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Reciprocal Regulation of β_1-Adrenergic Receptor Gene Transcription by Sp1 and Early Growth Response Gene 1: Induction of EGR-1 Inhibits the Expression of the β_1-Adrenergic Receptor Gene

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ABSTRACT

The β_1-adrenergic receptor (β_1-AR) plays a key role in regulating heart rate and contractility in response to catecholamines. Our studies have focused on defining the factors that regulate the expression of the β_1-AR gene. We determined that a 65-base-pair (bp) region in the β_1-AR promoter between bp −394 and bp −330 directs basal transcription. An element located between −377 and −365 can bind Sp1 and Sp3. In *Drosophila melanogaster* SL2 cells, Sp1 stimulated the expression of the β_1-AR promoter, whereas Sp3 was unable to activate transcription. Site-directed mutagenesis indicated that an intact Sp1-binding site is essential for maintaining the activity of the basal promoter. In addition to binding Sp family members, the nucleotides between −381 and −367 can bind the zinc-finger transcription factor Egr-1. The Egr-1 and Sp1 binding sites are partially overlapping and their binding sequence is conserved among mammalian β_1-AR genes. The induction of Egr-1 in rat neonatal ventricular myocytes with phorbol-12-myristate-13-acetate or in HeLa S3 cells by regulated expression of Egr-1 in a tetracycline-responsive promoter, suppressed expression from the β_1-AR promoter. Overexpression of Sp1 in SK-N-MC cells increased β_1-AR mRNA by 2.4-fold, whereas overexpression of Egr-1 reduced β_1-AR mRNA by 40%. Coexpression of Egr-1 with Sp1 reduced Sp1-mediated up-regulation of β_1-AR mRNA by 60%. Mutagenesis revealed that an intact Sp1-binding site is essential for observing transcriptional repression by Egr-1 and that Egr-1 suppressed the transcription of the β_1-AR gene by competing with Sp1 for binding to their overlapping sites. These results reveal a novel physiologically relevant transcriptional mechanism for reciprocal regulation of β_1-AR gene expression.

β-Adrenergic receptors (β-AR) are G_s-coupled receptors that transduce the binding of catecholamines into activation of adenylyl cyclase and elevation of cyclic AMP. The β_1-AR seems to be the dominant subtype of the β-AR family for mediating the chronotropic, inotropic, and lusitropic actions of catecholamines in the heart. Targeted disruption of the β_1-AR gene produced mice that lacked chronotropic and inotropic responsiveness to catecholamines, further demonstrating the importance of β_1-AR for proper cardiac function (Rohrer et al., 1996). In addition to the β_1-AR, two additional subtypes of β-AR have been cloned. The three subtypes of β-AR are products of separate genes and are unusual in that they are intronless or, as in the case of the β_3-AR, contain a very small intron (Kobilka et al., 1987; Machida et al., 1990; Granneman et al., 1993; Searles et al., 1995). The 5′-flanking region of mammalian β-AR reveal G+C-rich sequences which lack consensus TATA boxes to initiate transcription (Kobilka et al., 1987; Searles et al., 1995; Evanko et al., 1998). The major transcriptional start site (TSS) of the rat β_1-AR gene is at −253 relative to the first base of the initiation codon,[1] whereas that of the human β_2-AR gene is at −219, suggesting that similarities exist in their transcriptional initiation (Kobilka et al., 1987; Searles et al., 1995).

The expression of the β_1-AR is very low in tissues as well as in cultured primary ventricular myocytes (Bahouth et al., 1997a) and in cell lines that express the β_1-AR endogenously such as rat C6 glioma and human neuroepithelioma SK-N-MC cells (Bahouth et al., 1997b, 2001; Esbenshade et al., 1992). Promoter studies have shown that the 3-kb rat β_1-AR promoter has very low inherent ability to drive transcription compared with shorter promoter sequences (Searles et al.,

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[1] All numbers are relative to the first base of the initiation codon to facilitate comparisons among the various mammalian β_1-AR promoters.

ABBREVIATIONS: β-AR, β-adrenergic receptor; TSS, transcriptional start site; kb, kilobase; DMEM, Dulbecco's modified Eagles medium; SDM, Schneider's *Drosophila* medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; PMA, phorbol-12-myristate-13-acetate; EMSA, electrophoretic mobility shift assay; SV40, simian virus 40; GPCR, G protein-coupled receptor.

