatch CRE (TGApTGTCA) located at −444 to −437 upstream from the ATG start site. Interestingly, this CRE is also identified at a similar location (−443 to −438) in the 5′ flanking region of the human α_{1B}-AR gene (23). Previous data demonstrated that deletion of the region −432 to −460, which contains the CRE, abolished the P2 promoter activity in Hep3B and DDT_1MF-2 cells, suggesting that the CRE is critical for basal P2 promoter activity. To further assess the precise functional role of this CRE, we prepared a mutated P2/CAT construct ($P2_{CREm}$/CAT) in which the sequence TGATGTCA is changed into TGATAGCA. This mutated sequence has been shown to block forskolin-mediated activation of the human vasoactive intestinal polypeptide gene promoter (24) and the insulin-like growth factor binding protein 1 gene promoter (25). DDT_1MF-2 cells were then transfected with P2/CAT or $P2_{CREm}$/CAT constructs. After 60 hr, the cells were harvested, and the CAT activities were measured. Drugs were added at the time the cells were plated or 6 hr before harvesting. As shown in Fig. 4, the basal activity of $P2_{CREm}$ was

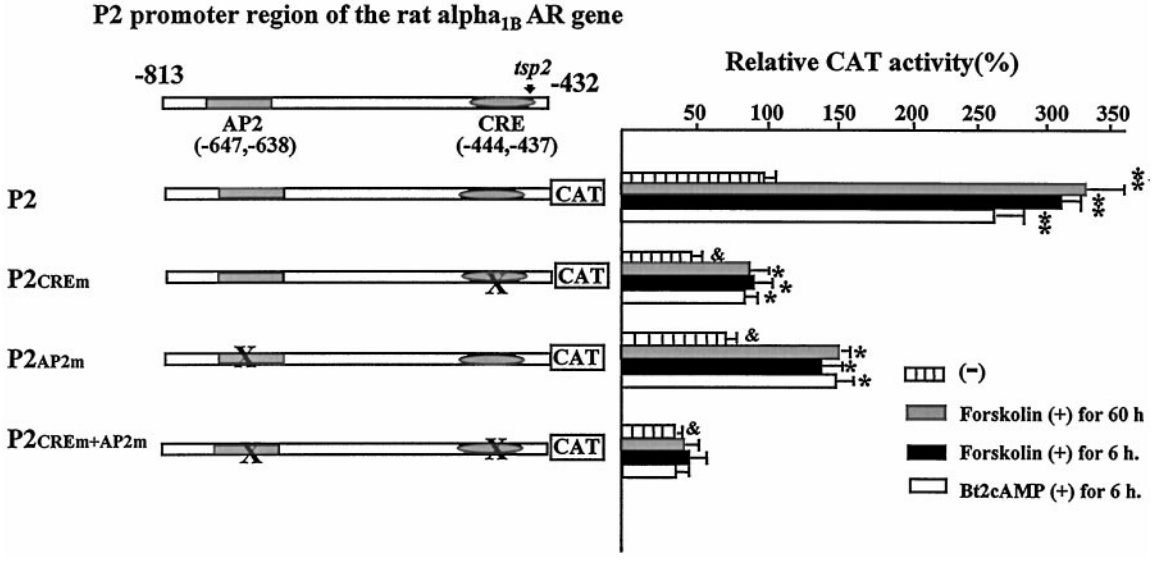

Fig. 4. Effects of site-directed mutations of the CRE and AP2 element on activation of the P2 promoter by forskolin and IBMX. DDT_1MF-2 cells were transfected with P2, $P2_{(CREm)}$, $P2_{(AP2m)}$, or $P2_{(CREmplusAP2m)}$/CAT constructs by using the Lipofectin reagent. After 16 hr, the medium was replaced with fresh medium without DNA and Lipofectin, and cells were grown for an additional 60 hr, after which they were harvested and used to measure CAT activity. Forskolin (1 μM) plus IBMX (0.25 mM) were added at the time of plating of the cells or 6 hr before harvesting. Bt2cAMP (1 mM) plus IBMX (0.25 mM) were added at 6 hr before harvesting. In all experiments, 1 μg of β-galactosidase vector (Promega) was cotransfected to allow for adjustments for transfection efficiency. CAT activity is expressed as a percentage of the control, as established by the use of wild-type P2 promoter/CAT constructs. Values are mean ± standard error for five independent experiments. *, $p < 0.05$; **, $p < 0.01$, significant difference from the activity of the same construct in the absence of forskolin or Bt2cAMP plus IBMX. &, $p < 0.05$, significant difference from the activity of the wild-type P2 promoter activity in the absence of forskolin or Bt2cAMP plus IBMX.

