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Dexamethasone Stimulates Human A_1 Adenosine Receptor (A_1AR) Gene Expression through Multiple Regulatory Sites in Promoter B

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ABSTRACT

The expression of the human A_1 adenosine receptor gene is controlled by two promoters, promoters A and B, and they are located 600 base pairs apart. The characteristics of the two promoters differ by the activity of expression, tissue specificity, and the potential regulatory elements around them. Promoter A is more active but its expression is observed only in selected tissues, whereas promoter B is constitutively expressed but at much reduced levels. In Chinese hamster ovary (CHO) cells transiently transfected with plasmids containing either promoter linked to a reporter gene, dexamethasone (dex) can stimulate (or enhance) the expression of promoter B much more effectively than that of promoter A. Mutation and deletion studies on plasmids containing promoter B have shown that the stimulation is mediated through multiple regulatory sites, including a serum response element, AP1, and TATA box. However, a single-glucocorticoid response element monomer-binding site between promoters A and B does not have significant contribution to dex-regulated expression. The interactions between glucocorticoid receptor (GR) and some regulatory sites are probably occurring via this protein (GR) interacting with other DNA-binding proteins because there is no GR DNA-binding sequence in the sites studied. The stimulation can be eliminated by mifepristone, an antagonist of GR, indicating the involvement of GR in gene regulation. In addition, dex treatment also stimulated the expression of A_1 adenosine receptors in CHO cells transfected with the plasmids containing contiguous genomic sequences of promoter B or promoters A and B linked to the receptor-coding sequence. When promoter A is active and both promoter A and B are present in a construct, dex treatment induced a much smaller percentage of stimulation.

The glucocorticoid family of steroid hormones affects transcriptional expression of many genes through interaction with its intracellular receptor, the glucocorticoid receptor (GR). Upon binding to the receptor, the complex can enter the nucleus and bind to the appropriate DNA sequence or other factors to regulate gene expression (McEwan et al., 1997). GR belongs to a superfamily of nuclear receptor transcription factors that share a common molecular structure organization containing a ligand-binding domain at the C terminus and a DNA-binding domain at the N-terminal side of the ligand-binding domain. The receptor-binding sequence on the target DNA molecule, known as glucocorticoid response element (GRE) is a hexamer (TGTTCT) for monomer-binding or a 15-base pair (bp) sequence for homodimer binding (Chalepakis et al.,1990). The sequences flanking the GRE, although not conserved, are also important for GR binding. Besides affecting gene expression through direct binding to GRE, the receptor (GR) may also exert its influence through protein-protein interaction with other transcription factors (McEwan et al., 1997). Depending on the type and target of the GR interaction, it can up- or down-regulate the affected gene expression (Diamond et al., 1990).

The expression of human A_1 adenosine receptor (A_1AR) gene is controlled by two separate promoters, promoter A and promoter B, which are about 600 bp apart (Ren and Stiles, 1995). Promoter B and exon 1B are part of intron 1A when promoter A is active. Computer analysis of the sequence surrounding both promoters revealed only one GRE monomer-binding site between promoter A and B. There is also an AP1 site within promoter B region that has been shown to be a target of GR interaction (Jonat et al., 1990; Teurich and Angel, 1995; Garlow and Ciaranello, 1995).

In DDT_1 MF-2 smooth muscle cells, treatment with the synthetic glucocorticoid dexamethasone (dex) caused increased expression of A_1ARs, whereas the adenosine A_2 receptor was down-regulated (Gerwins and Fredholm, 1991). Similar results were also obtained in rats wherein dex treat-

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ABBREVIATIONS: A_1AR, A_1 adenosine receptor; dex, dexamethasone; GR, glucocorticoid receptor; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; RU486, mifepristone; SRE, serum response element; GRE, glucocorticoid response element; CHO, Chinese hamster ovary; PCR, polymerase chain reaction.

ment of adrenalectomized rats showed a marked increase in A_1AR but not A_{2a}AR in brain tissue (Svenningsson and Fredholm, 1997). In this report, we present data showing that dex increases the transcriptional expression of luciferase reporter gene directed by the human A_1AR promoter and that the stimulation can be eliminated by the GR antagonist mifepristone (RU486), indicating direct GR involvement. Dex stimulation of gene expression is much more effective through promoter B than promoter A. Mutation and deletion studies showed that the dex stimulation involves multiple nuclear-binding sites. We also document that the ability of dex to stimulate the enhanced production of A_1AR is dependent on whether one or both of the promoters and intervening sequences are present in the construct transfected into Chinese hamster ovary (CHO) cells. The A_1AR expression was enhanced when only promoter B was present. But when both promoters were present and active in the same plasmid, dex was much less effective. This has direct implication as to why glucocorticoid-stimulated A_1AR response is tissue-specific.

Materials and Methods

Cell Culture and Transfection. The CHO cells do not express any A_1ARs and they were used in our previous study of the receptor promoter activity because the promoter constructs transfected into CHO cells showed higher activity than those transfected into other cells. The transfected CHO cells also showed good response to the dex treatment. Therefore, CHO cells were chosen in this report to study the effect of dex on promoter activity.