1995, Bahouth et al., 1997b). It is likely that low basal expression of the β_1-AR is caused by inhibitory domains or to a weak basal promoter. The first possibility was addressed by deletion studies from the 5′ end of the promoter. Shortening the 3-kb promoter to less than 1 kb substantially increased its activity in a variety of transiently transfected cell lines (Searles et al., 1995; Bahouth et al., 1997a,b). To characterize the basal promoter of the rat β_1-AR gene and to measure its relative transcriptional potency, we generated deletion mutants of the 5′-flanking sequence and then linked them to the expression of the gene for firefly luciferase. These constructs extended to bp −126 to avoid interference by regulatory sequences down-stream from bp −125 (Bahouth et al., 1997a,b). These analyses localized the sequence of the core promoter of the rat β_1-AR gene between −394 and −330 and identified two transcription factors that are involved in reciprocal regulation of the transcription of the β_1-AR gene.

Materials and Methods

Culture of Ventricular Myocytes. For each cell preparation, ventricles were isolated from 1- to 3-day-old Sprague-Dawley rats under aseptic conditions. Rat neonatal ventricular myocytes were dissociated with pancrelipase and isolated by discontinuous Percoll gradient centrifugation (Bahouth et al., 1997a). Using this procedure, highly enriched populations of ventricular myocytes (>95%) were obtained. Isolated myocytes were cultured on collagen-coated plates a density of 2×10^6 cells per 60-mm plate in 68% DMEM, 17% medium-199, 10% horse serum, and 5% fetal bovine serum and antibiotics. The next day the medium was aspirated and replaced with 80% DMEM and 20% medium-199.

Construction of β_1-AR Promoter-Luciferase Chimera and Luciferase Assays. Progressive deletions within the *Kpn*I-*Sac*II genomic fragment of the rat β_1-AR encoding the sequences between −1251 to −126 were generated either by restriction enzymes or by PCR and then ligated into the promoterless pGL3*basic* luciferase expression plasmid. Each 60-mm plate was transfected with 5 μg of plasmid DNA composed of 3.8 μg of the smallest vector (pGL3*basic*), 1 μg of carrier pGEM7ZF$^+$ DNA, and 0.2 μg of CMV-pRL (*Renilla reniformis* luciferase vector for correcting firefly luciferase activity) by the calcium phosphate precipitation technique (Bahouth et al., 1997a,b). 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 with pGEM7ZF$^+$. In all experiments, the pGL3*control* vector, which is a luciferase vector driven by the SV40 promoter and enhancer sequences, was transfected in equimolar amounts to the pGL3*basic* vector. Luciferase assays were performed using the Dual Luciferase Assay System (Promega Corp., Madison, WI). The luminescence of each construct is expressed as a percentage of the expression of pGL3*control* after normalizing for transfection efficiency. Each construct was transfected into three 60-mm plates and these transfections were replicated for each cell type in a minimum of three separate experiments ($n \geq 9$). The values from all experiments were combined and subjected to analysis of variance using Microsoft Excel (Microsoft Corp., Redmond, WA). Significance was determined by Student's t test ($p = 0.05$).

Culture and Transient Transfection of SL2 Cells. *Drosophila melanogaster* SL2 cells were cultured at room temperature in Schneider's *Drosophila* medium (SDM) supplemented with 10% FBS. DNA (1.5 μg per 35-mm plate) was mixed with 100 μl SDM and 9 μl of CellFECTIN solution (Invitrogen, Carlsbad, CA) for 15 min. DNA consisted of 0.5 μg of β_1-AR promoter luciferase vector, 0.1 μg pRL-CMV *R. reniformis* luciferase vector and the balance was composed of either carrier DNA or the appropriate Sp expression vector as described in the legend of Fig. 3. To the DNA-CellFECTIN mixture, 0.8 ml of SDM with 1.5% FBS was added, and the DNA/liposome complexes were layered over the cells for 5 h. After transfection, the DNA containing medium was replaced with 2 ml of SDM with 10% FBS for 36 h. Thereafter, the cells were harvested and luminescence was determined. The Sp1 and SP0 vectors under the control of the actin 5C promoter were obtained from R. Tjian (Kadonga et al., 1987; Courey and Tjian, 1988) and the other Sp vectors were provided by Guntram Suske and are described in Dennig et al. (1995).

