

## Transcriptional Down-Regulation of the Human $\alpha 2C$ -Adrenergic Receptor by cAMP

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### ABSTRACT

The heterologous regulation of the  $\alpha 2C$ -adrenergic receptor ( $\alpha 2C$ -AR) was investigated in the HepG2 cell line. Binding of [ $^3H$ ]MK912 ( $\alpha 2$ -antagonist) to membranes from cells submitted to various treatments showed that exposure to insulin, phorbol 12-myristate 13-acetate, or dexamethasone did not affect receptor density. On the other hand, treatment with forskolin resulted in a large reduction of  $\alpha 2C$ -AR number. The effect of forskolin was mimicked by 8-br-cAMP and was abolished by the protein kinase A inhibitor, H89. The action of cAMP was slow ( $t_{1/2} = 23$  h), dose-dependent, and additive to the receptor down-regulation elicited by the  $\alpha 2$ -agonist, UK14304. Furthermore, the diminution of receptor was not caused by an increased rate of its degradation but resulted from a decrease in the steady state amounts of  $\alpha 2C4$ -mRNA. As assessed by experiments in the presence of actinomycin D, the stability of

$\alpha 2C4$ -mRNA was not affected by 8-br-cAMP or forskolin. By contrast, the activity of a luciferase construct containing the entire promoter region of the  $\alpha 2C4$  gene (1.9 kilobase pairs) was inhibited, indicating that the primary mechanism of action of the two compounds is at the transcriptional level. Deletions in the 5'-end of this construct showed that the elements responsible for cAMP responsiveness lie within a 242-base-pair fragment of the gene promoter (nucleotides  $-236/+6$  relative to transcription start). Band-shift experiments indicated that nuclear factors bind to this region in a cAMP-dependent manner. The determination of the actual *cis*- and *trans*-acting elements involved will be the object of future investigation, but the present study provides evidence for transcriptional regulation of human  $\alpha 2C$ -AR by cAMP.

The  $\alpha 2$ -adrenergic receptors ( $\alpha 2$ -ARs) are G protein-coupled receptors that play a key role in the control of numerous physiological functions, such as renal  $Na^+$ -reabsorption, insulin secretion, platelet aggregation, or neurotransmitter release at sympathetic nerve endings (for review, see Ruffolo et al., 1993). Molecular cloning has now definitively established that this receptor family consists of three highly homologous subtypes encoded by distinct intronless genes. In man, the genes coding the  $\alpha 2A$ -,  $\alpha 2B$ -, and  $\alpha 2C$ -AR subtypes were designated  $\alpha 2C10$ ,  $\alpha 2C2$ , and  $\alpha 2C4$ , respectively (Kobilka et al., 1987; Regan et al., 1988; Lomasney et al., 1990). In addition to differences in affinity for various ligands (Bylund et al., 1994),  $\alpha 2$ -AR subtypes also diverge from each other in their tissue distribution, intracellular trafficking, and subcellular targeting as well as in their ability to undergo desensitization. All these discrepancies are consistent with growing evidence indicating that each receptor subtype is

endowed with discrete functions in vivo (Link et al., 1996; MacMillan et al., 1996).

The precise roles of the  $\alpha 2C$ -AR are still unclear in man, but it fulfills all the above-cited criteria of discrimination. From a pharmacological point of view,  $\alpha 2C$ -AR is distinguishable from  $\alpha 2A$  and  $\alpha 2B$  by its sensitivity to prazosin (Bylund et al., 1994) and its remarkably high affinity for MK912 (Schaak et al., 1997a). According to binding studies and to measurement of  $\alpha 2C4$ -mRNA level, its expression is primarily restricted to a limited number of tissues including brain, kidney, aorta, and spleen (De Vos et al., 1992; Peralá et al., 1992; Berkowitz et al., 1994). Finally, in contrast to  $\alpha 2A$  and  $\alpha 2B$ , which are strictly membrane-located and rapidly desensitized on exposure to agonist,  $\alpha 2C$ -AR is refractory to desensitization (Eason and Liggett, 1992) and exhibits both membranous and intracellular localization (Von Zastrow et al., 1993). As assessed by transfection experiments, this peculiar behavior is likely the consequence of the incapacity of G protein-coupled receptor kinase to phosphorylate  $\alpha 2C$ -subtype (Jewell-Motz and Liggett, 1996).

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**ABBREVIATIONS:** AR, adrenergic receptor; AP-1, activator protein-1; bp, base pair(s); PMA, phorbol 12-myristate 13-acetate; 8-br-cAMP, 8-bromo-cAMP; 8-br-cGMP, 8-bromo-cGMP; H89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; pKS+, pBlueScriptII KS+; RPA, RNase protection assay; TM buffer, Tris/MgCl<sub>2</sub> buffer; CRE, cAMP response element.

Compared with our knowledge of the regulation of the  $\alpha 2C$ -AR at the post-transductional level, our understanding of the mechanisms responsible for its tissue-specific distribution and for the control of its expression at the transcriptional level is virtually nonexistent. As yet, the lack of a suitable in vitro cellular system natively expressing this receptor subtype has been a major stumbling block in analyzing the molecular mechanisms controlling  $\alpha 2C4$  gene transcription. The recent recognition that the human hepatoma HepG2 cell line exhibits  $\alpha 2C$ -ARs now allows investigation in this direction (Schaak et al., 1997a). The use of this cell line already permitted us to define some characteristics of  $\alpha 2C4$  gene organization (Schaak et al., 1997b). Its transcription is initiated at a unique start site located 891 bases upstream of the ATG start codon through the activity of a promoter region that contains a nonconventional TATA box and several Sp1 sites, but lacks a CAAT box. Analysis of the sequence upstream of this region also indicated the presence of putative sites for other transcription factors, including upstream stimulatory factor (USF) or activator protein-1 (AP-1). The functional importance of these elements, however, remains to be demonstrated. More recently, HepG2 was also used to re-examine the homologous regulation of the  $\alpha 2C$ -AR (Cayla et al., 1999). In agreement with previous observations in transfected cells, the receptor was found refractory to desensitization after short-term exposure to  $\alpha 2$ -agonist. However, long-lasting treatment induced a sharp down-regulation because of an increased rate of receptor degradation.