CHO cells were grown in Dulbecco's modified Eagle's medium/F-12 medium (catalog no. 11330-032, Gibco Laboratories, Gaithersburg, MD) plus 10% fetal bovine serum in a 37°C incubator containing 5% CO_2. For luciferase reporter gene plasmid transfection, cells were transferred to 6-well plates the day before transfection at about 5.2×10^5/well cell density. Transient transfection using lipofectamine (catalog no. 18324-012, Gibco Laboratories) was carried out according to the manufacturer's instruction. For the plasmids with luciferase reporter gene, 10 ng/well plasmid pSVβ containing β-galactosidase gene was cotransfected to normalize the transfection efficiency differences among samples. Since the optimal amount of plasmid DNA used in the transfection experiment is about 1 μg/well, the plasmid Pmt B/−422 and its base substitution mutants were transfected into cells with this amount and the equimolar amount of deletion mutants were used. The total amount of plasmid DNA for deletion mutants was held constant by adding empty pCMV5 vector which has a similar size. Equimolar amounts of plasmids containing promoter A were also used in the transfection experiments. Forty-eight hours after transfection, cells were washed twice with serum-free medium (2 ml/well) and incubation was continued in the serum-free medium (Dulbecco's modified Eagle's medium/F-12) plus 0.1% bovine serum albumin (catalog no. A-8806, Sigma, St. Louis, MO) with or without 100 nM dex (Sigma). Some samples were treated with both dex and RU486 (500 nM, Sigma).

Luciferase and β-Galactosidase Assays. Twenty-four hours after the dex treatment, cells were processed for luciferase and β-galactosidase assays according to Promega's protocol for luciferase assay system with reporter lysis buffer. For each well of cells, 0.2 ml of 1× lysis buffer was used. Luciferase activity was measured as luminescence with a BioOrbit 1251 luminometer (Pharmacia LKB, Piscataway, NJ). The activity of β-galactosidase was measured according to the standard method (Ausubel et al., 1987). The luciferase activity from different samples was normalized by β-galactosidase activity obtained from the same quantity of sample. Each treatment had triplicate samples and all experiments were repeated at least three times. Within each experiment, the promoterless vector pBLPniF (a gift from Dr. W. E. Kraus, Duke University Medical Center) was also transfected into CHO cells as a background control. The promoter activity for each construct was calculated by subtracting the background activity from the total activity and then dividing by the activity with the promoterless construct. Therefore, the background luciferase activity obtained from the promoterless plasmid pBLPniF was set as one and in this way the variations between experiments were minimized. The results are presented as fold expression.

[^{3}H]-8-cyclopentyl-1,3-dipropylxanthine (DPCPX) Binding Assay. The CHO cells transfected with plasmids PmtB-A_1AR, PmtAB-A_1AR, pCMV5/huA1, and pCMV5/Ex4-6 were processed for receptor-binding assay according to the protocol described previously (Olah et al., 1992) with the radioligand [^{3}H]DPCPX (NEN Life Science Products, Boston, MA) at 8 nM.

Total RNA Isolation and RNA Slot-Blot. About 72 h after transfection (24 h after dex treatment), the cell culture medium was removed from the transfected CHO cells and 7 ml of Trizol reagent (Gibco) was added to extract total RNA following the manufacturer's instruction. The pelleted total RNA was resuspended in water and A_{260} was used to estimate RNA concentration.

The RNA slot-blot procedure follows the standard protocol (Ausubel et al., 1987). After UV cross-linking, the membrane was prehybridized in 5× SSC (0.75 M NaCl and 0.075 M sodium citrate), 5× Denhardt's solution, 50% formamide, 1% SDS, and 100 μg/ml yeast tRNA at 60°C for 3 h. The full-length purified RNA probe was added to the hybridization solution and incubation continued overnight at 60°C. The following day, the membranes were washed once with 2× SSC, 0.1% SDS at room temperature, twice with 0.2× SSC, 0.1% SDS at room temperature and twice with 0.1× SSC/0.1% SDS at 60°C. Each wash lasted about 15 min. The membranes were then sealed in a plastic bag and exposed to film. The bands on the developed film were scanned using the densitometer (Bio-Rad model 620 densitometer; Bio-Rad, Hercules, CA) to estimate the amount of mRNA. The experiments were repeated three times and the average increase are presented in *Results*.

RNA Probe Synthesis. The DNA template for RNA probe synthesis was created by polymerase chain reaction (PCR) using a 3′ end primer containing a sequence of T7 promoter which would direct the synthesis of an antisense RNA. The template for probe B-luc was amplified based on the plasmid PmtB/−422, which contained a fraction of luciferase coding sequence plus a fraction of promoter B insert in the plasmid.

The RNA probe B-luc was synthesized with Ambion's MAXIscript (Austin, TX) in vitro transcription kit. The resulting ^{32}P-labeled RNA probes were gel-purified on a 5% acrylamide/8 M urea gel to select the full-length molecules. The RNA probes eluted from the gel were directly used for Northern blot hybridization.

Plasmid Construction. Expression plasmids containing human A_1AR gene promoter A or promoter B or both were constructed based on similar procedures described previously (Ren and Stiles, 1995). Some plasmids have been used in previous studies such as PmtA/−897 (=pBLPniF/PmtA), PmtA/−253, PmtB/−422 (=pBLPniF/PmtB), PmtB/−129, PmtB/−29, PMTBMUT (Ren and Stiles, 1995), pCMV5/huA1 (Ren and Stiles, 1994a), and pCMV5/Ex1b−3 (=pCMV5/Ex4-6; Ren and Stiles, 1994b). The following list of plasmids were constructed during this study.