Site-Directed Mutagenesis of β_1-AR Genomic Fragments. Mutagenesis of the *Kpn*I-*Sac*II genomic fragment of the β_1-AR gene was performed based on the methods described in the Transformer site-directed mutagenesis manual (CLONTECH, Palo Alto, CA). The sequence of the mutagenic primer for site directed mutagenesis of the triplet between −380 and −378 was 5′-pGGGACACCATTGTAAAGGGGCGTGCCTTG-3′; for mutating the sequence between −371 and −369 was 5′-pTTGTTCGGGGGCGAAACTTGGCGACGATTG-3′; and for mutating the sequence between −374 and −372 was 5′-pCCATTGTTCGGGGAAATGCCTTGGCGACG-3′. The underlined oligonucleotides indicate the mutated sequence. Plasmid DNA was sequenced by automated dye-termination sequencing using a primer 5′-TCTGGAAGAAGCCTGAGCAG corresponding to the sequence between −519 and −500 in 5′-flanking region the β_1-AR gene. Using the mutagenized *Kpn*I-*Sac*II fragment as template, the −3311 to −126 and −484 and −126 genomic fragments were prepared and subcloned into pGL3*basic* to generate the desired vector.

Gel Electromobility-Shift Assays (EMSA). Three double-stranded oligomers with CTAG overhangs representing the wild-type and mutated sequences between −385 and −365 in the β_1-AR promoter were synthesized. These were the *wild type* sequence 5′-ATTGTTCGGGGGCGTGCCTTG-3′; the *mut-Egr-1* oligomer 5′-ATTGTaaaGGGGCGTGCCTTG-3′, in which the Egr-1 site was mutated; and the *mut-Sp1* oligomer 5′-ATTGTTCGGGGGCGaaaCTTG-3′, in which the Sp1-binding site was mutated. These oligomers were labeled with Klenow enzyme and [α-^{32}P]dCTP and combined with nuclear extracts for 20 min in a binding buffer composed of 80 mM KCl, 10 mM HEPES, pH 7.1, 1 or 2 μg of poly(dI-dC), and 10% glycerol (Bahouth et al., 1997a). The resulting complexes were resolved on 5% nondenaturing acrylamide gels in 25 mM Tris, 200 mM glycine at 4°C (Bahouth et al., 1997a). Nuclear extracts prepared from rat neonatal ventricular myocytes were prepared as described in Bahouth et al. (1997a). Sp1 was purchased from Promega Corp. Egr-1 cDNA cloned in pRSET-A (Cui et al., 1996), was obtained from N. Mackman (Scripps Research Institute, La Jolla, CA). Hexahistidine-tagged Egr-1 expressed in bacteria was partially-purified by affinity chromatography with nickel nitriloacetic acid resin (Cui et al., 1996).

DNase Footprinting Assay. The *Pst*I Genomic fragment between −484 and +269 was ^{32}P-labeled on one end as described in Bahouth et al. (1997b). Each ^{32}P-labeled DNA fragment (20,000 cpm) was incubated with purified transcription factor (10 ng) in binding buffer composed of 20 mM HEPES, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 50 mM NaCl, and 1 μg of poly(dI-dC) for 30 min at 0°C (Bahouth et al., 1997b). Digestion with 0.03–0.1 units of DNase I was allowed to proceed for 45 s, followed by adding 150 mM NaCl, 0.7% SDS, 15 mM EDTA, and 30 μg of yeast tRNA. The samples were extracted and subjected to electrophoresis on 6% acrylamide in 8 M urea gels. The protected DNA sequences were identified by running a separate lane containing a G sequence ladder generated by cleaving the ^{32}P-DNA fragment with piperidine as described previously (Bahouth et al., 1997b).

Generation of HeLa Cells with Tetracycline-Regulated Expression of Egr-1. HeLa S3 cells harboring a stably integrated copy of the regulator plasmid pTet-Off, were purchased from CLONTECH. pTet-Off contains a fusion of the Tet repressor and the carboxyl-terminal 130 amino acids of VP16 as well as a G418 resistance cassette (Gossen and Bujard, 1992; Yin et al., 1996). Transcription of the gene under the control of the tetracycline responsive element is inhibited by tetracycline or its analog doxycycline. Con-

versely, transcription of the gene of interest is active and maintained so long as tetracycline is absent (Gossen and Bujard, 1992; Yin et al., 1996). pTet-Off-HeLa S3 cells were cultured in DMEM plus 10% FBS supplemented with 600 μg/ml G418 and 5 ng/ml doxycycline.

