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Promoter Analysis of the Rat β_1-Adrenergic Receptor Gene Identifies Sequences Involved in Basal Expression

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SUMMARY

The β_1-adrenergic receptor (β_1-AR) mediates several functions of catecholamines in the heart, including the stimulation of heart rate and contractility. The expression of the rat β_1-AR gene was assessed by transiently transfecting chimeric genes containing the β_1-AR promoter, driving the luciferase reporter gene into various cell lines. β_1-AR/luciferase vectors containing 3 kb of the 5′-flanking region and extending to −126 relative to the start site of translation were expressed at high levels in ventricular myocytes, SK-N-MC cells, and HepG2 cells. The addition of 26 nucleotides from −125 to −100 to the −3311 β_1-AR/luciferase chimeric gene reduced expression in myocytes and SK-N-MC cells while eliminating expression in HepG2 cells. This element is located 125 base-pairs 3′ to the transcriptional start site. The mutation of four nucleotides between −121 and −118 diminished the inhibitory effect of this element. The inhibitory activity of the −125 to −100 sequence was completely dependent on promoter context and positioning. In addition to this 3′ element, sequences between −3311 and −2740 in the 5′-flanking region of the β_1-AR gene were required for the full transcriptional suppression. Using DNase I footprinting and gel mobility assays, it was determined that within the 26-bp region, rat heart nuclear proteins bound to two sites between nucleotides −123 and −112 and −106 and −100. Therefore, appropriate basal expression of the β_1-AR gene involves widely separated sequences 3′ and 5′ to the transcriptional start site.

Catecholamines exert many of their important physiological functions by interacting with membranous β-ARs. The activation of these receptors is transduced by the GTP-binding regulatory protein G_s into stimulation of adenylyl cyclase activity, which in turn increases intracellular levels of cAMP (1). β-ARs are subdivided into three pharmacologically distinct but structurally related receptors (2–5). These receptors are products of separate genes and are unusual in that the β_1-AR and β_2-AR genes are intronless, whereas the β_3-AR gene contains a very short intron (2, 4, 6). The gene for the β_2-AR has been extensively studied including identification of the TSS, sequences involved in basal expression, and hormone-responsive elements for glucocorticoids and cAMP (3, 7–9). The 5′-flanking regions for the human, nonhuman primate (rhesus macaque), rat, mouse, and ovine β_1-ARs have been cloned (10–15). Analogous to the β_2-AR, the early promoter regions of these genes are rich in GC sequences and lack a consensus TATA box. Several start sites for transcription were identified for the rat β_1-AR at ~−250 and ~−280 bp 5′ to the ATG that initiate translation (12, 16). The TSS of the human β_1-AR was located −253 relative to the ATG (17), and the TSS for the human and rat β_2-ARs also occurred ~250 bp 5′ to the ATG (3, 7). Therefore, similarities exist in the transcriptional initiation of β_1- and β_2-AR genes in humans and rodents. On the other hand, the TSS of β_1-AR genes in mouse and ovine species occurred at −415 and −660, respectively, demonstrating that there is variation among the species in the transcriptional initiation of β_1-AR genes (13, 14).

Another hallmark for the expression of the β_1-AR versus the β_2-AR genes is the tissue and developmental specificity of their expression. The β_1-AR gene is exclusively expressed in cerebral cortex, salivary glands, adipose cells, and VM, whereas the β_2-AR is present in lung, skin, skeletal muscle, and liver (18). Identification of promoter elements involved in basal and tissue-specific expression of these genes is an important avenue for characterization of this regulation. Another factor regulating the density of β_1- and β_2-ARs is their mRNA abundance in various tissues. The level of β_1-AR mRNA is inherently lower than that of β_2-AR mRNA in tissues expressing both subtypes of β-AR (2). However, in tissues containing both mRNAs, the relative receptor abun-

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ABBREVIATIONS: β_1-AR, β_1-adrenergic receptor; TSS, transcriptional start site; VM, ventricular myocytes; PEPCK, phosphoenolpyruvate carboxykinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TR, thyroid hormone receptor; SV40, simian virus.

dance is not reflected in the mRNA concentration. For example, in primary heart cultures containing 80% myocytes (β_1 expressors) and 20% nonmyocytes (β_2 expressors), the ratio of β_1-AR to β_2-AR is 5:1 (19). However, 75% of the β-AR mRNA represents the β_2 isoform, suggesting that the translational efficiencies of the β_1- and β_2-AR mRNAs may be different. Similarly, the density of β_1-AR mRNA in adipose tissue and brain is low despite the abundant expression of β_1-AR in these tissues (20). Therefore, both transcriptional and translational control mechanisms contribute to the abundance of β_1- and β_2-ARs.

In previous studies, the promoters of the rat β_1-AR and ovine β_1-AR were ligated to reporter genes and transfected into various cells lines (12, 21). With the use of these approaches, broad regions in each promoter that were involved in regulating basal transcription were identified. The goal of the current study was to identify more precisely the elements involved in appropriate basal expression of the rat β_1-AR gene. Using β_1-AR/luciferase chimeric genes in transient transfections, we identified elements responsible for the low level of β_1-AR transcription. These elements located between -3311 and -2741 and -125 and -100 function coordinately to decrease β_1-AR gene expression.

Materials and Methods

Construction of chimeric luciferase reporter plasmids. A 9.8-kb *Eco*RI genomic fragment of the rat β_1-AR encoding 3.3 kb 5′ to the initiator ATG, 1.5 kb of coding sequence, and 3.8 kb of the 3′ region was excised from pUC118 with *Eco*RI (10). Digestion of the *Eco*RI genomic fragment with *Sac*II generated a 3.2-kb fragment containing the entire 5′-flanking region of the β_1-AR gene (GenBank accession numbers D00634 and U58651). The β_1-AR *Kpn*I/*Sac*II segment extending from -1251 to -126, relative to the initiator ATG, was subcloned into pBluescript SK$^+$ and then excised with *Kpn*I and *Sac*I and cloned into pGL3*basic* (Promega, Madison, WI). pGL3*basic* is a promoterless luciferase expression plasmid containing a multiple cloning site 5′ to the luciferase insert. To generate the -1251 to -100 pGL3*basic* plasmid, a 28-bp double-stranded oligomer (GGCTGCCCTGACCTGGCCGCGACCTCGC) encoding the region from -125 to -100 in the β_1-AR promoter and flanked with *Sac*II-compatible ends was ligated into the *Sac*II site. This insertion created the sequence of the β_1-AR promoter from -1251 to -100 driving the luciferase reporter gene. The -3311 to -126 and -3311 to -100 plasmids were generated by ligating the *Eco*RI/*Kpn*I fragment extending from -3311 to -1250 5′ to the *Kpn*I site in the appropriate pGL3*basic* plasmid.