~50% of the activity of wild-type P2, and incubation with forskolin for 60 or 6 hr stimulated the $P2_{CREm}$/CAT activity only 1.6 ± 0.2- and 1.7 ± 0.2-fold, respectively, which are significantly lower values than the 3.3 ± 0.3- and 3.1 ± 0.25-fold stimulation of wild-type P2/CAT. Incubation with Bt2cAMP for 6 hr also enhanced the $P2_{CREm}$/CAT activity by 1.5 ± 0.23-fold, which is significantly lower than the 2.5 ± 0.35-fold stimulation of wild-type of P2/CAT. These results suggest that the CRE mediates both basal and cAMP-induced P2 promoter activity in DDT_1MF-2 cells.

The above findings demonstrate that although mutation of the CRE leads to a major loss in the ability of forskolin or Bt2cAMP to induce P2 promoter activity, significant stimulation remains. This suggests that either the mutated CRE can still weakly support cAMP-induced gene transcription or there is an additional element involved in this induction. The former possibility is very unlikely because this mutated CRE was unable to compete with the CRE for binding CREB (Fig. 5). Sequence analysis reveals that the P2 promoter region contains an AP2 element, which has been reported to mediate basal as well as cAMP-induced transcription in many genes (18). This led us to examine whether the AP2 element within the P2 promoter also mediated basal and cAMP-induced P2 promoter activity. The mutated $P2_{AP2m}$/CAT construct was prepared by mutating the AP2 element (CCCCTGGGGA) within the P2 promoter into the sequence CTAAATTCGGA. We also prepared a mutated $P2_{CREmplusAP2m}$/CAT construct by introducing both CRE and AP2 site mutations into the P2/CAT construct. The P2/CAT, $P2_{AP2m}$/CAT, or $P2_{CREmplusAP2m}$/CAT was transfected into DDT_1MF-2 cells. As shown in Fig. 4, the activities of the $P2_{AP2m}$/CAT and $P2_{CREmplusAP2m}$/CAT were ~70% and ≈35%, respectively, of the wild-type P2 promoter activity. Although forskolin could still stimulate the $P2_{AP2m}$/CAT activity by 1.9 ± 0.3-fold (60-hr incubation) or 1.8 ± 0.3-fold (6-hr incubation), these changes are significantly smaller than the respective 3.3 ± 0.3- or 3.1 ± 0.25-fold stimulations of wild-type P2/CAT. Incubation with Bt2cAMP for 6 hr also enhanced the $P2_{AP2m}$/CAT activity by 1.9 ± 0.25-fold, which is, again, significantly less than the 2.5 ± 0.35-fold stimulation of wild-type of P2/CAT. Mutations of both the CRE and AP2 elements, as in the $P2_{CREmplusAP2m}$/CAT construct, completely abolished the ability of forskolin and Bt2cAMP to stimulate P2 promoter activity. These results clearly indicate that both the CRE and AP2 elements mediate basal as well as cAMP-induced P2 promoter activity in DDT_1MF-2 cells.

Protein binding to the CRE in the rat α_{1B}-AR gene. Our earlier footprinting studies using liver nuclear extracts identified three footprints in the P2 promoter: footprint I (−432 to −452), footprint II (−490 to −540), and footprint III (−609 to −690). Examination of the sequence of footprint I reveals a one-mismatch CRE. To further characterize the proteins that interact with this region, DMSA was initially performed using liver nuclear extract plus ^{32}P-labeled oligonucleotide I. As shown in Fig. 5A, the labeled oligonucleotide I bound a complex specifically (*lane 1*) as it was competed away by unlabeled oligonucleotide I (*lane 2*). This complex was also strongly reduced by a CRE consensus oligonucleotide (*lane 5*) but not by mutated oligonucleotide Im or AP1, AP2, or Sp1 consensus oligonucleotides. CRE is known to bind the CREB (18) and ATF-1 (18). To identify which of these two factors binds to the CRE in oligonucleotide I, a DNA mobility supershift assay was performed using liver nuclear extract. As shown in Fig. 5B, the DNA/protein complex was supershifted by anti-CREB antibody (*lane 4*) but not by anti-ATF1 (*lane 3*) or NF1 (*lane 2*). These results suggest that the CRE in the rat α_{1B}-AR gene binds CREB in liver nuclear extract.