Egr-1 and ETTL (ETTL contains Egr-1 cDNA minus 2 zinc fingers and is therefore inactive) cDNAs were provided by Vikas Sukhatme (Sukhatme et al., 1988). Egr-1 and ETTL cDNAs were excised with *Eco*RI and cloned into the pTRE vector. The pTRE vector is a reconstituting vector that contains the necessary components to obtain regulated expression of Egr-1 or ETTL by doxycycline. HeLa S3 cells were transfected using the LipofectAMINE transfection reagent (6 μl/μg of DNA; Invitrogen) with Egr-1-pTRE and pTK hygromycin (at 10:1 ratio) for the selection of double-stable Tet-Off HeLa cells stably expressing either the Egr-1 or the ETTL gene under the control of the tetracycline responsive element. Clonal cell lines for either Egr-1 or ETTL were selected by their ability to suppress Egr-1 mRNA or ETTL mRNA expression in the presence of doxycycline and to express these mRNAs in the absence of doxycycline.

Double-stable Tet-Off HeLa cells were cultured DMEM plus 10% FBS, 600 μg/ml G418, 200 μg/ml hygromycin, and 5 ng/ml doxycycline until they were ~80% confluent, then were switched to DMEM supplemented with 1 ng/ml doxycycline and 0.5% FBS to suppress endogenous and exogenous Egr-1 (Cao et al., 1993). After 2 days, the cells were transiently transfected with 1.9 μg per 35-mm plate of the appropriate wild-type or mutagenized β_1-AR–promoter-luciferase plasmid and 0.1 μg of CMV-pRL *R. reniformis* vector using LipofectAMINE. After 5 h, the medium was aspirated and the cells were cultured overnight in DMEM + 0.5% FBS and 5 ng/ml doxycycline, to allow for cell recovery. The cells were then washed extensively with phosphate-buffered saline (to remove doxycycline) and recultured for 1.5 h in DMEM with 0.5% FBS, but without doxycycline to induce Egr-1 transcription. After 90 min, 5 ng/ml of doxycycline was added to suppress Egr-1 or ETTL transcription, and the cells were harvested 24 h later to measure the luciferase activity. A set of control plates which contained 5 ng/ml doxycycline to continuously suppress Egr-1 or ETTL expression were run in parallel.

Culture and Transient Transfection of SK-N-MC Cells. SK-N-MC cells are human neuroepithelioma cells that endogenously express the β_1-AR (Esbenshade et al., 1992; Bahouth et al., 2001). A mammalian expression vector for Sp1 was generated from pPac Sp1 as follows: a forward primer, 5′-GCAATGAACTCGTACTTTGGAACAGGC-3′, and reverse primer, 5′-TCAGAAGCCATTGCCACTGATATTAAT-3′, were used to amplify the 2.1-kb Sp1 cDNA fragment (Kadonga et al., 1987). The PCR-generated cDNA was cloned into the PCR 2.1 TOPO vector (Invitrogen), excised with *Kpn*I and *Xba*I and cloned into the mammalian expression vector pcDNA 3.1(Invitrogen). The mammalian expression vectors for Egr-1, pCMV-Egr-1 and for ETTL, pCMV-ETTL were described previously (Sukhatme et al., 1988). SK-N-MC cells were cultured in DMEM supplemented with 10% FBS. For transfection, SK-N-MC cells were cultured in DMEM and transfected with 5 μg of each vector per 150-mm plate (10 μg DNA per plate) by the LipofectAMINE method for 6 h. After 6 h, the medium was aspirated, and the cells were cultured in DMEM supplemented with 10% FBS for 48 h. Total cellular RNA was extracted by the RNA-STAT 60 method and 25 μg or RNA was subjected to Northern blotting and transferred to Nytran filters as described previously (Bahouth et al., 2001). The blot was prehybridized for 6 h in Ultrahybrid solution (Ambion, Austin, TX), then incubated in Ultrahybrid solution containing 2×10^6 cpm/ml of radiolabeled human β_1-AR probe for 16 h at 42°C. The β_1-AR probe was a 227-bp fragment from human β_1-AR cDNA corresponding to the sequences +522 to +748 (Bahouth et al., 2001). To control for the variability in RNA loading, the blot was stripped and reprobed with a 103-bp human cyclophilin cDNA probe corresponding to the sequences between +38 and + 140 (Ambion). The cpm in each band was counted by electronic autoradiography in the InstantImager (Packard Bioscience, Meriden, CT) and the data for each condition subtracted from background.