The PEPCK genomic fragment was generated by digesting a PEPCK-CAT vector with *Xho*I and *Bgl*II. The resulting 550-bp fragment contained nucleotides -490 to $+73$ of the promoter of the PEPCK gene (22, 23). The pGL2*basic* vector was digested with *Nhe*I and *Bgl*II, and the 550-bp insert was subcloned to generate the PEPCK-pGL2 plasmid. PEPCK-pGL2 was digested with *Hin*dIII, and a 32-bp double-stranded oligomer (AGCTTGGCTGCCCTGACCTGGCCGCGACCTCA, in which the underlined sequences encode the region from -125 to -100 in the β_1-AR gene flanked with *Hin*dIII-compatible ends) was ligated in front of the PEPCK sequence. Plasmids containing a single copy of the -125 to -100 sequence in either orientation were identified by sequencing. To relocate the -125 to -100 segment to another region in the β_1-AR promoter, the promoter sequences between nucleotides -1251 and -126 were excised with *Kpn*I and *Hin*dIII and subcloned into pGEM7ZF$^+$. The resulting pGEM plasmid was digested with *Xma*I at base -345 in the 5′-flanking region of the β_1-AR, and a 32-bp double-stranded oligomer encoding the region from -125 to -100 in the β_1-AR gene and flanked with *Xma*I-compatible ends was ligated. Plasmids containing a single copy of the -125 to -100 segment in either orientation were identified through sequencing. To clone the entire 5′-flanking region into these plasmids, an *Eco*RI/*Kpn*I fragment encompassing the nucleotides from -3311 to -1251 of the β_1-AR gene was ligated into the pUC-based plasmids. The entire 3.3-kb 5′-flanking region was excised from these plasmids with *Xho*I and *Hin*dIII and subcloned into pGL2*basic* to generate chimeric reporter plasmids.

To introduce mutations into the -125 to -100 region, eight pairs of 28-bp oligonucleotides containing the appropriate mutations and flanked with *Sac*II-compatible ends were synthesized. The annealed oligonucleotides were subcloned into the *Sac*II site at -126 in the [-3311, -126]pGL3*basic* and [-1251, -126]pGL3*basic* vectors. Plasmids containing a single copy of the insert in the proper orientation were identified through sequencing.

Thirteen deletion mutants of the -3311 to -1251 segment of the β_1-AR 5′-flanking region were generated through polymerase chain reaction amplification. For each mutant, a 30-bp primer encoding the desired sequence in the 5′-flanking region and flanked with an *Xho*I site and backward primer 5′-GAACTCAGAGAATGACGCTTCAGAC complementary to the sequence extending from -1203 to -1227 was used. The polymerase chain reaction product for each reaction was subcloned into the pGEM-T plasmid (Promega) and characterized through sequencing. The insert was excised from pGEM-T by *Xho*I and *Kpn*I, which cleave from the 5′ end to -1252, and subcloned into the [-1251, -100]pGL3*basic* plasmid. All constructs were verified through dideoxy sequencing and restriction digests. The nomenclature of the β_1-AR constructs is [5′ end, 3′ end] to indicate the 5′ and 3′ boundaries of a DNA. The numbers within parentheses reveal the localization of each segment relative to the start site of translation of the β_1-AR (12).

Cell transfections and luciferase assays. Ventricles were excised from 1–3-day-old rats, and VM were prepared and cultured as previously described (19). HepG2 and H4IIE cells were cultured in 3 ml of Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, whereas SK-N-MC cells were cultured in medium containing 10% fetal bovine serum. Plasmids were introduced into these cells by calcium phosphate precipitation (22). HepG2 and H4IIE cells were seeded at a density of 4×10^5 cells/60-mm dish, whereas VM and SK-N-MC cells were seeded at a density of 1.5×10^6 cells/60-mm dish. After 24–48 hr, each dish was transfected with 10 μg of plasmid DNA composed of 5 μg of the smallest vector (pGL3*basic*), 3 μg of carrier pGEM7ZF$^+$ DNA, and 2 μg of SV40/β-galactosidase (pSV-βgal; Promega) as a transfection control in a total volume of 1 ml. In all transfections, the amount of each β_1-AR/luciferase construct was increased to an equivalent molar ratio of pGL3*basic,* and the balance of the DNA was adjusted to 8 μg with pGEM7ZF$^+$. Cells were exposed to the calcium phosphate precipitates for 16–20 hr, washed twice with phosphate-buffered saline, and recultured for an additional 48 hr. Cells were harvested in 250 μl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, and 1% Triton X-100), and the lysates were clarified by centrifugation. Luciferase assays were performed using 20 μl of lysate and 100 μl of luciferase assay reagent (Promega), which were injected automatically into a Turner-20 luminometer. β-Galactosidase assays were performed using 150 μl of lysate and the *o*-nitrophenyl-β-D galactopyranoside substrate as previously described (24). Protein was measured in 5 or 10 μl of extract using a detergent-compatible protein assay (BioRad, Hercules, CA).

In all experiments, the appropriate pGL*control* vector, which is a luciferase vector driven by the SV40 promoter and enhancer sequences, was transfected in equimolar amounts to the promoterless pGL*basic* vector. For each batch of cell lysates, a standard curve was generated by measuring the luminescence generated by 5–5000 fg of firefly luciferase. The luminescence of each sample was converted to pg of firefly luciferase/mg of protein using the standard curve that was generated for each batch of independent measurements (25).

These data provide a measure for the absolute luciferase activity of each construct in each cell type to compare the relative activities of the constructs among the different cell lines. Moreover, for each cell type, the luminescence of each construct was expressed as a percentage of the expression of pGL*control* after normalization for pSV-βgal (26). Each construct was transfected into three 60-mm dishes, and these transfections were replicated for each cell type in a minimum of three separate experiments (nine experiments). The values from all experiments were combined and subjected to analysis of variance with the use of Microsoft Excel. Significance was determined by Student's t test ($p = 0.05$).

Primer extension analysis. An antisense oligonucleotide denoted GLprimer2 (Promega), which is 5′-CTTTATGTTTTTGGCGTCTTCCA-3′ and corresponds to positions +111 to +89 relative to the multiple cloning site in pGL3*basic* (+2 and +24 in the luciferase gene), was 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. RNA was prepared from VM that were transfected with chimeric β_1-AR/luciferase vectors (19). Ten micrograms of RNA and labeled primer was denatured for 10 min at 80° and then annealed for 45 min at 45°. The extension reaction was conducted at 42° using AMV Reverse Transcriptase for 20 min (Boehringer-Mannheim, Indianapolis, IN). The extension reaction was terminated by the addition of 5 μg of salmon sperm DNA and heat denaturation at 75° for 10 min followed by the addition of DNase-free RNase for 10 min at 37° (Boehringer-Mannheim). The extension reactions were treated with phenol/chloroform (1:1) and then ethanol-precipitated. The extension products (~300 bp) were denatured and analyzed on an 8% acrylamide sequencing gel containing 7 M urea. No extension products were observed on RNA extracted from VM that were not transfected with β_1-AR/luciferase vectors.