The aim of the present work was to study the heterologous regulation of the  $\alpha 2C$ -AR in HepG2. We show that cell exposure to forskolin or cAMP analogs causes a significant reduction of receptor expression. This effect is correlated with a decrease of the amount of  $\alpha 2C4$ -mRNA, which, according to measurement of the activity of a luciferase construct containing the promoter region of the  $\alpha 2C4$  gene, is the consequence of an inhibition of gene transcription. Transfection experiments with constructs containing different fragments of the 5'-flanking region of  $\alpha 2C4$ -gene demonstrates that the *cis*-acting elements responsible for cAMP-dependent regulation are located within a 242-base-pair (bp) fragment of the promoter. Taken together, these observations bring new insights into the mechanisms whereby the expression of the human  $\alpha 2C$ -AR is transcriptionally regulated.

## Materials and Methods

**Drugs and Reagents.** [ $^3H$ ]MK912 (79–80.5 Ci/mmol) was from New England Nuclear (Boston, MA). [ $\alpha$ - $^{32}P$ ]UTP and [ $\alpha$ - $^{32}P$ ]dATP were purchased from ICN (Costa Mesa, CA). Phentolamine and UK14304 were generously donated by Ciba-Geigy (Basel, Switzerland) and Pfizer (Sandwich, UK), respectively. Dexamethasone, human recombinant insulin, phorbol 12-myristate 13-acetate (PMA), forskolin, 8-bromo-cAMP (8-br-cAMP), 8-bromo-cGMP (8-br-cGMP), actinomycin D, and all other chemicals were from Sigma (St. Louis, MO). *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89) was from Calbiochem (San Diego, CA), fetal calf serum (FCS) from Gibco-BRL (Cergy Pontoise, France). The pCRE-Luc construct was obtained from Stratagene (La Jolla, CA). The vectors, pGL3-Basic and pGL3-Promoter, were from Promega (Madison, WI).

**Cell Culture and Treatments.** The human hepatocarcinoma cell line HepG2 was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 100  $\mu$ g/ml streptomycin, 100 IU/ml penicillin, 2 mM glutamine, and 10% FCS. Unless otherwise

specified, all treatments were performed on confluent attached cells and in the absence of FCS. At zero time of the treatment, the hormone or drug to be tested was added to the culture from frozen stock solution. At the indicated time, the medium was removed, the cell-layers rinsed twice with PBS, and rapidly frozen at  $-80^{\circ}C$  until analysis.

**Synthesis of  $\alpha 2C4$  and  $\beta$ -Actin Riboprobe.** The probe for the detection of  $\alpha 2C4$  mRNAs was obtained by subcloning a 370-bp fragment (*Sma*I-*Mae*III), corresponding to nucleotides 1014/1382 of the  $\alpha 2C4$  coding region, into pBlueScriptII KS+ (pKS+; Stratagene). The  $\beta$ -actin probe was obtained by polymerase chain reaction and cloned into the *Eco*RV site of pKS+, the amplified fragment (236 bp) corresponds to nucleotides 415/650 of the cDNA (exon 3). For synthesis of the radiolabeled probes, the two plasmids were linearized with the appropriate restriction enzyme and antisense RNAs were synthesized in the presence of [ $\alpha$ - $^{32}P$ ]UTP using T3 RNA polymerase (Promega).

**RNA Preparation and RNase Protection Assays (RPA).** Cellular RNAs were isolated using the guanidinium isothiocyanate/phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). RPA were performed as described previously but with slight modifications (Schaak et al., 1997b). Lyophilized RNAs (100  $\mu$ g) were taken up in 30  $\mu$ l of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM 1,4-piperazinediethanesulfonic acid, pH 6.7) containing an excess of  $^{32}P$ -labeled riboprobe. The samples were heated to  $95^{\circ}C$  for 5 min and then immediately placed at  $55^{\circ}C$  for 14 h. Nonhybridized probe was eliminated by the addition of 0.3 ml of Tris/EDTA/NaCl buffer (10 mM Tris-HCl, 5 mM EDTA, 300 mM NaCl, pH 7.5) containing RNase A (40  $\mu$ g/ml) and RNase T1 (2  $\mu$ g/ml). After 2 h at  $37^{\circ}C$ , 5  $\mu$ l of proteinase K (10 mg/ml) were added and the samples further incubated for 15 min at  $37^{\circ}C$ . Carrier tRNA (10  $\mu$ g) and 0.3 ml of solution D (4 M guanidinium isothiocyanate, 0.1 M 2-mercaptoethanol, 0.5% w/v sarkosyl, 25 mM sodium citrate, pH 7.0) were added to each tube and protected hybrids were precipitated with isopropyl alcohol. After washing with 70% ethanol, RNA pellets were dissolved in 10  $\mu$ l of sample buffer (97% deionized formamide, 0.1% SDS, 10 mM Tris-HCl, pH 7.0) and loaded onto a 5% acrylamide gel containing 7 M urea. The amounts of protected probe were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Receptor Quantification.** The number of  $\alpha 2$ -ARs was measured on crude membrane preparations using [ $^3H$ ]MK912 (Pettibone et al., 1989). Frozen cells were harvested in 25 ml of Tris/EDTA buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5), then disrupted using a Dounce homogenizer and centrifuged at 39,000g for 10 min. The particulate fraction was washed in Tris/EDTA buffer and the final crude membrane pellet was taken up in the appropriate volume of Tris/MgCl<sub>2</sub> (TM) buffer (50 mM Tris-HCl, 0.5 mM MgCl<sub>2</sub>, pH 7.5). Total binding was measured by incubating 100  $\mu$ l of cell membrane with the radioligand in a total volume of 400  $\mu$ l of TM buffer. After a 45-min incubation at  $25^{\circ}C$ , bound and free radioactivity were separated by filtration through GF/C Whatman filters using a Millipore Manifold Sampling unit. The filters were rapidly washed with ice-cold TM buffer and membrane-bound radioactivity was determined by liquid spectrometry. Specific binding was defined as the difference between total and nonspecific binding measured in the presence of  $10^{-5}$  M phentolamine. For saturation studies, the final concentrations of [ $^3H$ ]MK912 ranged from 0.04 to 3 nM. Saturation isotherms were analyzed using the EBDA-LIGAND computer programs (McPherson, 1985). Protein concentration was estimated according to Bradford's method using BSA as standard (Bradford, 1976).