The construction of the plasmid is as follows. The plasmid pBLPniF was digested with *Sal*I and *Eco*RI to remove the luciferase gene and to be ready for the insertion of genomic sequence of both promoters A and B including intronic structure plus A_1AR coding sequence. Two fragments were created by PCR as plasmid inserts. The upstream fragment was amplified from the genomic sequence, which started from −1032 nucleotides of the transcription start site of promoter A and ended at the *Sma*I site of the A_1 receptor-coding sequence. The downstream fragment was amplified from the cDNA clone 7A (Ren and Stiles, 1994) with the 3′ primer containing a poly(A) signal at the end of coding sequence to skip the 3′ untrans-

lated sequence. The two fragments were ligated into the digested plasmid.

The plasmid PmtB-A_1AR has the same insert as PmtAB-A_1AR except that the 5′ end of the insert is at −422 of promoter B. Therefore, the promoter A is excluded from the plasmid.

The plasmids with extended 5′ flanking sequence (PmtA/−3180) of promoter A were constructed with PCR fragments amplified from genomic sequence and replaced the insert of Pmt A/−897.

The plasmids containing mutations at GRE, serum response element (SRE), AP1, and TATA box sites were constructed with PCR fragments according to the protocol described previously (Ren and Stiles, 1995). The selected mutants were sequenced to confirm the mutation. The sequence comparisons between wild type and mutants are illustrated in Table 1.

Results

Dex Stimulates the Expression of a Luciferase Reporter Gene Controlled by the Human A_1AR Promoter. The promoters of the human A_1AR gene are organized in such a way that promoter B is about 600 bp downstream of promoter A and that it is part of intron 1A (Fig. 1). By examining the available 5′ flanking sequences for both promoters, only one GRE monomer-binding site was discovered about 379 bp upstream from the promoter B transcription start site and 221 bp downstream from the promoter A transcription start site. The AP1 site within the promoter B area has been demonstrated to be important for the basal activity of promoter B (Ren and Stiles, 1995). There are two SREs upstream of promoter B, SRE-1 at −113/−106 and SRE-2 at −69/−62. The sequences at the two SREs are almost identical and in the same orientation (Fig. 1).

The 5′ end of the insert for plasmid PmtB/−422 (=pBLPniF/PmtB) is at −422 of promoter B and the 5′ ends of the inserts for a group of plasmids containing promoter A are extended a variety of distances up to −3180 nucleotides of the promoter A transcription start site (PmtA/−253, PmtA/−897, and PmtA/−3180). When those plasmids were transfected into CHO cells and treated with 100 nM dex in serum-free medium for 24 h 2 days after transfection, the luciferase expression when driven by promoter B alone increased about 88% compared with the untreated cells. However, the luciferase expression when driven by promoter A (PmtA/−897) had little change when treated with dex (Fig. 2). When the 5′ flanking sequence of promoter A was extended to about 3 kb (PmtA/′3180), promoter A expression had only a 10% increase in activity. Although the absolute increase in activity by dex treatment in promoter A is about the same as that in promoter B, the percentage of change of activity in promoter B is much greater than that in promoter A because the basal level of promoter A is much higher than that of promoter B. This suggests that dex regulation of human A_1AR gene expression affects the transcriptional activity of promoter B much more than that of promoter A when the promoters are present separately.

TABLE 1

Consensus, wild-type, and mutant sequences of the transcription factor-binding sites for the promoter B of human A_1AR

Binding Site	Consensus	Wild-type	Mutant
GRE	AGAWCAGW[a]	AGAACAGT	TAGCATCT
SRE-1	CRCSSCAS[a]	CGCCCCAG	TTACGTAC
SRE-2	CRCSSCAS[a]	CACCCCAG	CTCATCAT
AP1	TGASTMA	TGAGTCT	CGTGGAT
TATA box	TATAAT	TTTAAAA	GCTGACT

W = A or T; R = A or G; S = C or G; M = A or C. The mutant sequences have been analyzed by computer to ensure that there are no known DNA-binding elements being created due to mutation.

[a] Reversed orientation.

The slot-blot hybridization of total RNA isolated from the CHO cells transiently transfected with PmtB/−422 showed that the transcript from promoter B increased by about 45% after dex treatment but the stimulation was reduced when RU486, a GR antagonist, was included with dex (Fig. 3). Although the increase of mRNA is slightly smaller than the increase in luciferase activity after dex treatment, this is direct evidence of transcriptional regulation of A_1AR gene expression by dex.

The DNA-Binding Sites SRE, AP1, and TATA Box are Responsible For Most of the Dex-Stimulated Promoter Activity. Because there is one GRE site upstream of promoter B, the previously used deletion mutant PmtB/−129 was used to determine whether it is responsible for the dex-stimulated expression (Fig. 4A). The results showed that the basal activity of promoter B doubled, indicating a possible involvement of an inhibitory element in the sequence segment and that this sequence including the GRE monomer-binding site accounts for only a small part of the stimulation. In addition, the base substitution mutant PmtB/GRE showed a reduction in basal level expression so the percent increase due to dex-mediated stimulation did not change. Thus, this GRE monomer-binding site probably does not have any significant contribution to the dex-stimulated promoter activity and other site(s) must be involved in the mechanism.