Preparation of rat heart nuclear extract. Left and right ventricles from 20 rats (21–24 days old) were cut into small pieces and suspended in 12 volumes of homogenization buffer composed of 10 mM HEPES, pH 7.6, 25 mM KCl, 2 M sucrose, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride (27). The suspension was homogenized for 12 sec in a Brinkman Polytron (Brinkman Instruments, Westbury, NY) at setting 5 and homogenized further with six strokes using a Teflon/glass homogenizer. The homogenate was layered over a 10-ml cushion of homogenization buffer and centrifuged at 24,000 rpm for 30 min at 0° in an SW 27 rotor. The nuclear pellet at the bottom of each tube was resuspended in 2 volumes of nuclear lysis buffer composed of 10 mM HEPES, pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride and lysed by 20 strokes in a Teflon/glass homogenizer. The nuclear suspension was diluted to ~10 A_{260} units/ml of nuclear lysis buffer, and a 0.1 volume of 4 M ammonium sulfate was added dropwise. The suspension was mixed by inversion at 4° for 30 min, and the viscous lysate was centrifuged at 50,000 rpm for 60 min in a Ti80 rotor at 0° to pellet chromatin. To the supernatant, 0.3 g/ml of solid ammonium sulfate was added, and the solution was mixed by inversion at 4° for 20 min. The suspension was centrifuged at 10,000 rpm in a swinging bucket JS-13 rotor for 40 min at 4°. The protein pellet was dissolved in 1 ml of dialysis buffer (25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride) and dialyzed twice at 4° for 1.5 hr each time against 500 volumes of dialysis buffer. The nuclear extract was centrifuged at 14,000 rpm in a microfuge at 4°, and the supernatant was stored in small aliquots in liquid nitrogen. Rat liver nuclear extract was prepared according to the method of Gorski *et al.* (27).

Gel mobility assays. Double-stranded oligomers containing a 4-bp overhang were labeled with Klenow enzyme and [α-^{32}P]dCTP (28). The binding reactions were performed at 0° for 20 min in a binding buffer composed of 80 mM KCl, 10 mM HEPES, pH 7.1, and 10% glycerol. Each binding mixture contained 1 μg of poly(dI-dC) as nonspecific competitor and proteins as indicated. The resulting complexes were resolved on 5% nondenaturing acrylamide gels in 25 mM Tris and 190 mM glycine at 4° (29). The oligomer derived from the β_1-AR sequence between −125 and −100 was CTAGAGGCTGCCCTGACCTGGCCGCGACCTCT (the underlined oligonucleotides represent those sequences in the β_1-AR gene). The relative concentration of double-stranded oligomers of the wild-type sequence between −125 and −100 and its mutants was determined by labeling 1 μl of oligomers annealed by Klenow enzyme and [α-^{32}P]dCTP (28). Because 5′ to 3′ extension by Klenow polymerase occurs only on double-stranded oligonucleotides, the relative incorporation of the label into each oligomer indices the relative concentration of double-stranded DNA among the different samples.

DNase footprinting assays. The *Sac*I genomic fragment encompassing nucleotides −3220 to +397 of the β_1-AR promoter was digested with *Pst*I, and the 753-bp *Pst*I fragment extending from −484 to +268 was subcloned into the *Pst*I site in the multiple cloning region of pGEM3ZF$^+$. The 753-bp *Pst*I fragment was digested with *Nar*I, and a 184-bp segment extending from −158 to +27 was cloned into the *Acc*I site of pGEM3ZF$^+$ plasmid. The *Nar*I fragment containing plasmid was digested with *Hind*III, dephosphorylated, and labeled with γ-^{32}P-ATP and T4 polynucleotide kinase (28). The insert was released from the plasmid with *Bam*HI and purified by electrophoresis on 1% agarose gels. The ^{32}P-labeled DNA fragment (20,000 cpm) was incubated in the absence or presence of nuclear extract (5–10 μg) and 1 μg of poly(dI-dC) in a binding buffer composed of 20 mM HEPES, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 50 mM NaCl for 30 min at 0° (30). Digestion with 0.03–0.1 unit of DNase I was allowed to proceed for 45 sec, followed by the addition of 150 mM NaCl, 0.7% sodium dodecyl sulfate, 15 mM EDTA, and 30 μg of yeast tRNA. The samples were extracted once with phenol-chloroform and ethanol-precipitated. Samples were resuspended in electrophoresis buffer and subjected to electrophoresis on 6% acrylamide in 8 M urea gels (30). The protected DNA sequences were identified from the autoradiogram of the gel. The sequence of the protected DNA was determined by running a separate lane containing a G sequence ladder generated according to the piperidine cleavage method of Maxam and Gilbert (31).

Results

Transfection analysis identifies an element important for proper expression of the β_1-AR gene. Our initial experiments were designed to delineate regions in the β_1-AR promoter that are involved in regulating basal expression of the gene. The β_1-AR 5′-flanking region contains three unique restriction sites: *Sac*II site at −126 relative to the ATG initiating translation, *Kpn*I site at −1251, and *Eco*RI site at −3311 (4). The *Kpn*I/*Sac*II and *Eco*RI/*Sac*II fragments were linked to the reporter gene for firefly luciferase. Transient transfections were carried out in four cell lines from different tissues to assess absolute levels of expression as well as tissue-specific expression (Fig. 1). VM were prepared from ventricles of newborn rats by Percoll density gradient centrifugation and were composed of >90% myocytes that express β_1-AR (19). SK-N-MC cells are human neuroblastoma cells that express β_1- and β_3-ARs (32). HepG2 human hepatoma and H4IIE rat hepatoma cells are of liver origin and express β_2-AR (2, 33). Sequences extending from −1251 to −126 in the β_1-AR promoter were ligated into the promoterless pGL3*basic* vector and cotransfected with the pSV-βgal vector into these cells. The relative amount of luciferase activity of the −1251 to −126 construct after correction for the transfection efficiency using β-galactosidase was 52 ± 11 pg/mg of protein in HepG2 cells, 41 ± 9 pg/mg in VM, and 31 ± 10 pg/mg in SK-N-MC cells. Furthermore, the activity of this vector was ~40% of that of the SV40-driven pGL3*control*