**Reporter Gene Constructs.** The  $\alpha 2C4$ -promoter/luciferase constructs are numbered relative to the translation start site of the  $\alpha 2C4$  gene. They were generated from the promoterless vector pGL3-Basic, as follows. The *Bam*HI-*Pst*I fragment, corresponding to nucleotides -2806/-886 and ending six bases downstream of the transcription start site, was first subcloned into the *Bam*HI and *Pst*I sites of pKS+. It was then excised with *Bam*HI-*Hind*III and inserted into the *Bgl*II-

*Hind*III sites of pGL3-Basic, generating the construct named pGL3C4 -2806/-886. The series of 5'-end deleted constructs (pGL3C4 -1799/-886, pGL3C4 -1633/-886 and pGL3C4 -1340/-886) was obtained using the *Sma*I site in the polylinker of pGL3C4 -2806/-886 and the *Sma*I, *Stu*I, or *Dra*I blunt sites located in the  $\alpha 2C4$  fragment. Similarly, deletion by *Sac*I resulted in the generation of a construct termed pGL3C4 -1127/-886. The shortest construct in this study (pGL3C4 -1044/-886) was generated by polymerase chain reaction using the sense primer 5'-CATGGTACCCCGAGCCGCCGTGCTGC-3', creating a *Kpn*I site at position -1044 and an antisense primer located in the luciferase sequence. The amplification product was cut with *Kpn*I and *Pst*I and ligated into pGL3 digested with the same enzymes. Mutated versions of the pGL3C4 -1127/-886 construct having the TGCCATCA sequence deleted or mutated into a canonical cAMP response element (CRE) were generated using the Quick Change Site-directed Mutagenesis kit from Stratagene. All constructs were verified by sequencing.

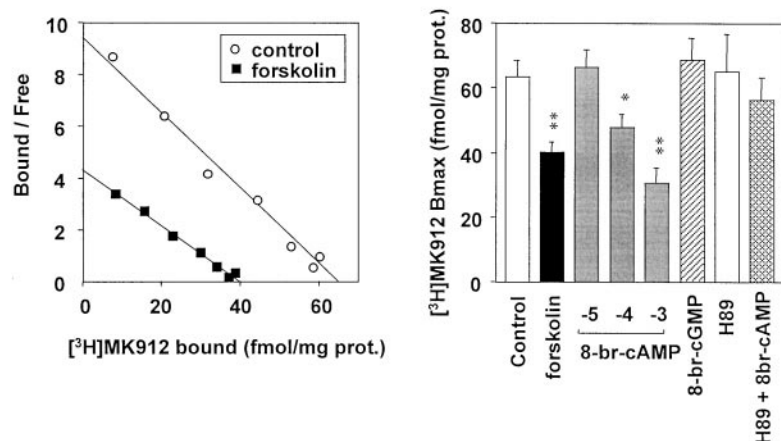
**Cell Transfection and Measurement of Luciferase Activity.** HepG2 and JEG-3 cells were transfected using either the calcium phosphate method (Ausubel et al., 1994) or the Fugene-6 transfection reagent (Boehringer-Mannheim, Meylan, France). Transfections were performed in 30-mm diameter dishes. Cells were kept in the presence of the precipitate or Fugene/DNA complex for 8 h. They were placed in fresh medium, grown for an additional 24-h period and finally treated with 8-br-cAMP or not. Cells were harvested 12 h after treatment and luciferase activity was measured using Promega's luciferase assay.

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays.** Nuclear extracts were prepared basically as described previously (Schreiber et al., 1989). The following oligonucleotides were used: oligonucleotide A (5'-CTGCGAGAGGTT-GCTCTGCCATCAGGCCATGGACCCCGAG-3') spans the region -1076/-1037 of the  $\alpha 2C4$  gene sequence. Oligonucleotide B (5'-GGTTGCTCTGCCATCAGGCCAT-3') corresponds to nucleotide -1068/-1047. Oligonucleotide M (5'-GGTTGCTCTCAGCACAGGC-CAT-3') is identical with B but with the sequence TGCCATCA mutated into TCAGCACA. Oligonucleotide R (5'-TGGAGCTCCAC-CGCGGTGGCGGCCGCTCTAGAACTAGT-3') corresponds to the polylinker of the pRS series and was used as a nonspecific competitor of random sequence. Oligonucleotides A, B, or M (100 ng) were labeled in 15  $\mu$ l of Tris-HCl buffer (50 mM, pH 7.5) containing 10 mM  $MgCl_2$ , 5 mM dithiothreitol, 50  $\mu$ g/ml BSA, 20  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]dATP, and 2 IU of Klenow polymerase. Nuclear proteins (4  $\mu$ g) and labeled double-strand probe (20,000 cpm) were incubated for 15 min at room temperature in a 15- $\mu$ l final volume of HEPES buffer (40 mM, pH 7.9) containing 75 mM KCl, 0.4 mM EDTA, 1.5 mM dithiothreitol, 6% Ficoll 400, 1.5 mM  $MgCl_2$  and 1.5  $\mu$ g of poly(dI-dC). DNA/protein complexes were separated on a 5% polyacrylamide gel and shifted probes were detected by autoradiography for 1 to 3 days. For competition assays, the cold competitor was added together with the labeled probe.