The deletion of the two SREs (PmtB/−39) significantly reduced the effect of dex (PmtB/−39 in Fig. 4A). Mutations were also made at these two sites separately to determine which SRE is more effective (Table 1 and Fig. 4B). The base substitution mutation at SRE-1 alone (PmtB/SRE1) had little effect on the dex-stimulated activity but the same base substitution mutation at SRE-2 alone caused almost complete elimination of basal activity (data not shown), indicating that this site is important for promoter B basal activity. To evaluate the role of SRE-2 on the effect of dex treatment, a different mutant (PmtB/SRE2) was selected (Table 1). The new mutation was made at nonconserved bases of SRE and caused a slight increase of basal activity but significantly reduced the dex-stimulated activity (Fig. 4B). This result indicates that SRE-2 is partially responsible for GR stimulation. When the AP1 site alone was mutated (PmtB/AP1 MUT in Fig. 4B), the basal activity was significantly reduced and the reduction of dex stimulation also occurred but percentage-wise not as much as SRE-2 mutant. The double mutant PmtBMUT has both the TATA box and AP1 sites mutated. In this case, the basal activity of promoter B was greatly reduced and the dex-stimulated activity was not significant, indicating the involvement of TATA box in the process (Fig. 4B). The base substitution mutant GSAMUT has all three sites GRE, SRE-2, and AP1 mutated and the result showed that a major part of the dex-stimulated expression was then eliminated. However, a noticeable amount of dex stimulation was still present with GSAMUT-transfected cells. In contrast, the 5′ deletion mutant PmtB/−24 (Fig. 4A), which had all of the sequences 5′ to the AP1 removed, showed essentially no dex stimulation although it has the intact AP1 site, again indicating the role of the TATA box in the mechanism. Finally, when all four sites, GRE, SRE-2, TATA box, and AP1, were mutated (PmtB/GSTAMUT), no significant level of

the dex-mediated stimulation of promoter B activity was observed even though the mutant plasmid had the same length insert as the wild-type PmtB/−422. This evidence suggests that dex stimulation of A_1AR gene promoter B expression is through multiple sites and probably involves general transcription assembly.

Dex Treatment also Stimulates A_1AR Gene Expression as Measured by [^{3}H]DPCPX Binding. Because human A_1AR gene expression is controlled by two separate promoters, the relationship between the two promoters will determine how mRNA and receptor are expressed. To study the expression pattern of promoters A and B in a more physiological setting, two plasmids PmtAB-A_1AR and PmtB-A_1AR with inserts containing genomic sequence of either promoter B alone or both promoters A and B together along with the first exons, introns plus the receptor-coding sequence were constructed to transfect CHO cells. The transcripts produced from these plasmids would have to be pro-