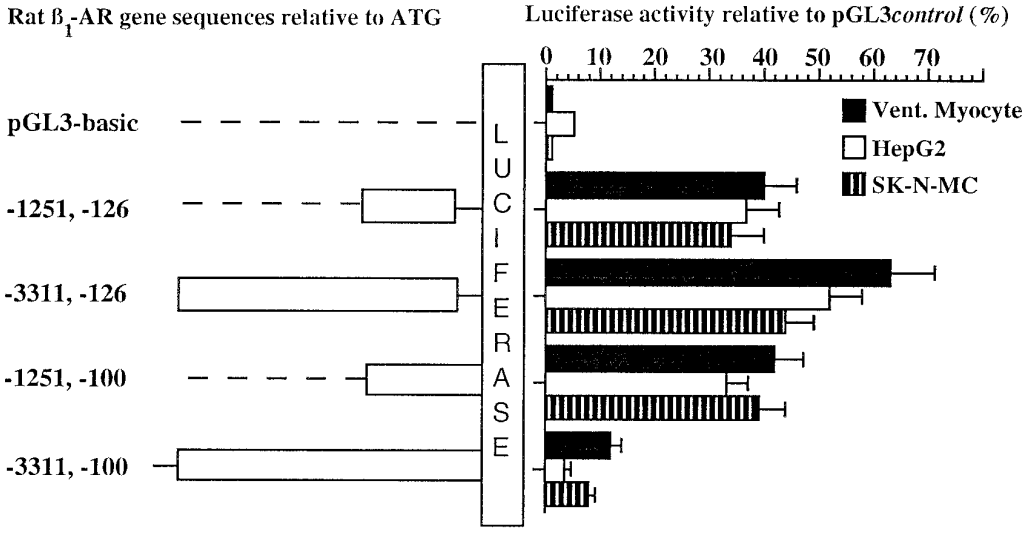

Fig. 1. Expression analysis of the rat β_1-AR gene in mammalian cells. *Left,* sequences in the 5′-flanking region of the β_1-AR gene that were ligated into the promoterless pGL3*basic* vector. VM (*Vent. Myocyte*), HepG2 cells, and SK-N-MC cells were transiently transfected with 5 μg of equivalent of pGL3*basic* vector and 2 μg of SV40-βgal as described in Materials and Methods. Cells were harvested at 48 hr after transfection and assayed for luciferase activity. Luciferase activity was corrected for β-galactosidase to account for variations in transfection efficiency. *Right,* expression of the chimeric β_1-AR/luciferase constructs relative to the expression of the SV40-driven luciferase plasmid pGL3*control*. Values are presented as mean ± standard error of three to six transfections. All transfections were performed in triplicate.

plasmid, in which the luciferase reporter gene is driven by the SV40 promoter/enhancer unit. The −1251 to −126 construct was expressed at high levels, which does not reflect the expected transcriptional activity for the β_1-AR gene.

To determine whether regulatory sequences were contained in the more 5′ flanking region of the promoter, an additional 2 kb of the 5′-flanking sequence was added to the −1251 to −126 vector. The activity of the resulting −3311 to −126 construct was increased in all the cell types under study (Fig. 1). Therefore, we directed our efforts to incorporating more of the 3′-flanking region. Two additional constructs, extending from −1251 to −100 and −3311 to −100, were tested. The shorter form of the promoter was abundantly active in all cell types. The activity of the −3311 to −100 vector was absent in HepG2 cells because its expression was less than that of the promoterless pGL3*basic* plasmid (Fig. 1). The activity of this construct in VM and SK-N-MC cells was <10% of the activity of pGL3*control* vector. This level of expression is within the anticipated parameters for the activity of this gene on the basis of its mRNA levels in VM and SK-N-MC cells. The absolute levels of expression of the −3311 to −100 construct in VM and SK-N-MC cells were 2.5 ± 0.3 and 1.4 ± 0.2 pg/mg, respectively. Primer extension analysis of RNA extracted from VM revealed proper transcriptional initiation from the four constructs that were analyzed in Fig. 1 (data not shown).

To test whether the −125 to −100 element could suppress transcription in a rat cell line that does not express β_1-AR, the five constructs shown in Fig. 1 were transfected into a rat H4IIE hepatoma cells. In H4IIE cells, the −1251 to −126 and −1251 to −100 vectors were expressed at approximately 3% of the pGL3*control* luciferase vector. The −3311 to −126 vector was also expressed at 3% of the pGL3*control* vector. In this cell line, the −3311 to −100 vector containing the −125 to −100 element was not expressed. Therefore, the −125 to −100 element also suppresses transcription completely in a rat non-β_1-AR-expressing cell line. These transfections were repeated three times (nine experiments). It is impossible to say whether the lower expression of the −1251 to −126 vector relative to pGL3*control* reflects low expression of the β_1-AR or more efficient expression of the SV40 promoter/enhancer of pGL3 in H4IIE cells. Therefore, the region extending from −125 to −100 contains a suppressing domain. However, this domain exerts its effect only in the context of the 3-kb β_1-AR promoter, suggesting coordinate regulation of 5′ and 3′ elements in the promoter. Transcription was not only reduced in β_1-AR-expressing cells but also extinguished in the nonexpressing rat and human hepatoma cell lines, suggesting that these sequences may contribute to the tissue-specific expression of the β_1-AR gene.

Characterization of the activity of the nucleotides between −125 and −100 in the β_1-AR promoter. We examined whether the region extending from −125 to −100 acts in either an orientation-specific or a position-specific manner to suppress transcription (Fig. 2A). When placed in its normal position in the promoter extending to −3311, the sequence from −125 to −100 inhibited the expression of the β_1-AR gene in both orientations. Relocation of this element from −126 into position −345 in the 5′-flanking region of the β_1-AR restored full activity to the promoter, indicating that appropriate localization is important for the function of this element. Next, we tested whether the −125 to −100 sequence can inhibit the expression of a gene that uses a different set of regulatory proteins or factors (Fig. 2B). The PEPCK gene has a TATA box and is regulated by a variety of transcription factors (22, 23). The activity of the PEPCK-pGL2 construct in HepG2 cells was 38 pg/mg of protein. Insertion of the −125 to −100 sequence of the β_1-AR in front of the PEPCK 5′-flanking region had no effect on PEPCK expression, indicating that inhibition by this element may be specific for the β_1-AR promoter.

The next series of experiments were designed to identify which nucleotides within the −125 to −100 region participate in the inhibitory effect. The sequence of the nucleotides