## Results

**Effect of Various Culture Conditions on  $\alpha 2$ -AR Expression.** Studies carried out on various cellular models of human or rodent origin have demonstrated that the expression of the  $\alpha 2A$ -AR subtype is affected by different agents such as forskolin (Sakaue and Hoffman, 1991), insulin (Devedjian et al., 1991), PMA (Reutter et al., 1997), and dexamethasone (Hamamdzic et al., 1995). As a first effort to define the environmental factors that may interfere with  $\alpha 2C$ -AR expression, HepG2 cells were exposed to these four compounds. Measurement of [ $^3$ H]MK912 binding on membrane prepared from cells treated for 48 h indicated that neither insulin (100 nM), dexamethasone (10  $\mu$ M), nor PMA (100 ng/ml) modified receptor density (not shown). In contrast, exposure to forskolin (10  $\mu$ M) induced a significant reduction of [ $^3$ H]MK912  $B_{max}$  (Fig. 1). To verify that the effect of forskolin was cAMP-mediated, HepG2 cells were incubated in the presence of the cell-permeable cyclic-nucleotide analogs 8-br-cAMP and 8-br-cGMP. As shown in the right panel of Fig. 1, treatment with 8-br-cAMP but not 8-br-cGMP provoked a dose-dependent decrease in receptor density. Moreover, pretreatment with the PKA inhibitor H89 abolished the effect of 8-br-cAMP. In HepG2, the  $\alpha 2C$ -AR was recently demonstrated to undergo down-regulation in response to agonist exposure (Cayla et al., 1999). Cells were thus exposed to forskolin in combination with UK14304 ( $\alpha 2$ -agonist) to see whether the effect of the two compounds were additive (Table 1). After 48 h of treatment, forskolin and UK14304 caused decreases of  $35 \pm 9$  and  $51 \pm 12\%$  in receptor number, respectively. A more pronounced reduction ( $68 \pm 4\%$ ) was observed when the two were combined. Additivity was also observed when the  $\alpha 2$ -agonist was combined with 8-br-cAMP. Taken together, these data demonstrate that the attenuation of  $\alpha 2C$ -AR expression by forskolin is mediated via the classical cAMP/PKA signaling pathway. They also suggested that forskolin and the  $\alpha 2$ -agonist act via independent mechanisms. In an effort to elucidate these mechanisms, we first analyzed the kinetics of 8-br-cAMP action.

**Kinetics of Receptor Decrease and Measurement of  $\alpha 2C4$  mRNA Levels.** The time course of the onset of receptor decrease induced by 8-br-cAMP is depicted in Fig. 2. In clear contrast to that observed with 10  $\mu$ M UK14304, which caused a 50% down-regulation within 6 h, the effect of 8-br-



**Fig. 1.** Effect of various treatments on  $\alpha 2C$ -AR density. Left, HepG2 cells were incubated for 48 h in serum-free DMEM containing 10  $\mu$ M forskolin (■) or not (○). Membranes were prepared and used for [ $^3$ H]MK912 binding assay as described under *Materials and Methods*. The amount of specifically bound radioligand was determined using  $10^{-5}$  M phentolamine to estimate nonspecific binding. The data from a typical experiment, in which each point is the mean of duplicate determinations, are displayed as Scatchard plots. Right, HepG2 cells were treated as above but with 10  $\mu$ M forskolin, 8-br-cAMP (0.01 to 1 mM), 1 mM 8-br-cGMP, 10  $\mu$ M H89, or 10  $\mu$ M H89 plus 1 mM 8-br-cAMP. Membranes were prepared and assayed for their capacity to bind [ $^3$ H]MK912. The values of receptor density were derived from analysis of saturation isotherms according to a one-component model and are expressed as femtomoles per milligram of protein. Results are the means  $\pm$  S.E.M. of four determinations (\*\* and \* indicate values significantly different from controls at  $P < .01$  and  $P < .05$ , respectively).



cAMP was apparent only for periods of incubation longer than 12 h. The maximal decrease occurred after 36 h of treatment, 23 h being necessary for the effect of 8-br-cAMP to reach its half-maximum. Identical kinetics were observed with 10  $\mu$ M forskolin (not shown). The down-regulation of  $\alpha$ 2C-AR induced by UK14304 is the consequence of an increased rate of degradation of the receptor protein without modification of the steady-state level of its mRNA (Cayla et al., 1999). Receptor half-life and the amounts of  $\alpha$ 2C4-mRNA were measured to see if the cAMP effect resulted from a similar mechanism. Receptor stability was examined as follows: after incubating the cells for 12 h with 1 mM 8-br-cAMP, the protein synthesis inhibitor, cycloheximide (50  $\mu$ g/ml) was added and the disappearance of  $\alpha$ 2C-AR was appreciated by measuring [ $^3$ H]MK912 binding over a period of 24 h. It was seen that the  $\alpha$ 2C-AR half-life in cells treated with the cAMP analog ( $12.2 \pm 2.5$  h) was not significantly different from that in control cells ( $13.5 \pm 1.5$  h) (data not shown). The steady-state amounts of the  $\alpha$ 2C4-mRNA were measured by RPA on cellular RNA extracted from HepG2 cells incubated or not for 24 h in the presence of the different drugs. As shown in Fig. 3, a significant decrease was ob-