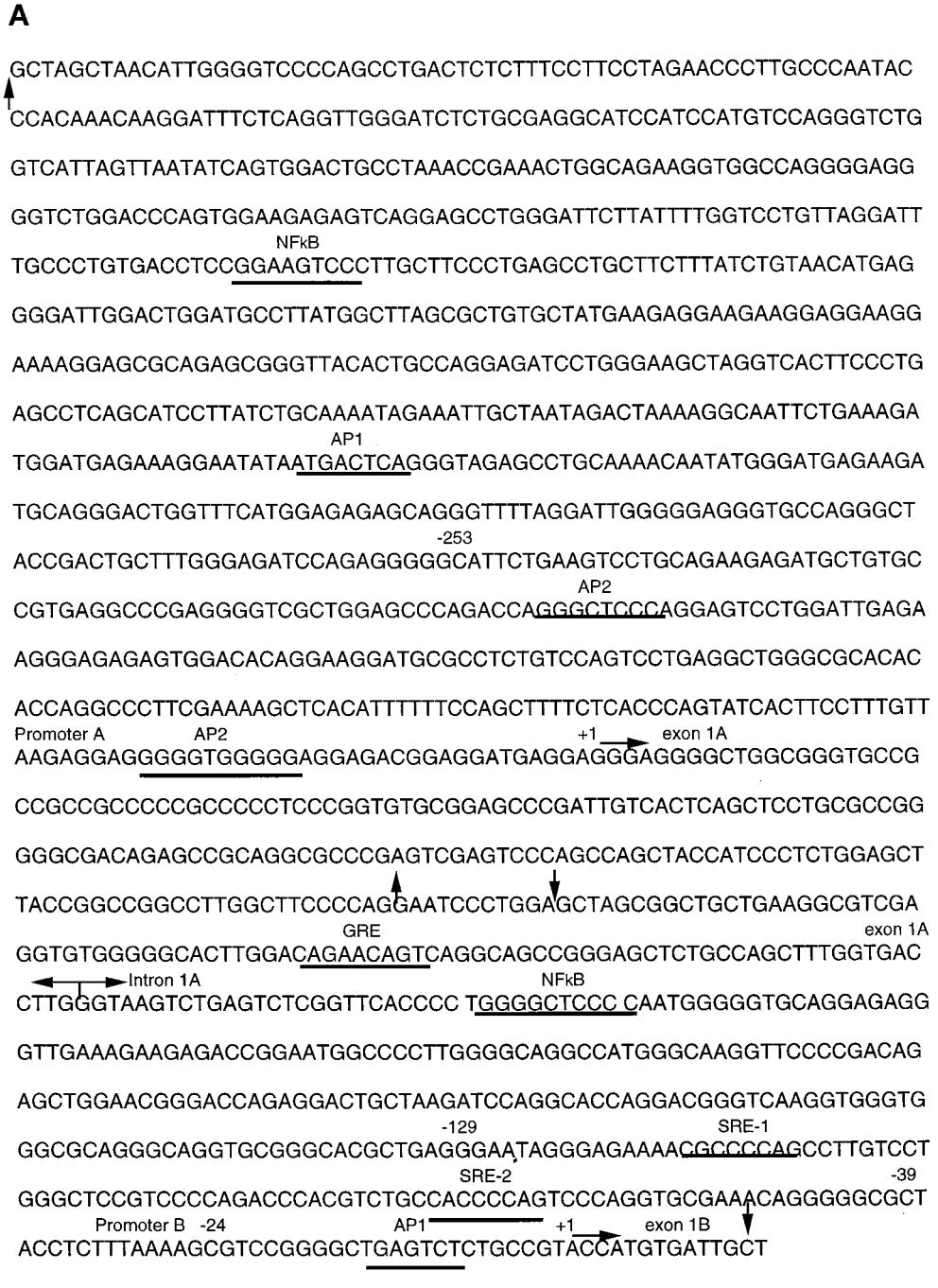

Fig. 1. A, human A_1AR gene promoters and their flanking sequences. The transcription start sites for promoters A and B are labeled +1 and they are about 600 bp apart. The transcriptional factor-binding sites are underlined. The arrows pointing upward or downward indicate the boundaries of plasmid inserts for PmtA/−897 or PmtB/−422, respectively. The horizontal arrows indicate the boundaries of exons and intron. The 5′ ends of deletion mutants for promoters A and B are also labeled as negative numbers. B, the simplified structure of human A_1AR promoters and regulatory elements.

cessed to excise the intervening introns. The results in Fig. 5A showed that the A_1 receptor-binding activity was stimulated by dex treatment and that this stimulation was eliminated by RU486, a GR antagonist. When the PmtAB-A_1AR-transfected cells were treated with dex, only a small increase of the receptor-binding activity was observed (Fig. 5B) when both promoters were active as determined by reverse transcription-PCR (data not shown). At the same time, the pCMV5/huA1-transfected cells were used as a negative control and showed no response to dex treatment. The results show that dex stimulation of promoter B works on both luciferase reporter gene expression and the expression of A_1ARs and that the stimulation is indeed regulated by GR. The results also show that when promoter A is active, the dex treatment is much less effective. In addition, when the CHO cells transfected with the plasmid pCMV5/Ex1b-3 were treated with dex, no stimulation of receptor expression was observed (data not shown) although the transcript produced by this plasmid is about the same as PmtB-A1AR, indicating that the dex stimulation is promoter-dependent but not related to translation.

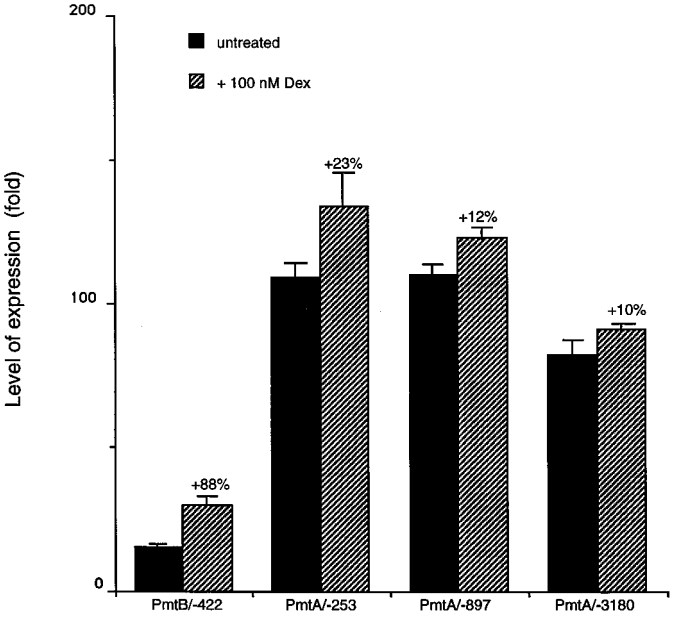

Fig. 2. Comparison of the effect of dex on promoter A and promoter B. The luciferase activities presented are normalized with the formula (total activity−background)/background. The dex treatment caused negligible change of background luciferase activity (<1%) from the promoterless construct pBLPniF (data not shown). The normalized luciferase activities from cells treated with dex were compared with that from cells without dex treatment and the percent increase of activity is indicated for each construct ($n \geq 3$).

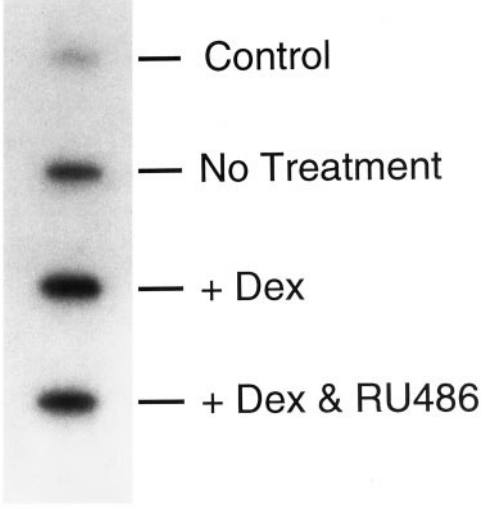

Fig. 3. Northern blot hybridization of total RNA slot-blot from the CHO cells transfected with PmtB/−422. Total RNA was isolated from the transfected CHO cells 3 days after transfection and 24 h after dex (100 nM), RU486 (500 nM), or dex plus RU486 treatments. About 5 μg of total RNA per sample was applied to the slot-blot membrane. Control is the total RNA from untransfected plain CHO cells. The probe (B-luc) was the ^{32}P-labeled antisense RNA covering part of luciferase coding sequence and exon 1B sequence of A_1 receptor gene from plasmid PmtB/−422. The bands on X-ray film were densitometer scanned to estimate the amount of mRNA in each sample. This experiment was repeated at least three times and the estimated average increase of signal due to dex treatment is about 45%.