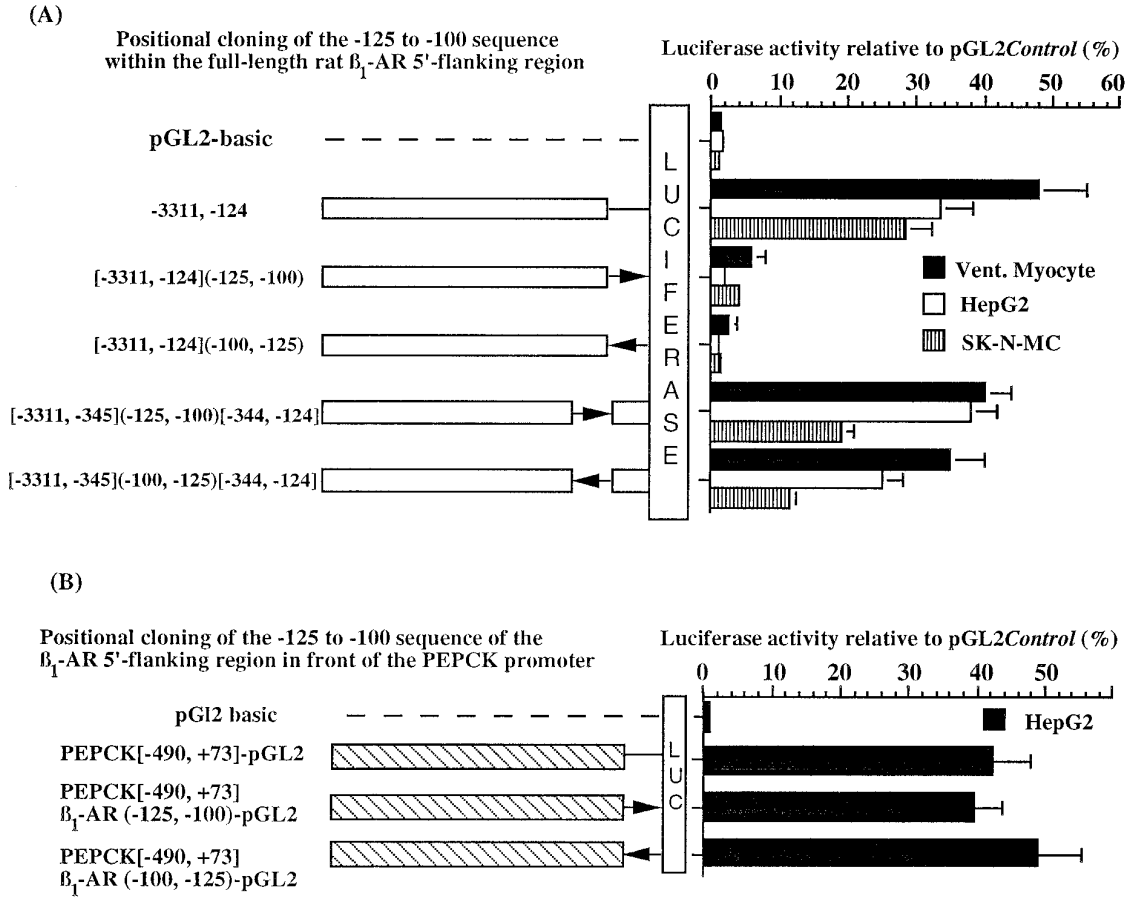

Fig. 2. Expression analysis of the nucleotides between −125 and −100 in regulation of β_1-AR and PEPCK gene expression. *Left,* sequences in the 5′-flanking region of the β_1-AR and PEPCK genes. *Arrow direction*, orientation of the nucleotides between −125 and −100 in the promoter (i.e., ⇆, 5′ to 3′ orientation; ⇄, 3′ to 5′ orientation). *Right,* expression of the β_1-AR and PEPCK constructs relative to the SV40-driven pGL2*control* vector. A, Expression of the β_1-AR constructs relative to pGL2*control* in VM (*Vent. Myocyte*), HepG2 cells, and SK-N-MC cells was determined as described in the legend of Fig. 1 and in Materials and Methods. B, Expression of the −490 to + 73 sequence of the PEPCK promoter in the absence or presence of the nucleotides between −125 and −100 in the β_1-AR 5′-flanking region in HepG2 cells was determined. The activities of the pGL2*control* vectors in VM, HepG2 cells, and SK-N-MC cells were 31 ± 5, 39 ± 6, and 19 ± 4 pg/mg, respectively. Values are presented as mean ± standard error of three transfections. All transfections were performed in triplicate.

between −125 and −100 is outlined in Fig. 3a. A series of 4-bp mutations was introduced across this region in the context of the −3311 to −100 β_1-AR promoter (Fig. 3b). These mutant promoters were ligated to the luciferase gene and transfected into the three cell types used in Fig. 1. Mutation of the sequences between −121 and −118 (domain D) almost restored full promoter activity, whereas mutations of domains C and B between −116 and −113 and −111 and −108, respectively, had no effect on basal activity. Mutation of domain A between nucleotides −105 and −102 caused a modest increase in the activity of the promoter compared with the 5-fold increase that was generated by the −121 to −118 mutation in domain D. The double mutants (in which domains C and A or domains B and A were mutated) and the triple mutant (in domains C, B, and A) were less active than the single mutant in domain A.

To determine whether mutations in the −125 to −100 region could affect transcription from the truncated promoter, the wild-type or mutated sequences in the −125 to −100 region were ligated in-frame in front of the β_1-AR promoter encompassing the nucleotides between −1251 and −100 (Table 1). The constructs without or with the wild-type sequence between −125 and −100 were equally as active as those containing the mutated sequences. These data confirm the observation that the inhibitory activity of the −125 to −100 domain is dependent on the sequences from −3311 to −1251 in the 5′-flanking region.

Transfection analysis of the progressively truncated 5′-flanking region of the β_1-AR gene. To further characterize the interplay between the −3311 to −1251 region and the −125 to −100 domain in inhibition of the expression of the β_1-AR promoter, a series of 13 progressive truncations in the promoter between −3311 and −1251 were generated. These fragments were ligated to the β_1-AR promoter extending from the *Kpn*I site at −1251 to −100 and tested for luciferase activity in transient transfection assays in VM and HepG2 cells (Fig. 4). Truncation of the β_1-AR 5′-flanking region from −3311 to −3009 slightly increased the expression in VM. The activity of the β_1-AR promoter in HepG2 cells also increased modestly with each progressive truncation. Deletion of the bases between −2870 and −2740 significantly increased the expression of luciferase in VM and HepG2 cells. Excision of these 130 bases increased the expression by 3-fold in both cell types and boosted the expression to ~50% of pGL3*control*. Expression increased by an additional 2-fold in VM when the bases between −2740 and