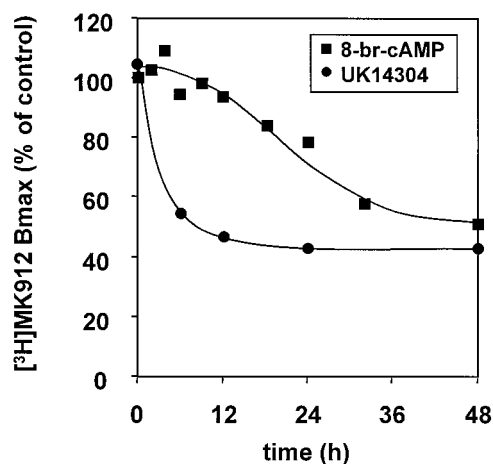
TABLE 1

Effect of combined exposure of HepG2 cells to UK14304 with forskolin or cAMP

HepG2 cells were exposed to 10  $\mu$ M forskolin, 1 mM 8-br-cAMP, 1  $\mu$ M UK14304, 10  $\mu$ M forskolin plus 1  $\mu$ M UK14304, or 1 mM 8-br-cAMP plus 1  $\mu$ M UK14304. After a 48-h period of treatment, membranes were prepared and assayed for their capacity to bind [ $^3$ H]MK912. The values of receptor density were derived from analysis of saturation isotherms according to a one-component model. Results are the means  $\pm$  S.E.M. of three determinations. Statistical analysis was performed using Student's *t* test.

Treatment	[ $^3$ H]MK912 $B_{\max}$	
	-1 $\mu$ M UK14304	+1 $\mu$ M UK14304
	<i>fmol/mg protein</i>	
None	55 $\pm$ 4	27 $\pm$ 6**
Forskolin	36 $\pm$ 5	18 $\pm$ 4*
8-br-cAMP	31 $\pm$ 4	13 $\pm$ 2**

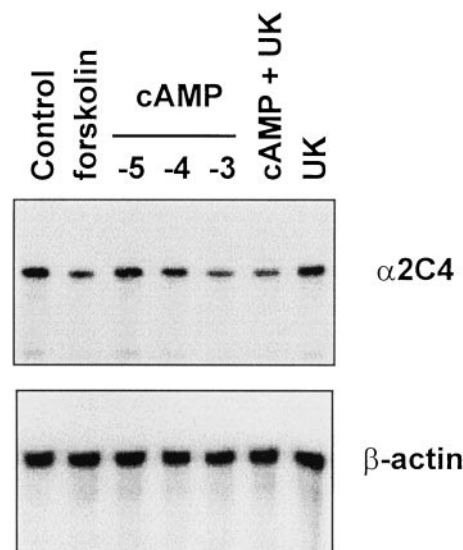
\*\* and \* indicate a significant diminution of  $B_{\max}$  value by UK14304 compared with the corresponding control, at  $P < .02$  and  $P < .05$ , respectively.



**Fig. 2.** Kinetics of the decrease of  $\alpha$ 2C-AR number. HepG2 cells were exposed to 1 mM 8-br-cAMP (■) or 1  $\mu$ M UK14304 (●) for various periods of time ranging from 0 to 48 h. Membranes were prepared and assayed for [ $^3$ H]MK912 binding as described under *Materials and Methods*. The values of receptor density were derived from analysis of saturation isotherms according to a one-component model and are expressed as a percentage of control (untreated cells). Data represent the mean of two independent experiments.

served in cells exposed to forskolin or 8-br-cAMP. On the basis of four determinations and after normalization versus  $\beta$ -actin, the fall in  $\alpha$ 2C4-mRNA represented 30% for 10  $\mu$ M forskolin and 44% for 1 mM 8-br-cAMP. The effect of 8-br-cAMP was dose-dependent; it can be noted that the extent of the mRNA decrease closely matched the decrease in receptor expression as assessed by binding studies. As expected, UK14304 alone did not alter the amounts of mRNA. Furthermore, when applied in combination with 8-br-cAMP, it did not cause any additional reduction in the  $\alpha$ 2C4-mRNA level.

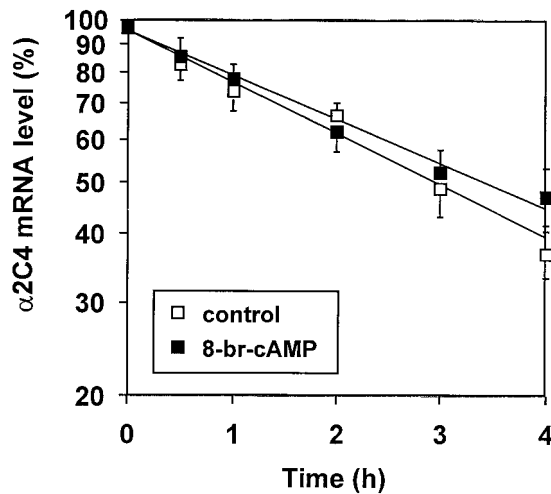
**Forskolin and 8-br-cAMP Inhibit  $\alpha$ 2C4 Gene Transcription.** To clarify the mechanisms whereby the steady state levels of receptor mRNA are altered, we first examined the effect of 8-br-cAMP on the half-life of the  $\alpha$ 2C4 transcripts. To do so, HepG2 cells were incubated in medium containing the transcription inhibitor actinomycin D, either alone or in combination with 1 mM 8-br-cAMP; the disappearance of  $\alpha$ 2C4 mRNA was then monitored over a 4-h period. As illustrated in Fig. 4, the degradation rate of  $\alpha$ 2C4 mRNA was not affected, the half-life being  $3.4 \pm 0.4$  h in cAMP-treated cells versus  $2.9 \pm 0.2$  h in control cells (mean  $\pm$  S.E.M.,  $n = 3$ ). The transcriptional activity of the  $\alpha$ 2C4 gene promoter was also investigated by transfecting HepG2 cells with a luciferase construct (pGL3C4 -2806/-886) containing 1921 bases of the promoter region of  $\alpha$ 2C4 gene. As shown in Fig. 5, treatment of the cells for 12 h with 8-br-cAMP strongly inhibited the luciferase activity. Such an effect was observed neither with the corresponding promoterless vector pGL3-Basic nor with the pGL3-Promoter. Under identical experimental conditions, 8-br-cAMP induced a huge increase of the activity of the pCRE-Luc, a vector in which the reporter gene under the control of a promoter that comprises a TATA-box and four canonical CREs. Similar results were obtained with JEG3 cells, demonstrating that the observations made on HepG2 were not restricted to this specific cell line. Additional experiments indicated 1) that