Discussion

The expression of the human A_1AR gene is controlled by two separate promoters and the transcripts they produce differ only by the first nontranslated exons. When promoter A is active, promoter B and exon 1B are part of intron 1A and are spliced out after transcription. In addition, the transcriptional activity of promoter A is much higher than that of promoter B when they were tested separately (Ren and Stiles, 1995) and the reason for that is not clear. Because the sequences surrounding the two promoters are quite different, their regulation patterns may also be different. For example, a purine-rich sequence at the transcription start site of promoter A is very important for the promoter activity and binds to nuclear protein(s) preferentially in single-stranded form (Ren and Stiles, 1998). This sequence is not present in promoter B. Instead an AP1 site next to the transcription start site of promoter B and an SRE at −69/−62 are important for the promoter B activity. Moreover, both promoters A and B have a nuclear factor κB (NFκB) site located upstream of the promoters. The NFκB site upstream of promoter A is involved in the stress-related stimulation of A_1AR expression, whereas the NFκB site upstream of promoter B has no effect (Nie et al., 1998).

Because glucocorticoid (dex) stimulation of A_1AR expression has been reported in DDT_1 MF-2 smooth muscle cells (Gerwins and Fredholm, 1991) and rat brain (Svenningsson and Fredholm, 1997), it was important to learn how the human A_1AR gene responds to dex treatment at the molecular level. A computer search of the 5′ flanking sequences for both promoters revealed only one GR monomer-binding site between promoters A and B. Although the homodimer of GR seems to be the most efficient binding format for GR function (Chalepakis et al., 1990), the GR monomer binding can also affect gene expression. Our results show that although the deletion of a sequence segment including the GRE at −379/−372 of promoter B reduced the level of dex stimulation more than the base substitution mutation at GRE (Fig. 4), this GRE monomer-binding site does not contribute a significant amount of GR-regulated activity increase. Since a major part of the dex stimulation was still present after GRE mutation or deletion, other transcription factors and/or sites may interact with GR without GRE involvement. This type of protein-protein interaction has played a major role in hormone-mediated transcriptional regulation involving nuclear

receptor transcription factors including GR (McEwan et al., 1997). A primary target of GR interaction in promoter B is the AP1 site, an interaction that has been reported in many studies (Diamond et al., 1990; Jonat et al., 1990; Garlow and Ciaranello, 1995; Teurich and Angel, 1995). When the AP1 site alone was mutated (PmtB/AP1 MUT in Fig. 4B), a small percentage of reduction of dex stimulation was observed after a drop of basal activity (Fig. 4B). This result may indicate a partial role for the AP1 site in the GR-mediated increase in transcriptional activity. When both the TATA box and AP1 site, which are in close proximity and are the major regulatory components of promoter B, were mutated (PmtBMUT in Fig. 4B), dex stimulation was further reduced with a similar level of basal activity as that of PmtB/AP1 MUT. This result may suggest the interaction of GR with the general transcription initiation complex associated with the TATA box. In human osteocalcin gene promoter, the GR-binding sequence and TATA box sequence overlap. Thus, the competitive binding of GR at the TATA box caused negative regulation of expression (Meyer et al., 1997). The interaction between GR and TATA box in the human A_1AR promoter B could be protein-to-protein because there is no GR-binding site involved.

The results from deletion mutants (PmtB/−129 and PmtB/−39) in Fig. 4A indicate that the sequence between −39 and −29 of promoter B caused a reduction of dex stimulation. Within this area, there are two serum response elements SRE-1 (−113/−106) and SRE-2 (−69/−62). When they were individually mutated to test which one caused the reduction of dex stimulation, mutations in SRE-1 resulted in a slight increase of basal activity but little change in dex-mediated stimulation (Fig. 4B), whereas the same sequence mutation in SRE-2 caused a complete elimination of basal activity of promoter B (data not shown). This SRE site is important to the basal activity of promoter B as we indicated in our previous study (Ren and Stiles, 1995). To study the effect of dex