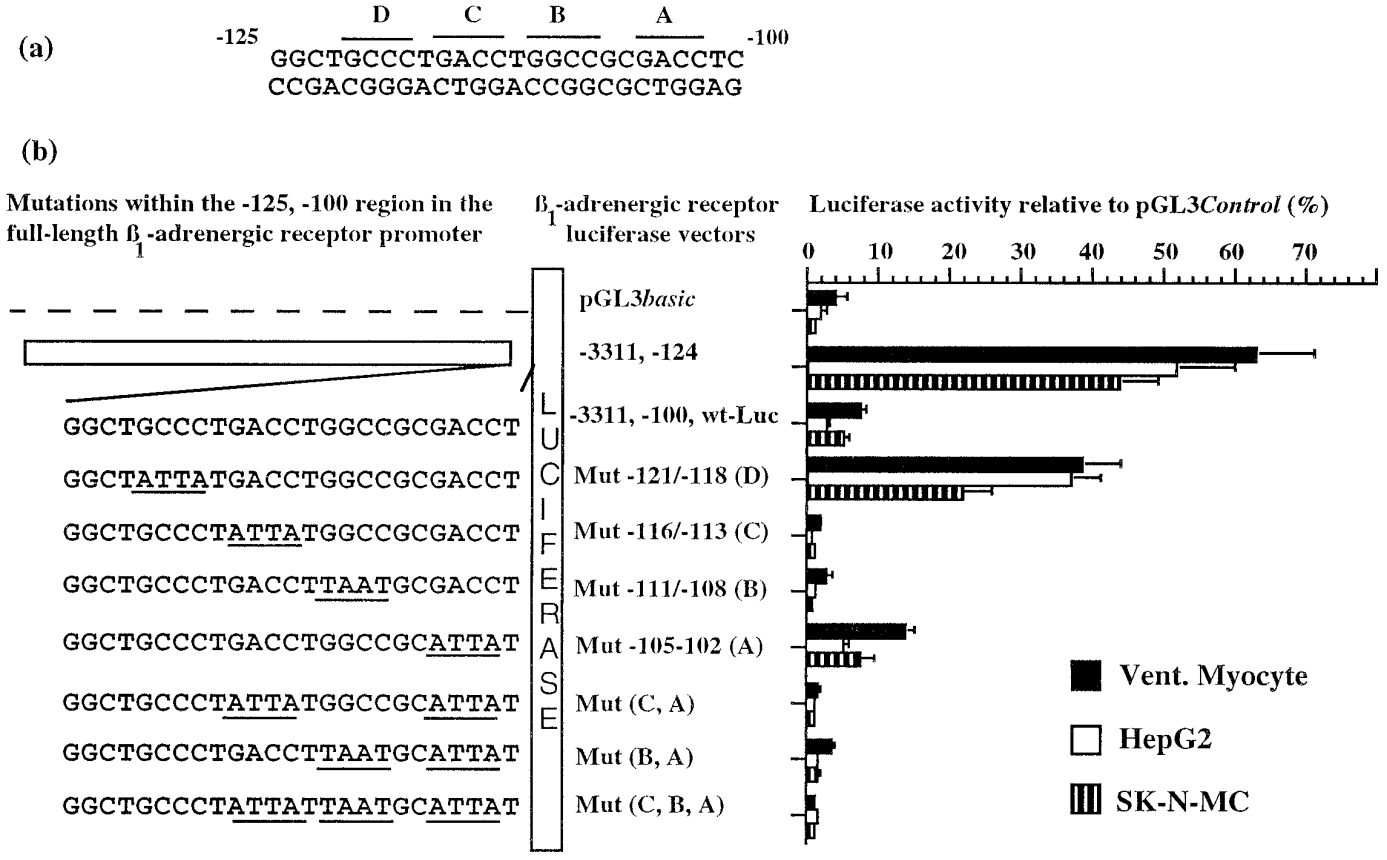

Fig. 3. Identification of nucleotides between −125 and −100 that are involved in regulating β_1-AR expression. a, Sequence of the nucleotides between −125 and −100 in the 5′-flanking region of the rat β_1-AR gene. *Underlined*, position of each 4-bp mutation. b, *left,* sequences that were ligated in front of the −3311 to −126 cassette of the β_1-AR gene. These sequences were subcloned into the luciferase expression vector and transfected into VM (*Vent. Myocyte*), HepG2 cells, and SK-N-MC cells. *Right,* expression of the chimeric β_1-AR/luciferase constructs relative to the expression of the SV40-driven luciferase plasmid pGL3*control*. Values are presented as mean ± standard error of three transfections. All transfections were performed in triplicate.

TABLE 1

Effect of mutating the −125 to −100 sequence on the expression of −1251 to −126 fragment of the β_1-AR promoter

The sequences between the nucleotides −1251 and −126 of the β_1-AR 5′-flanking region were cloned into the promoterless pGL3*basic* luciferase vector. The plasmid was cleaved at the *Sac*II site, and the wild-type nucleotides between −126 and −100 (wt −125, −100) or the −125 to −100 sequence containing the base-pair changes outlined in Fig. 3 (*Mut*) were ligated into the vector. HepG2 and SK-N-MC cells were transiently transfected with 5 μg equivalent of pGL3*basic* vector and 2 μg of SV40-*βgal* as described in Experimental Procedures. The activity of the SV-40 luciferase plasmid pGL3*control* in HepG2 and SK-N-MC cells was 150 ± 20 and 60 ± 9 pg/mg, respectively. The data represent the percentage expression of the β_1-AR construct relative to the expression of the pGL3*control* plasmid. Values are mean ± standard error for the combined results of two transfections. Each transfection was performed in triplicate.

Vector	Relative luciferase activity	
	HepG2	SK-N-MC
	%	
pGL3*basic*	1 ± 0.2	0.2 ± 0.1
−1251, −126	38 ± 6	45 ± 7
−1251, −100	51 ± 9	54 ± 8
−1251, −100–[Mut −121/−118] (D)	24 ± 4	17 ± 4
−1251, −100–[Mut −116/−113](C)	38 ± 5	43 ± 6
−1251, −100–[Mut −111/−108](B)	100 ± 11	105 ± 15
−1251, −100–[Mut −105/−102](A)	30 ± 5	28 ± 4
−1251, −100–[Mut −116/−113, −105/−102](C, A)	50 ± 9	61 ± 8
−1251, −100–[Mut −111/−102](B, A)	54 ± 5	92 ± 10
−1251, −100–[Mut −116/−102](C, B, A)	40 ± 6	38 ± 4

−2109 were excised. The activity of the β_1-AR promoter extending from −2109 to −100 in VM and HepG2 cells was 120% and 70%, respectively, relative to the SV40 luciferase vector. Continued deletion from −2109 to −1969 reduced the expression to ~30% in both cell types, suggesting the presence of a stimulatory element or elements in this region. The data indicate that the 5′-flanking region between −3311 and −1251 contained negative and positive regulatory elements that exert profound effects on β_1-AR expression.

DNase I footprinting analysis of the early promoter region of the β_1-AR gene. The next experiments were designed to determine whether nuclear proteins could bind to