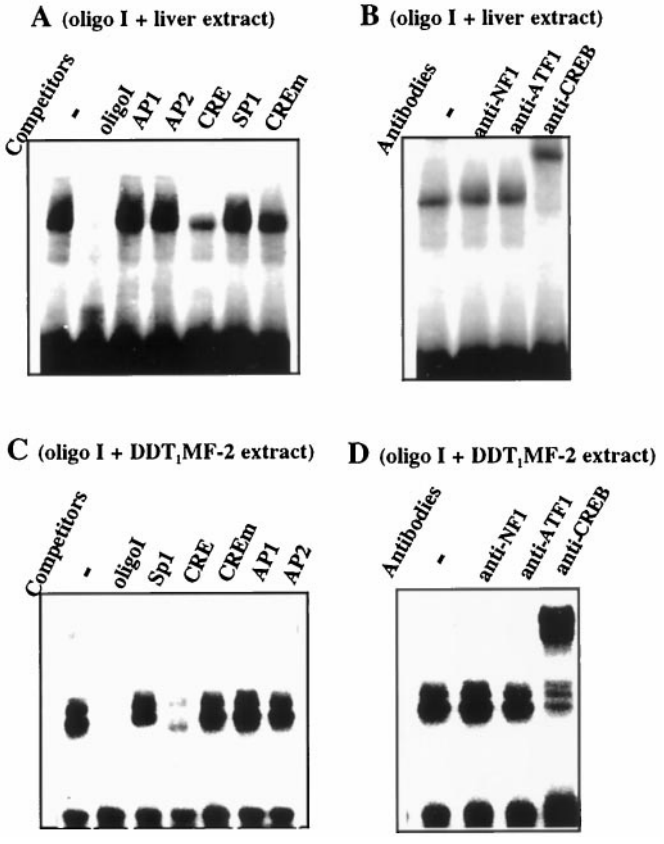

Fig. 5. DMSA of the specific proteins interacting with the CRE within the P2 promoter region. DMSA were performed with 1 ng of ^{32}P-labeled oligonucleotide I plus 10 μg of liver nuclear extract (A) or 5 μg of DDT$_1$MF-2 nuclear extract (C) in the absence (*lane 1*) or presence of 100 ng of different competitor oligonucleotides (indicated *above the lanes*). Supershift analysis of the specific protein binding to the ^{32}P-labeled oligonucleotide I using 2 μg of rat liver nuclear extract (B) or 5 μg of DDT$_1$MF-2 nuclear extracts (D) and the indicated antibodies. The nuclear extracts were preincubated with no antibody (−) or with anti-CREB, anti-ATF, or anti-NF1 antibody for 30 min at 25°.

Since the role of CRE in mediating basal and cAMP-induced transcription of the α_{1B}-AR gene was explored in DDT$_1$MF-2 cells, we wondered whether binding of CREB to the CRE can be also demonstrated using nuclear extracts from DDT$_1$MF-2 cells and ^{32}P-labeled oligonucleotide I. As shown in Fig. 5C, the oligonucleotide I bound two complexes specifically (*lane 1*) as they were competed away by unlabeled oligonucleotide I (*lane 2*). These complexes were also abolished by a CRE consensus oligonucleotide (*lane 4*) but not by mutated oligonucleotide Im or AP1, AP2, or Sp1 consensus oligonucleotides. The supershift assay in Fig. 5D clearly indicates that these complexes contain CREB as they are supershifted by anti-CREB antibody (*lane 4*) but not by anti-NF1 (*lane 2*) or ATF1 antibodies (*lane 3*).

Discussion

The results reported here demonstrate that forskolin rapidly induces the transcription and expression of the α_{1B}-AR gene in DDT$_1$MF-2 cells by increasing the activity of the dominant P2 promoter of the α_{1B}-AR gene and that both a CRE and an AP2 element within the P2 promoter region contribute to the basal as well as the cAMP-induced transcription. This AP2 element was shown to bind a purified AP2 protein in previous footprinting analysis (7), and the CRE was shown to bind CREB from either rat hepatocytes or DDT$_1$MF-2 cells in the present report. These data demonstrate that both CREB and AP2 proteins bind to the dominant P2 promoter of the α_{1B}-AR gene and mediate the basal and forskolin-induced transcription of this gene. This effect of forskolin is cAMP mediated in that it could be blocked by the cAMP-dependent protein kinase inhibitor U89 (Fig. 1C) and mimicked by the cAMP analog Bt2cAMP (Fig. 1B).