Results

Transcriptional Regulation of the Rat β_1-AR Gene by Sp1. To define the region within the β_1-AR gene that is involved in regulating basal expression, deletion mutants of the 5′-flanking sequences between −1251 to −126 were constructed and linked to the expression of the firefly luciferase reporter gene. Progressive deletions from the 5′ end indicated that the minimal promoter of the β_1-AR gene lies within a 65-bp region between −394 and −330 (Fig. 1A). The first 26-bp of the minimal promoter between −394 and −368 accounted for about 45 ± 5% of the basal activity. Deleting this construct down to −349 resulted in an additional 35 ±

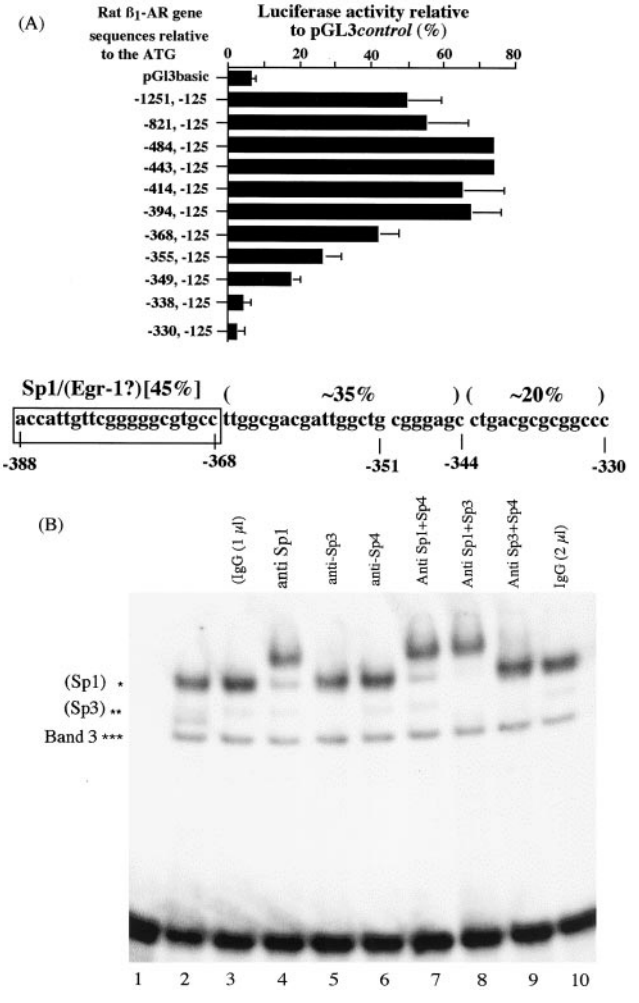

Fig. 1. Characterization of the early promoter region of the rat β_1-adrenergic gene by transient transfections and gel-shift assays. A, the sequences in the β_1-AR promoter shown in the left side were ligated into the multiple cloning region of the promoterless firefly luciferase vector pGL3*basic* and transfected into ventricular myocytes. The activity of luciferase relative to the SV40-driven firefly luciferase vector pGL3*control* was determined. Transfections were performed in triplicate and replicated five times, $n = 15$. The sequence of the core promoter of the rat β_1-AR gene and the percentile activity of its segments are shown. B, a ^{32}P-labeled oligomer corresponding to the sequence between −385 and −365 in the rat β_1-AR promoter was incubated with 1 μl of nuclear extract prepared from rat neonatal ventricular myocytes that were cultured in serum-free medium (lanes 2–10) and with IgG or immunoglobulins against Sp family members as indicated for 20 min. The complexes were resolved on 5% nondenaturing polyacrylamide gels in Tris/glycine and visualized by autoradiography. * and **, major and minor binding complexes of Sp1 and Sp3, respectively. ***, faster migrating band 3.

5% decline in activity, and the remaining 20% of the activity was lost when the promoter was truncated to −330.