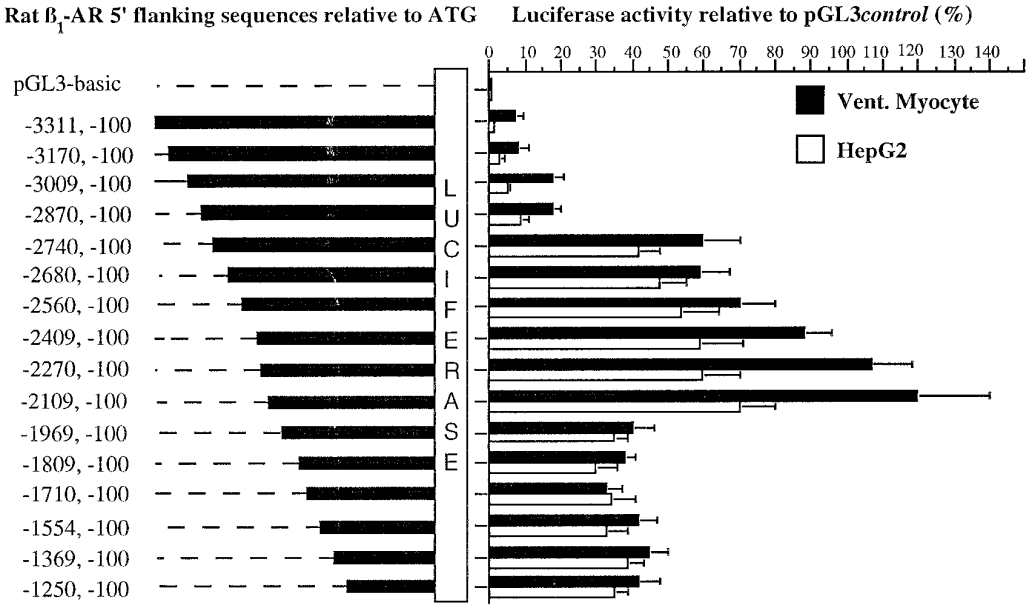

Fig. 4. Expression analysis of progressively truncated 5′-flanking regions of the β_1-AR gene. *Left,* rat β_1-AR 5′-flanking sequences that were subcloned into the pGL3*basic* luciferase expression vector and transfected into VM (*Vent. Myocyte*) and HepG2 cells. *Right,* expression of the chimeric β_1-AR/luciferase constructs relative to the expression of the pGL3*control* plasmid. Values are presented as mean ± standard error of three or four separate transfections. All transfections were performed in triplicate.

any sites in the early promoter region of the β_1-AR gene. A 184-bp *Nar*I fragment containing the sequences between −158 and +27 was labeled on the top strand, and the binding of rat heart nuclear proteins to this fragment was analyzed by DNase I footprinting (Fig. 5). Protected footprints were localized to the binding regions between −123 and −112 and between −106 and −100 (Fig. 5). These results indicate that the inhibitory domain identified by transient transfections can bind proteins in rat heart nuclei and suggest that two nuclear factors might be binding to this region. No other protected regions were identified on this strand. Domains D and C are continuously protected in the DNase I footprint. Domain A is also protected in the footprint, while the nucleotides in domain B demonstrate no ability to interact with nuclear proteins.

Binding of rat heart nuclear proteins to the −125 to −100 sequence of the β_1-AR gene. Gel mobility assays were used to further characterize the interaction of rat heart nuclear proteins with the sequences between −125 and −100 (Fig. 6). To determine which nucleotides in the oligomer were required for binding of rat heart nuclear proteins, the wild-type oligomer as well as the oligomers containing the 4-bp mutations described in Fig. 3 were labeled and tested for their ability to bind nuclear proteins. As is shown in Fig. 6A, rat heart nuclear proteins formed two complexes, with the labeled wild-type oligomer containing the nucleotides between −125 and −100 (*lane 2*). The oligomer with a mutation in site D lost the ability to bind the more slowly migrating complex (*lane 3*). With the mutant C oligomer, a new complex was formed that migrated more quickly than the largest complex formed with the wild-type probe (*lane 4*). With mutant A, the more quickly migrating complex formed more strongly (*lane 5*). With the mutant B oligomer, the lower complex seemed to be migrating a little more quickly than the comparable band with the wild-type probe (*lane 6*). This observation suggests that these nucleotides might also contribute to the binding of nuclear proteins even though they are not protected in the DNase I footprinting assay. The data from the gel mobility assays confirmed the results found in the footprint assay: that at least two proteins can bind to this element. The binding of rat liver and rat heart nuclear extract was also compared (Fig. 6B). The liver nuclear extract forms only one complex with the wild-type oligomer (lane 8). Whether this protein represents the same or a different protein from the one in heart nuclear extract cannot be determined at this time.

Discussion

In this report, we present detailed analysis of elements that mediate the basal expression of the rat β_1-AR gene. The β_1-AR gene is expressed in a limited number of tissues and cell lines, including VM, rat C6 glioma cells, and SK-N-MC cells. Measurement of β_1-AR mRNA by DNA-excess solution hybridization in VM and rat glioma C6 cells revealed that β_1-AR mRNA is present at very low levels (19, 34). Our results indicate that basal expression of the β_1-AR gene is mediated by the interaction of two widely separated inhibitory domains. These elements are located ~−120 bp and ~−2800 bp 5′ of the translational start site. Our data suggest that this unusual promoter architecture is responsible for the low basal activity of the β_1-AR gene and may contribute to tissue-specific expression of the β_1-AR.

A previous study by Searles *et al.* (12) indicated that the transcriptional activity of the rat β_1-AR promoter extending from −3354 to −1 in rat C6 glioma cells was ~30% of the thymidine kinase promoter activity. Deletion of the 5′-flanking sequences from −3354 to −1064 increased the activity of these constructs to 70% of the thymidine kinase promoter. Activity of the promoter remained high with additional 5′ deletions of the promoter. In our studies, expression of the 3-kb rat β_1-AR promoter in VM and SK-N-MC cells was also quite low: ~10% of that of the more active SV40 promoter/enhancer construct. Therefore, our data and those of Searles *et al.* (12) are in agreement and indicate that the transcription from the 3-kb β_1-AR rat promoter is quite low. The ovine β_1-AR promoter has been used in transient transfections into a variety of cell lines (21). In this promoter, a vector containing −2333 bp of 5′ flanking sequence driving the CAT reporter gene was expressed a low levels, whereas a CAT vector

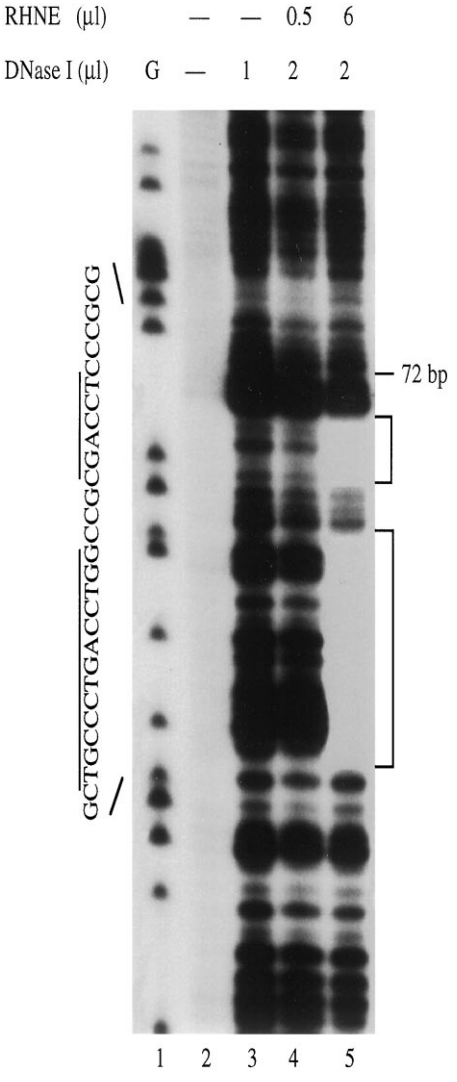

Fig. 5. DNase I footprinting of 5′-flanking regions in the β_1-AR promoter. To perform DNase I footprinting, the −158 to +27 fragment was cloned into the *Acc*I site in the polylinker of pGEM3ZF$^+$. The polylinker was cleaved at the *Hind*III site, dephosphorylated, labeled with T4 polynucleotide kinase, and then cleaved at the *Eco*RI site. The labeled fragment was isolated by gel electrophoresis and incubated (20,000 cpm) without (*lanes 2* and *3*) or with (*lanes 4* and *5*) 0.5 or 6 μg of rat heart nuclear extract (*RHNE*) protein, respectively, followed by DNase I digestion with 15 munits (*lane 3*) or 30 munits (*lanes 4* and *5*) as described in Materials and Methods. The localization of the DNase I footprint (*lane 5*) was determined using Maxam-Gilbert G ladders (*lane 1*). The migration of the 72-bp DNA molecular weight standard is indicated in the gel .