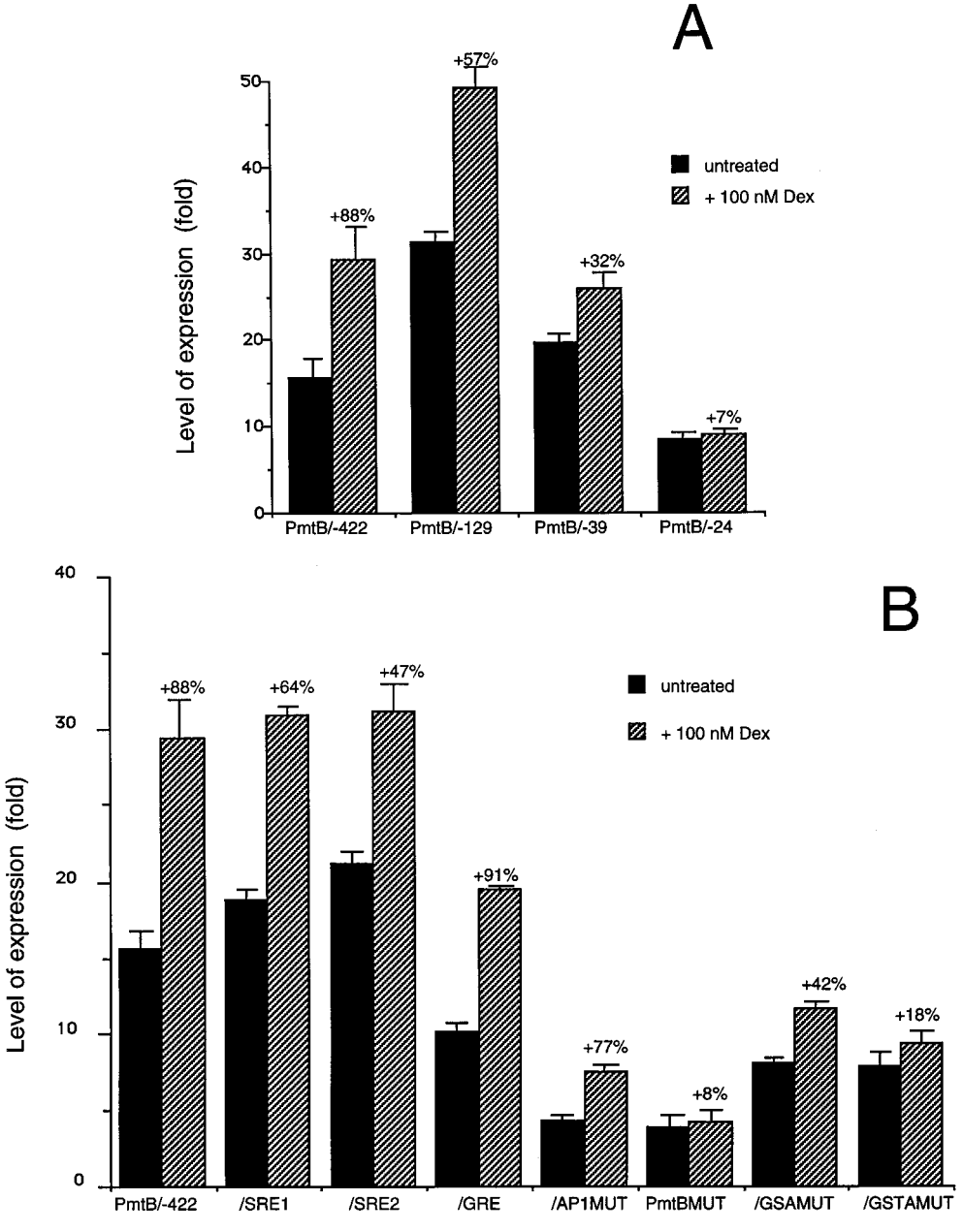

Fig. 4. Luciferase activity from mutant plasmids transfected cells compared with that from the wild-type plasmid PmtB/−422 transfected cells with or without dex treatment. The luciferase activities between samples with or without dex treatment from 5′ deletion mutants (PmtB/−129,/−39,/−24) (A) and base substitution mutants of promoter B (PmtBMUT, PmtB/GRE,/SRE1,/SRE2,/AP1 MUT,/GSAMUT, and/GSTAMUT) (B) were compared with the activity in wild-type plasmid PmtB/−422 as described in *Materials and Methods*. The results are presented as fold expression over background (pBLPniF). Three or more experiments were conducted for all mutants ($n \geq 3$).

on gene expression, a reasonable level of basal activity is needed. Thus, we changed the sequence mutation in SRE-2 in such a way that only the variable positions in the consensus sequence were mutated. A mutant, PmtB/SRE2, so selected showed a close to 50% decrease in dex stimulation. Glucocorticoid regulation of gene expression through direct binding on SRE has been reported in *c-fos* oncogene promoter (Karagianni and Tsawdaroglou, 1994), in which GR binding repressed SRE-dependent gene activation. The results in our study showed that the two almost identical SREs upstream of promoter B responded to dex treatment differently. SRE-1 has little effect but SRE-2 affects both basal and dex-stimulated activity. Whether GR affects A_1AR expression through direct binding to SRE-2 or through interaction with other transcription factors such as a serum response factor is not yet known.

According to the deletion and mutation studies, the SRE-2 and TATA box seem to be responsible for most of increase in the dex-stimulated activity, whereas the GRE monomer-binding site does not have much influence. The AP1 site is important for the promoter activity and is probably involved in the dex-mediated stimulation due to its location between the transcription start site and TATA box.

Because the transcription of promoters A and B seems to be regulated by different mechanisms, the relationship between the two promoters is important to the expression of the A_1AR gene. In our previous study we have found that the transcript from promoter A is only present in certain tissues, whereas the transcript from promoter B is present in all of the tissues that express A_1AR (Ren and Stiles, 1994a). Most of our work had tested the promoters in separate plasmids linked to a reporter gene. Therefore, it would be important to learn the effect of dex on the expression of A_1AR when the "complete" gene with its natural genomic sequence including introns was expressed. The inserts of PmtAB-A_1AR and PmtB-A_1AR contain the continuous genomic sequence of the A_1AR gene except intron 2, which disrupts the coding sequence; also, the 3′ untranslated sequence was removed. The CHO cells transfected with these plasmids express A_1AR as measured by the radioligand-binding assay (Fig. 5). The results clearly demonstrated the stimulatory effect of dex on the expression of A_1AR and the involvement of GR by RU486