To characterize the transcription factors that bind to the domain between −394 and −368 we performed EMSA between a ^{32}P-labeled double-stranded oligomer corresponding to the sequence between −385 and −365 and nuclear extract prepared from rat neonatal ventricular myocytes that were cultured in serum-free medium (Fig. 1B). The nuclear proteins formed three major complexes with the wild-type oligomer, whereby the more slowly migrating complex was the major forming complex. Because the domain between −394 and −373 is G+C rich, we used antibodies to transcription factors with preference to high G+C clusters. Antibodies to the Sp1 transcription factor supershifted the most slowly migrating complex, suggesting that transcription factors related to the Sp family were part of the complex over the oligomer between −385 and −365 (Fig. 1B, lane 4). The Sp family of transcription factors consists of four members, Sp1, Sp2, Sp3, and Sp4 (Suske, 1999). Sp2 expression is restricted to the CNS, whereas Sp1, Sp3, and Sp4 are widely distributed (Dennig et al., 1995). In addition to Sp1, the Sp3 antibody disrupted the minor band just below the Sp1 complex and, along with the anti-Sp1 antibody was able to supershift the entire Sp1 complex (Fig. 1B, lane 8). The antibody to Sp4 did not supershift, indicating that both Sp1 and Sp3 bind to the −385 to −365 region. Antibodies to Sp family members did not disrupt the binding to the faster migrating band 3 (Fig. 1B). Binding of nuclear proteins to band 3 was dependent on the concentration of poly(dI-dC) in the EMSA. Increasing the poly(dI-dC) from 1 to 2 μg/assay disrupted the binding to band 3, indicating that it represented a nonspecific protein-DNA complex.

The next experiment was designed to determine by DNase I footprinting whether the Sp1 transcription factor could bind to any sites in the basal promoter region of the β_1-AR gene. A 753-bp *Pst*I fragment containing the sequences between −484 and +269 was labeled on the top strand (5′ end) and the binding of Sp1 to this fragment was analyzed (Fig. 2 A). Protected footprints were localized to the region between −377 and −365 and to the region between −399 and −390. No other protected regions were identified (data not shown). Therefore, as suggested by the EMSA, Sp1 binds to a core sequence within the −394 and −368 domain. Based upon the results of transient transfection assays in Fig. 1, the activity of the β_1-AR promoter was unaffected when the region between −414 and −394 was deleted, indicating that the footprint between −399 and −390 is not critical for basal expression of the β_1-AR gene.

To determine the functional significance of Sp1 binding to the β_1-AR promoter, we performed transient transfection assays in *D. melanogaster* Schneider SL2 cells, which do not express Sp1 or the other Sp family members (Kadonga et al., 1987; Courey and Tjian, 1988; Dennig et al., 1995). SL2 cells were transfected with the −394,-126-pGL3, which contains the Sp1-binding site and with the −368, −126-pGL3 construct that lacks the Sp1-binding site (Fig. 3A). The cells were also transfected with the expression vector for Sp1, pPac Sp1, and with its inactive control pPac O (Courey and Tjian, 1988; Dennig et al., 1995). The data reveal that cotransfection of 0.1 or 0.3 μg of pPac Sp1 per plate increased the expression of the −394, −126-pGL3 construct by 6- and 17-fold, respectively. The expression of the −368, −126-pGL3 construct in which the Sp1-binding site was deleted was not stimulated by low concentrations of Sp1. At the highest Sp1 concentration used, there was a slight (~2-fold) stimulation of the −368, −126-pGL3 construct. This is expected because SL2 cells undergo a slight activation of general transcription when transfected with high levels of pPacSp1 (Kadonga et al., 1987).

Figure 3B shows the effect of lower amounts of Sp1 and Sp3 on transcription of the −394, −126-pGL3 construct in SL2 cells. Sp1 alone increased the transcription of by 10-fold, whereas Sp3 did not appreciably increase the expression of this construct. Sp1 and Sp3 together were not additive. Coexpression of Sp3 with Sp1 reduced the induction by Sp1 of β_1-AR transcription from 10- to 4-fold.

Characterization of Egr-1 Binding to the Early Promoter of the Rat β_1-AR Gene. Another transcription factor that binds to G+C rich elements is the early growth response gene 1 (Egr-1) factor (Sukhatme et al., 1988; Rauscher et al., 1990), also known as Zif268, NGF1-A, krox24, and TIS8. Egr-1 is a zinc-finger transcription factor that binds to a consensus sequence GCGGGGGCG (Lim et al., 1989). We observed that the sequence between −385 and −365 con-