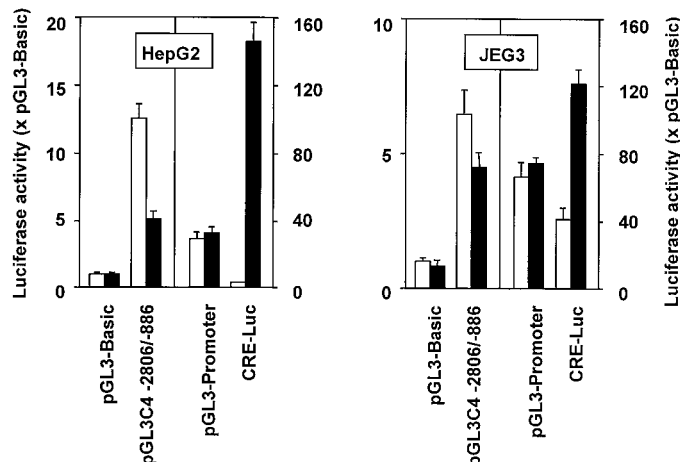
**Fig. 3.** Effect of forskolin, 8-br-cAMP, and UK14304 on  $\alpha$ 2C4 mRNA levels. HepG2 cells were incubated for 24 h with 10  $\mu$ M forskolin, 8-br-cAMP (0.01 to 1 mM), 1 mM 8-br-cAMP plus 1  $\mu$ M UK14304 or 1  $\mu$ M UK14304. Cellular RNA was extracted and hybridized with labeled antisense riboprobe for  $\alpha$ 2C4 (upper panel) or  $\beta$ -actin (lower panel). The samples were digested with a mixture of RNases and resistant hybrids separated by electrophoresis.

inhibition of the activity of pGL3C4 -2806/-886 was also observed after treatment of HepG2 with forskolin, and 2) that exposure to UK14304 neither affected the activity of this construct nor decreased its inhibition by forskolin (not shown). Taken together, these data show that the effect of cAMP is not caused by an enhanced rate of  $\alpha 2C4$ -mRNA degradation but that it is the consequence of attenuated transcription. They also indicate that the sequence element(s) conferring the negative response to the nucleotide are located within the 1.9 kb of the  $\alpha 2C4$  promoter.

**Localization and Identification of the Elements Conferring Responsiveness to cAMP.** A series of progressively 5'-deleted constructs was used to narrow down on the promoter region(s) responsible for the cAMP response (Fig.

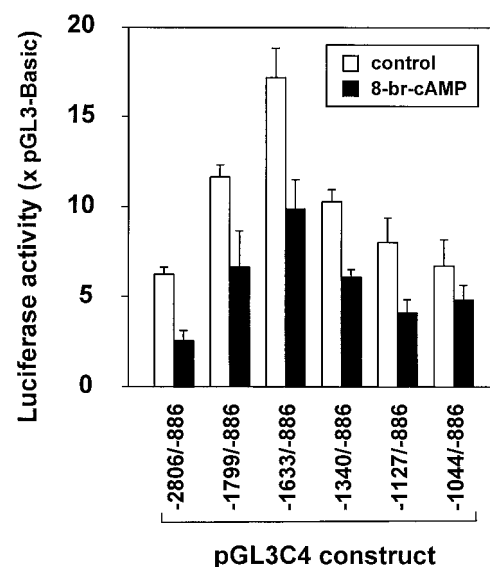


**Fig. 4.** Estimation of the  $\alpha 2C4$  mRNA half-life. HepG2 cells were placed in DMEM containing either 5  $\mu$ g/ml actinomycin D ( $\square$ ) or 5  $\mu$ g/ml actinomycin D plus 1 mM 8-br-cAMP ( $\blacksquare$ ). Cells were collected at the times indicated and the levels of  $\alpha 2C4$ -gene transcripts were quantified as described in the legend to Fig. 3. The amounts of  $\alpha 2C4$  mRNA are expressed as percentage of control. The data, which are the means  $\pm$  S.E.M. of two independent experiments with triplicate point determination, were fitted by least-squares linear regression analysis.



**Fig. 5.** Effect of 8-br-cAMP on the activity of luciferase constructs. HepG2 and JEG-3 cells were transfected with pGL3-Basic, pGL3C4 -2806/-886, pGL3-Promoter, and pCRE-Luc constructs. Eight hours after transfection, the cells were placed in fresh medium and grown for an additional 24-h period. They were then treated with 1 mM 8-br-cAMP (closed bars) or not (open bars) for 12 h and luciferase activity measured. Activities are expressed relative to that of the promoterless vector pGL3-Basic. Values are means  $\pm$  S.E.M. of six independent experiments.