Increasing the intracellular concentrations of cAMP results in concomitant rapid increases in α_{1B}-AR mRNA levels and binding site densities within 30 min, with a further rise in both parameters after 2 hr and sustained levels for up to 2 days. These changes are paralleled by progressive increases in the rate of transcription of this receptor gene to 2-fold by 1 hr and 4-fold the basal levels by 6 hr after forskolin treatment. These findings suggest that increased transcription is likely the major mechanism by which cAMP increases α_{1B}-AR gene expression. The molecular mechanisms underlying transcriptional activation by cAMP are not fully understood. Roesler *et al.* (18) divided cAMP-regulated genes into two main groups: group 1 genes regulated rapidly, and group 2 genes regulated more slowly by cAMP. The genes belonging in group 1 were further subdivided into group 1A genes containing the *cis*-acting CRE, such as the genes encoding P-enolpyruvate carboxykinase, somatostatin, and vasoactive intestinal peptide, and group 1B genes containing the AP2 element, such as the genes encoding metallothionein IIa, growth hormone, prolactin, and plasminogen activator. Two genes classified as members of group 1, the proenkephalin (26) and aquaporin 2 genes (27), are exceptional in that they contain both CRE and AP2 elements, although the nature of the proteins binding to these elements in the above two genes has not been verified directly. Both CRE and AP2 elements have been shown to mediate basal as well as cAMP-induced gene transcription, and the AP2 element has also been implicated in phorbol ester induction of gene transcription (18).

The results reported here indicate that the P2 promoter of the α_{1B}-AR gene is similar to the proenkephalin and aquaporin 2 genes (26, 27) in that it has typical features of both group 1A and 1B genes. First, both the transcription of the α_{1B}-AR gene and the expression of the α_{1B}-AR are stimulated rapidly (within 30 min) by increased intracellular cAMP. Second, the P2 promoter region contains both a CRE and an AP2 element, and both elements contribute to basal as well as to cAMP-induced P2 promoter activity, as demonstrated by mutational analyses. Third, binding of the CREB protein to CRE and of the AP2 protein to the AP2 element (7) could be documented by DMSA, DNA mobility supershift assay, and DNase I footprinting.

Treatment of DDT$_1$MF-2 cells by a phorbol ester was shown to induce the transcription of the α_{1B}-AR gene (12), probably via the AP2 element in the P2 promoter region. In addition to this AP2 element, there are several additional AP2 elements in the P1 and P3 promoter regions of the rat α_{1B}-AR gene (5, 6). The P1 promoter contains an AP2 element located between −144 and −135 (5), which was shown not to mediate the cAMP-induced transcription of the rat α_{1B}-AR gene in FRTL-5 thyroid cells (13). The P3 promoter region contains three AP2 elements (6), the roles of which have not yet been studied directly. However, forskolin significantly increases the expression of the 3.3-kb α_{1B}-AR mRNA species

(see Fig. 2), transcription of which is controlled by the P3 promoter (6). Thus, it is very likely that the AP2 elements in the P3 region are also involved in the cAMP-induced transcription of the α_{1B}-AR gene in DDT$_1$MF-2 cells.

It is generally believed that cAMP regulates gene transcription via activation of protein kinase A. Activated protein kinase A translocates into the nucleus and phosphorylates CREB or a closely related protein, ATF-1 (28–34). Phosphorylated CREB or ATF1 then binds to the CRE and stimulates gene transcription (31, 32). CREB is believed to mediate basal gene transcription via an activation domain distinct from that involved in cAMP-induced transcription (35). In certain genes, such as the *α-hCG* gene (36), CREB was found to control basal transcription in a tissue-specific manner, whereas cAMP-induced transcription was not tissue specific. Our mutational and gel shift analyses demonstrated that in DDT$_1$MF-2 cells, the CRE located at −437 to −443 bp in the α_{1B}-AR gene mediates both basal and cAMP-induced transcription via the P2 promoter through the binding of CREB. However, Kanasaki *et al.* (13) reported that this CRE mediated only cAMP-induced and not basal transcription of the α_{1B}-AR gene in FRTL-5 thyroid cells. In that study, a single transcription start point was identified at −173 bp upstream from the translation start site (13), and another group of researchers identified a single α_{1B}-AR mRNA species of 2.2 kb in the rat medullary thyroid carcinoma 623 cells (12). This suggests that the α_{1B}-AR gene promoter active in both FRTL-5 and 623 thyroid cells corresponds to the proximal P1 promoter identified earlier as a weak promoter in the liver (6). It is not clear why CRE controls basal promoter activity of P2 but not of P1. It is possible that due to its location near the 3′ end (around the transcription initiation site) of the P2 promoter region (6), the CRE is involved in the formation of the basal transcription machinery. In contrast, this same CRE is located upstream from the P1 promoter and at a distance of ~300 bp from the transcription initiation site, which may explain its lack of involvement in the basal activity of this latter promoter.