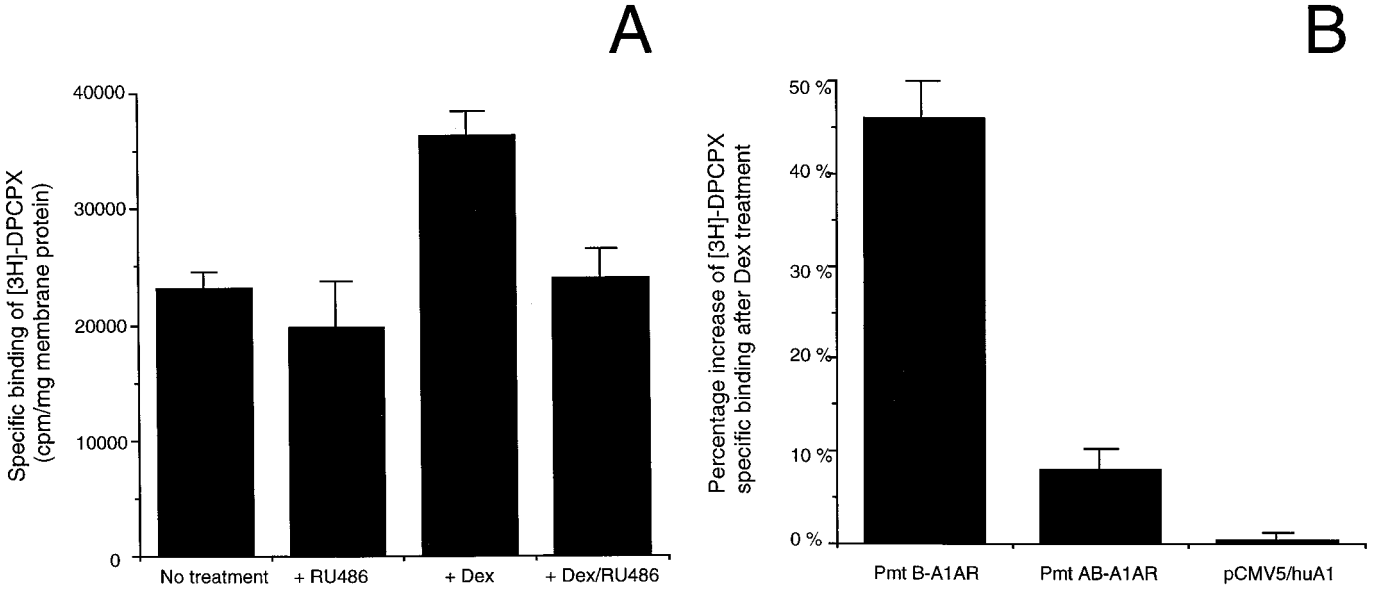

Fig. 5. Effects of dex treatment on human A_1AR expression controlled by promoter B, promoters A and B, or cytomegalovirus promoter. CHO cells were transfected with plasmids containing the genomic sequence of promoter B, promoters A and B, or cytomegalovirus promoter by lipofectamine. Dex (100 nM) and/or RU486 (500 nM) were used to treat the transfected cells 48 h after transfection in serum-free medium. Cell membranes were prepared for binding assay 24 h later as described in *Materials and Methods*. A, specific binding of [^{3}H]-DPCPX to the PmtB-A_1AR transfected CHO cells. B, the percentage of increase of specific binding activity due to dex treatment.

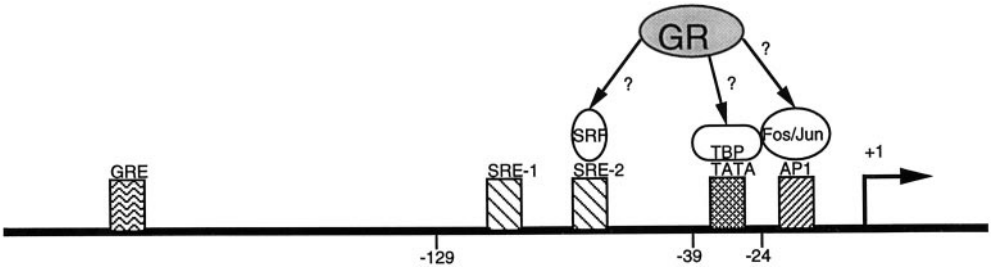

Fig. 6. Schematic display of regulatory sites in promoter B and their possible interaction with GR. Promoter B of the human A_1AR is represented by TATA box and its surrounding sequence. The +1 site is the transcription start site and the −24, −39, and −129 sites are the 5′ ends of corresponding deletion mutants. Various base substitution mutations have been made in those regulatory sites as indicated in Table 1 and the GR involvement in gene regulation was confirmed by RU486 inhibition of dex-stimulated expression.

reversal of the effect. In addition, the results also showed that dex treatment was most effective on the expression of promoter B when promoter A was not present. Therefore, in the tissues where promoter A is not active, dex treatment may be more effective.

The effect of GR regulation can be positive, such as that seen in the human A_1AR and rat serotonin-2 receptor (Garlow and Ciaranello, 1995) or negative (Goodman et al., 1996; Pei, 1996) depending on the binding sites and cell types. In addition, multiple binding sites are involved in GR-regulated expression in certain cases such as the human A_1AR promoter B and rat insulin-like growth factor binding protein-1 promoter (Suh et al., 1996).

In summary, the results of this study show that dex can stimulate the human A_1AR promoter B expression through multiple regulatory elements. The effect is mediated by GR and probably by protein-protein interaction (Fig. 6). We, of course, can not rule out that the dex also influences other genes that consequently have an effect on A_1AR expression. The expression from promoter A appears to be much less affected by the dex treatment. It appears that glucocorticoid responsiveness is complex and requires multiple DNA sites and will be tissue-specific depending on whether or not promoter A is expressed.

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