6). Deletion of fragments spanning from nucleotide -2806 to nucleotide -1128 did not significantly affect the extent of inhibition of luciferase activity by cAMP, the percentage decrease of pGL3C4 -1127/-886 activity (53%) being fairly similar to that of pGL3C4 -2806/-886 (41%). By contrast, the inhibitory effect was only 25% when the shortest construct pGL3C4 -1044/-886 was tested, suggesting that the -1127/-1045 region was at least partially involved in the cAMP effect. Gel retardation assays using oligonucleotides and extracts from HepG2 cells were thus performed to identify the nuclear proteins binding to this region. Experiments carried out with oligonucleotide A (Fig. 7, left) demonstrated that at least two protein factors bind to the -1076/-1037 region of the  $\alpha 2C4$  promoter. The intensity of the two shifted bands was increased when extracts were prepared from forskolin-treated cells. Moreover, binding was totally abolished in the presence of a 50-fold excess of cold oligonucleotide A, but not when oligonucleotide R was used as a competitor, implying that the observed binding is specific. In a preliminary attempt to further delineate the sequence responsible for protein binding, a shorter probe centered on oligonucleotide A was assayed. As shown in Fig. 7, middle panel, a shifted band was also observed when oligonucleotide B (nucleotide -1068/-1047) was used. Binding was enhanced by forskolin treatment in a time-dependent manner. In addition, this effect was abrogated in the presence of cycloheximide, indicating that de novo protein synthesis is required for its occurrence. Because oligonucleotide B contains a sequence (TGCCATCA) that resembles a CRE, a probe (oligonucleotide M) covering the same region but having this CRE-like element mutated into TCAGCACA was tested. Oligonucleotide M was shifted in a similar manner as oligonucleotide B (Fig. 7, right), suggesting that this octamer is not involved in protein binding. In agreement with this conclusion, additional transfection experiments demonstrated that the activities of versions of pGL3C4 -1127/-886 having



**Fig. 6.** Effect of 8-br-cAMP on the transcriptional activity of 5'-deleted  $\alpha 2C4$  promoter. HepG2 cells were transfected and treated with 1 mM 8-br-cAMP (closed bars) or not (open bars), as in the legend to Fig. 5. Deletions were numbered according to their location relative to the start codon. Activities are expressed relative to that of the promoterless vector pGL3-Basic. Values are means  $\pm$  S.E.M. of six independent experiments.

the TGCCATCA sequence deleted or mutated into a canonical CRE (TGACGTCA) were inhibited by cAMP to the same extent as the wild-type construct (not shown).

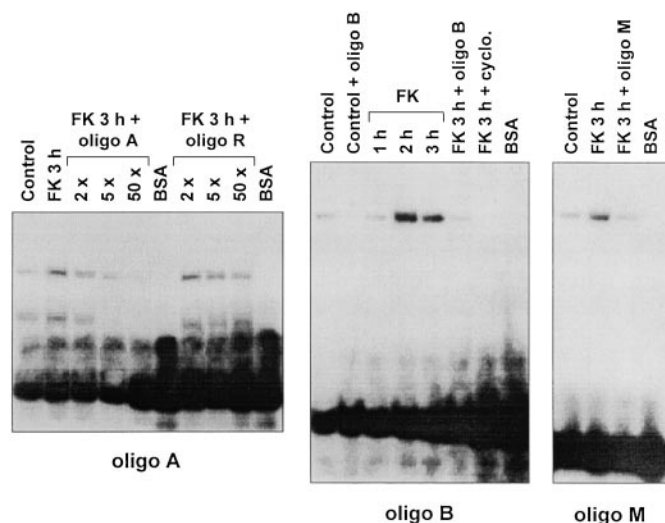
## Discussion

It is now firmly established that the expression of ARs is controlled by various mechanisms, including homologous regulation by agonists and heterologous regulation by hormones or cytokines (Collins et al., 1991; Hadri et al., 1997). Using the HepG2 cell line, we recently demonstrated that the  $\alpha$ 2C-AR undergoes down-regulation after exposure to  $\alpha$ 2-agonists as a consequence of increased degradation (Cayla et al., 1999). The aim of the present work was to define some of the other environmental factors that may affect expression of this receptor subtype. The preliminary experiments carried out on this model indicated that the heterologous regulation of  $\alpha$ 2C-AR diverges from that of  $\alpha$ 2A in several respects. Unlike  $\alpha$ 2A-AR in HT29 cells (Devedjian et al., 1991) or in rat astrocytes (Richards et al., 1987), expression of  $\alpha$ 2C-AR was not lowered after HepG2 exposure to insulin. Also, in contrast to what has been reported in the insulinoma cell lines RIN-5AH and HIT-T15 (Hamamdizic et al., 1995), dexamethasone failed to increase receptor expression. HepG2 certainly does not cause the lack of effect of insulin and dexamethasone, because this hepatocarcinoma cell line is well known to be sensitive to both compounds. Finally, conversely to what

was previously found in HT29 (Sakaue and Hoffman, 1991), treatment with forskolin decreased rather than increased receptor density in HepG2.

As demonstrated by the use of cyclic nucleotide analogs and H89, the inhibitory effect of forskolin on  $\alpha$ 2C-AR expression is triggered through the classical cAMP/PKA pathway. Furthermore, the decrease in receptor is not caused by an acceleration of its degradation, but solely reflects lowered amounts of its mRNA, which is the consequence of attenuated transcription of the  $\alpha$ 2C4 gene. The down-regulation induced by UK14304 was additive to that caused by forskolin. This may seem surprising because UK14304 can inhibit forskolin-induced cAMP production in HepG2 (Schaak et al., 1997a), so it would be rather expected that it attenuate the effect of forskolin. The levels of intracellular cAMP were measured to try to clarify this apparent paradox (Table 2). On the one hand, UK14304 has no incidence on basal cAMP level. This lack of effect matches with the failure of the  $\alpha$ 2-agonist to modify the transcriptional activity of the pGL3C4 -2806/-886 construct and with previous data indicating that the amount of receptor mRNA is unchanged by treatment with UK14304 (Cayla et al., 1999). On the other hand, UK14304 significantly attenuated cAMP production in forskolin-treated cells. The inhibition was partial, however, with the cAMP level in cells treated with forskolin plus UK14304 representing approximately 20-fold the basal level. It is conceivable that such an amount of cAMP is still sufficient to provoke inhibition of transcription. In support of this view, UK14304 did not affect either the attenuation of luciferase activity of the pGL3C4 -2806/-886 construct caused by forskolin or the decrease of  $\alpha$ 2C4-transcript induced by cAMP. Thus additivity of forskolin and UK14304 effects is undoubtedly caused by the combination of the action of cAMP on  $\alpha$ 2C4 gene transcription with that of the  $\alpha$ 2-agonist on receptor degradation.