The role of the AP2 element in basal and cAMP-induced gene transcription has not been subjected to the same detailed analysis as the role of the CRE. It has been proposed that increased intracellular levels of cAMP modify the transcriptional activation domain of the AP2 protein after it has bound to the AP2 element in the promoter/regulatory region of a gene. As a result of this modification, the binding protein could resemble basal transcription factors and alter protein/protein interactions (18, 38). The responsiveness of the AP2 element to cAMP and phorbol esters seems to be cell type specific (37, 38). For example, the induction of the human metallothionein II$_A$ gene by cAMP or phorbol esters was observed in HeLa but not HepG2 cells (37, 38), which could be due to the presence of the AP2 protein in the former but not the latter. Here, we show that the AP2 element in the P2 promoter mediates both basal and cAMP-induced promoter activity in DDT$_1$MF-2 cells, and Hu *et al.* (12) reported that a phorbol ester stimulated the transcription of the rat α_{1B}-AR gene in the same cells. However, Kanasaki *et al.* (13) reported that an AP2 element located within the α_{1B}-AR gene P1 promoter did not mediate cAMP-induced transcription and that a phorbol ester also did not induce the transcription of the α_{1B}-AR gene in FRTL-5 cells. These results suggest that the role of AP2 elements in transcriptional control of the α_{1B}-AR gene is cell type specific and depends on the presence or absence of the AP2 protein in a given cell type. Indeed, we found that only the CRE, not the AP2, element mediated basal as well as cAMP-induced P2 promoter activity in HepG2 liver tumor cells,[1] which may be due to the absence of the AP2 protein in these cells (37, 38).

Forskolin treatment of DDT$_1$MF-2 cells caused a similar increase in the expression of the 2.7- and 3.3-kb α_{1B}-AR mRNA species (Fig. 2). Our earlier study demonstrated that the 3.3-kb mRNA is generated by the distal P3 promoter located at −1107 to −1363 bp upstream from the translation initiation point (6). Because there are three AP2 elements immediately upstream from this region (6), it is tempting to speculate about their potential role in basal and cAMP-induced transcription of the α_{1B}-AR gene via the P3 promoter. Although these AP2 elements are not present in the 5′-flanking sequence reported by Kanasaki *et al.* (13), there is strong reason to believe that the sequence reported by these authors is incorrect upstream from the *Bam*HI site at −595 bp, from which point it diverges from the sequence we reported earlier (5, 6). Three lines of evidence suggest that our sequence is correct. First, we determined the genomic sequence of the rat α_{1B}-AR without using the *Bam*HI restriction site. Second, all promoter/CAT constructs that covered the *Bam*HI site were obtained through PCR using genomic DNA as template and primers based on our sequence, and their structures were verified by dideoxy sequencing (6–8). Third, our sequence was confirmed by other investigators, who found that the sequence of a domain upstream from the *Bam*HI site at −595 bp is identical to the sequence we reported (39, 40). In the study of Kanasaki *et al.* (13), a sequencing error may have arisen as a result of the use of the *Bam*HI fragment to obtain the genomic sequence, and this may be the reason for this discrepancy.

In summary, cAMP can rapidly stimulate α_{1B}-AR gene expression and transcription in DDT$_1$MF-2 cells. This induction is mediated in part via the activation of the major P2 promoter of the α_{1B}-AR gene. Mutational and CAT reporter gene analyses demonstrated that both a CRE and an AP2 element are involved in basal as well as cAMP-induced transcription of the α_{1B}-AR gene in DDT$_1$MF-2 cells, and the respective binding of CREB and the AP2 protein to these elements has been verified directly. Roles for CRE in mediating basal promoter activity and for the AP2 element in cAMP-induced promoter activity were not evident in FRTL-5 cells (13), which suggests that the regulation of α_{1B}-AR gene transcription by cAMP is cell specific. The P1 and P3 promoters of the rat α_{1B}-AR gene also contain AP2 elements, but the potential role of these elements in cAMP-induced transcription of the α_{1B}-AR gene in DDT$_1$MF-2 cells or in other cells expressing the α_{1B}-AR gene remains to be determined.

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