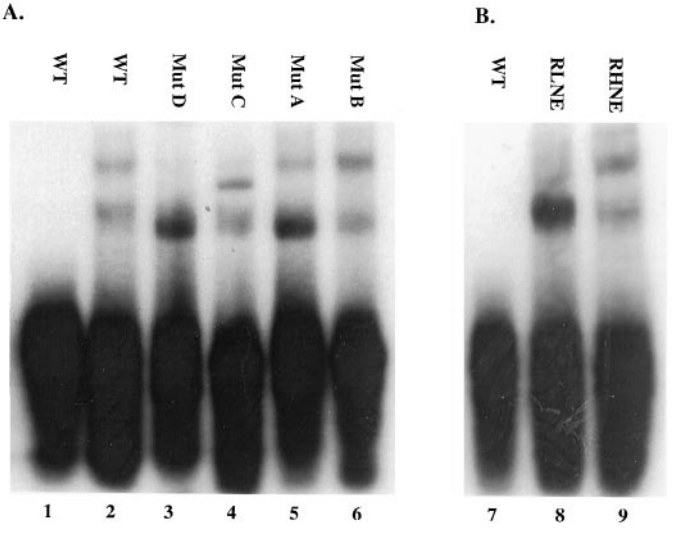

Fig. 6. Binding of nuclear proteins to the −125 to −100 element of the β_1-AR gene. Procedures for the gel mobility assay and the preparation of proteins from rat heart nuclei extract (*RHNE*) are described in Materials and Methods. A, Binding reaction mixture contained 25,000 cpm of ^{32}P-labeled oligomer representing the nucleotides between −125 and −100 in the β_1-AR promoter. *Lanes 2–6*, 1 μl of rat heart nuclei extract (1 μg/μl) was added to each binding reaction. *Lane 1*, no protein was added to the mixture. *Lanes 3–6*, oligomers containing a 4-bp mutation as defined in the legend to Fig. 3 were labeled and used in the binding reactions. The binding reactions were conducted for 20 min at 4°. The protein/DNA complexes resolved on a 5% nondenaturing acrylamide gel at 4°. B, Binding of rat heart nuclei extract and binding of rat liver nuclear extract (*RLNE*) to the wild-type (*WT*) −125 to −100 β_1-AR oligomer were compared. The binding reactions and gel mobility assay were conducted as described above. The dried gels were exposed to a Kodak XAR-5 film overnight with one intensifying screen.

containing −800 bp of the promoter was expressed at a 3-fold higher level. Like its rat counterpart, the ovine β_1-AR promoter has inhibitory sequences in the 5′ end.

Using serial deletions of the β_1-AR promoter ligated to the luciferase reporter gene, we have expanded previous observations and identified several regions between −3311 and −2740 that are important for the suppression of rat β_1-AR transcription. Primarily, a region between −2870 and −2740 is responsible for transcriptional suppression. However, the function of the upstream inhibitory domains is absolutely reliant on the presence of an inhibitory element located 3′ to the TSS. Removal of the 3′ inhibitory element between −125 and −100 or relocation of this element to a site 5′ to the TSS resulted in abundant expression in all cells that were analyzed, including hepatoma cell lines, which do not express β_1-AR mRNA or functional β_1-AR (2, 33). Deletion of the nucleotides between −125 and −100, however, did not alter the expression of constructs extending to −1251 (Fig. 1) or −484 (12). Therefore, the −125 and −100 element functions as a suppresser only in conjunction with other elements in the β_1-AR promoter and only when located 3′ to the TSS.

The sequences between −125 and −100 normalize the activity of the 3-kb promoter in two ways. First, they suppress the expression of the β_1-AR/luciferase chimera to ~6–10% of the activity of the SV40/luciferase vectors. These levels of activity are within the expected parameters for a gene that is expressed at low levels (35). The concentration of β_1-AR mRNA in VM is ~0.25 amol/μg of RNA, which corresponds to ~28 molecules/cell (16, 19). These mRNA levels are ≥100-fold lower than those of actively transcribed genes such as the apolipoprotein E gene in the liver (36). Second, the sequences between −125 and −100 inhibit the expression of the β_1-AR/luciferase construct in HepG2 and H4IIE cells, which do not express β_1-AR mRNA (2). This observation suggests that this element can inhibit transcription in all cell types and that in conjunction with other sequences, it may contribute to the tissue-specific expression of the β_1-AR. Phenomenologically, the expression of the construct extending from −3311 to +100 seems to be similar to the expression of the β_1-AR *in vivo*. However, more detailed analyses of these constructs *in vivo* is required to verify that the tissue-specific

expression of the β_1-AR promoter is under the regulation of this element.

In addition to the involvement of the −125 and −100 region in regulating basal expression, a second region that lies 3′ to the TSS between nucleotides −186 to −211 was identified by Searles *et al.* as an inhibitor of basal expression (12). These experiments were conducted in the context of a luciferase vector extending from −1 to −484 relative to the start of translation. Our experiments did not evaluate the contribution of this element, and the probe used in our DNase I footprinting studies did not cover this region. Therefore, it is not known whether this element can bind nuclear proteins. This site is not conserved among the 5′-flanking regions of the human, rat, and mouse β_1-AR genes, whereas the sequence in the −125 and −100 region of the rat β_1-AR gene is conserved among all the cloned β_1-AR genes (10–13). It will be interesting to evaluate the contribution of the −186 to −211 sequence in the context of the full 3.3-kb β_1-AR promoter.

Our results show that the −125 to −100 region can interact with two factors present in rat heart nuclear extract. We also found that the −125 to −100 element can bind the TR and mediate the thyroid hormone induction of the β_1-AR gene in VM.[2] Our data indicate that the complexes formed between rat heart nuclear extract and the −125 to −100 sequence in gel mobility assays do not contain the TR. However, during the preparation of nuclear extract, many nuclear factors are lost, so we cannot be certain that the factors binding to the −125 to −100 element in the gel mobility assays are binding *in vivo*. Our results indicate that this sequence is a multifunctional site that directs basal transcription and hormone responsiveness. It also raises the possibility that the TR could be one of the factors suppressing basal transcription of the β_1-AR gene because unliganded TR inhibits gene transcription (37). Future studies will be directed at identifying the factors that can bind the −125 to −100 element and characterizing their interactions with proteins on the more 5′ region of the gene.

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