The negative regulation of gene transcription by cAMP is far less well documented than positive regulation. According to our knowledge of the most-studied examples, inhibition of transcription by cAMP can be achieved through various mechanisms, all of which ultimately result in the alteration of the binding properties of transcription factors to promoter elements. In the genes for interleukin-2, interleukin-2 receptor, and malic enzyme (Tamir and Isakov, 1994; Mounier et al., 1997), inhibition by cAMP requires an AP-1 site and is caused by an alteration of Jun/Fos heterodimer binding. As demonstrated for other genes, including that encoding  $\alpha$ 1-AR (Pfeffer et al., 1998), the inhibitory effect of cAMP on transcription can be achieved through increased binding to CRE



**Fig. 7.** Binding of nuclear proteins to the  $\alpha$ 2C4 promoter region. Nuclear extracts were prepared from control and forskolin-treated HepG2 cells. Four micrograms of proteins were incubated with  $^{32}$ P-labeled double-strand oligonucleotide and DNA/protein complexes were separated on a 5% polyacrylamide gel. Left, oligonucleotide A (-1076/-1037) was used as probe. Control, extract from untreated cells; FK 3 h, extract from cells exposed to 10  $\mu$ M forskolin for 3 h; FK 3 h + oligo A, extract from cells exposed to forskolin for 3 h plus cold oligonucleotide A in 2 $\times$ , 5 $\times$ , and 50 $\times$  excess; FK 3 h + oligo R, extract from cells exposed to forskolin for 3 h plus cold oligonucleotide R in 2 $\times$ , 5 $\times$ , and 50 $\times$  excess. Middle, oligonucleotide B (-1068/-1047) was used as probe. Control, extract from untreated cells; Control + oligo B, extract from untreated cells plus cold oligonucleotide B in 100 $\times$  excess; FK, extract from cells exposed to 10  $\mu$ M forskolin for 1, 2, or 3 h; FK 3 h + oligo B, extract from cells exposed to forskolin for 3 h plus cold oligonucleotide B in 100 $\times$  excess; FK 3 h + cyclo, extract from cells exposed to forskolin for 3 h after pretreatment with cycloheximide 50  $\mu$ g/ml for 30 min. Right, oligonucleotide M was used as probe. Control, extract from untreated cells; FK 3 h, extract from cells exposed to 10  $\mu$ M forskolin for 3 h; FK 3 h + oligo M, extract from cells exposed to forskolin for 3 h plus cold oligonucleotide M in 100 $\times$  excess.

**TABLE 2**

Effect of UK14304 on intracellular cAMP level

HepG2 cells were detached by treatment with PBS-EDTA, resuspended and incubated for 15 min at 37°C in 200  $\mu$ l of HEPES-buffered DMEM containing either vehicle (basal), 1  $\mu$ M UK14304, 10  $\mu$ M forskolin, or 10  $\mu$ M forskolin plus 1  $\mu$ M UK14304. Concentrations of cAMP were measured by radioimmunoassay. Results are expressed as means  $\pm$  S.E.M. of eight determinations. Statistical analysis was performed using Student's *t* test.

Addition	cAMP Level pmol/mg cell protein
None	25 $\pm$ 4
UK14304	29 $\pm$ 6
Forskolin	1040 $\pm$ 234
Forskolin + UK14304	547 $\pm$ 234*

\* Significant diminution of cAMP level by UK14304 at *P* < .001.



of repressors of the CRE modulator protein family, such as CREM  $\alpha$  and  $\beta$  (CRE modulator) or ICER (inducible cAMP early repressor).

The promoter region of the  $\alpha 2C4$ -gene bears a putative AP-1-like sequence (TGATTCA), centered at position -1233 relative to the translation start codon, that contains one mismatch with respect to the consensus AP-1 site (TGA(G/C)TCA). Clearly, this AP-1-like element is not responsible for the cAMP effect on transcription because the activity of the construct pGL3C4 -1127/-886 was as sensitive to cAMP as the construct containing the entire promoter region. Moreover, the functional relevance of this site is questionable because, according to the present study, expression of  $\alpha 2C$ -AR is also insensitive to PMA treatment. Interestingly, the removal of nucleotide -1127/-1045 resulted in an attenuation of the effect of cAMP on luciferase activity, suggesting that this fragment is, to some extent, involved in the transcriptional effect of the nucleotide. In support of this view, nuclear factors bind the -1076/-1037 region of the  $\alpha 2C4$  promoter. The binding of these proteins is enhanced in a time-dependent manner after cell exposure to forskolin and protein synthesis is necessary for the increase of binding to occur. The use of a mutated oligonucleotide or of mutated luciferase constructs eliminates the possibility that the TGC-CATCA sequence at position -1060/-1053 is responsible for the effect of cAMP. However, the type of transcription factor(s) and the DNA sequence(s) accounting for band shift remain to be elucidated. It is also worth mentioning that the effect of cAMP was not totally abolished on the pGL3C4 -1044/-886 construct, suggesting that additional proximal elements contribute to the inhibitory effect of the nucleotide. Future studies will thus be necessary to clarify these issues.

In summary, the present work demonstrates that cAMP down-regulates the expression of  $\alpha 2C$ -AR in HepG2 cells as a result of inhibition of  $\alpha 2C4$ -gene transcription. This observation reveals a novel aspect of the mechanisms whereby this subtype may be regulated.

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