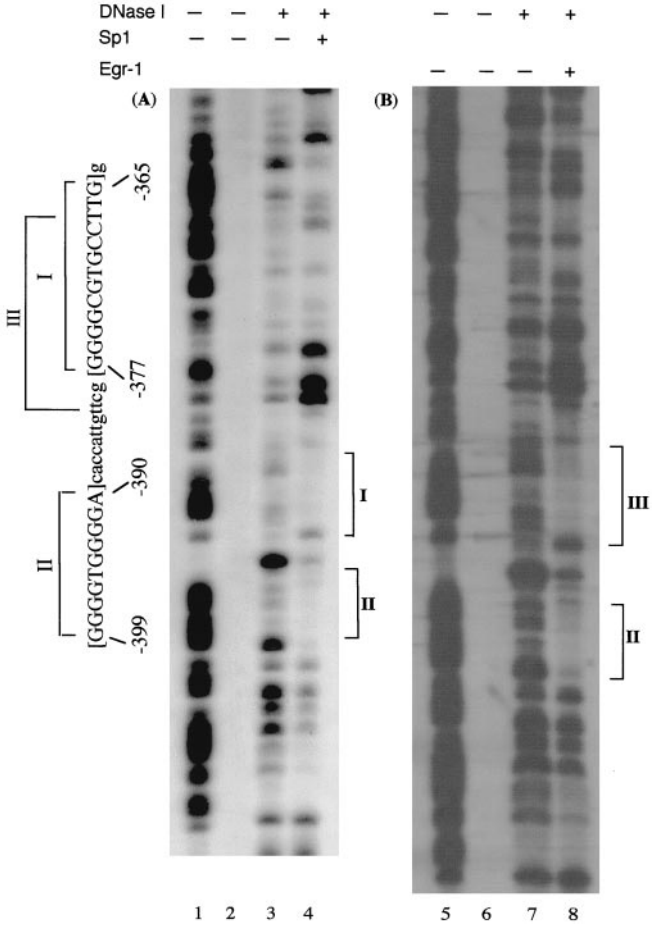

Fig. 2. DNase I footprinting of the β_1-adrenergic receptor promoter with Sp1 and Egr-1. The ^{32}P-labeled *Pst*I −484 to +268 β_1-AR genomic fragment (20,000 cpm) was incubated alone (lanes 2 and 6), with 30 μunits DNase I (lanes 3 and 7), or with 1 μl of either Sp1 (lane 4) or histidine-tagged Egr-1 (lane 5) and subjected to DNase I footprinting as described under *Materials and Methods*. The localization of the DNase I footprints was determined using Maxam-Gilbert G ladders (lanes 1 and 5). The sequences for protected bands I and II are outlined along the figure.

tained overlapping binding sites for Sp1 and Egr-1 (Fig. 1). In this region, the Sp1-footprint is between nucleotides −377 and −365, and the putative Egr-1 binding motif is between −380 and −372 (Figs. 1 and 4). Each site is imperfect by one nucleotide from the consensus Sp1 and Egr-1 binding sites.

We tested by EMSA whether Egr-1 could bind to the domain between −385 and −365. Partially purified hexa-histidine tagged human Egr-1 bound to the ^{32}P labeled oligomer corresponding to the sequence between −385 and −365 in the β_1-AR gene (Fig. 4A, lane 3). Nuclear extracts were prepared from rat neonatal ventricular myocytes that either were exposed or were not exposed to phorbol-12-myristate-13-acetate (PMA) for 1 h, a condition known to induce Egr-1 expression in quiescent heart cells (Chien et al., 1991; Knowlton et al., 1993). In control and PMA-treated nuclear extracts a slowly migrating band corresponding to Sp1 was present (Fig. 4A, lanes 1 and 2). In nuclear extracts prepared from PMA-treated myocytes an additional faster migrating band that comigrated with purified Egr-1 was evident (denoted with an asterisk in Fig. 4A, lane 2). To identify the sequences in the β_1-AR promoter which bind Egr-1, DNase footprinting between the ^{32}P-labeled 753-bp *Pst*I-fragment described earlier and purified hexa-histidine human Egr-1 was performed (Fig. 2 B). A major protected footprint was localized between −381 and −367, indicating that the Egr-1 binding site overlapped with the Sp1 binding site between −377 and −365.

To determine the contribution of the individual Sp1 and Egr-1 sites to transcription factor binding in EMSA assays, we mutagenized the nonoverlapping sequences between the Egr-1 and Sp1 sites in the fragment corresponding to the sequence between −385 to −365 (Khachigian et al., 1995; Cui et al., 1996). This approach involved mutagenesis of the three nucleotides in the Sp1-binding site that are not shared with Egr-1 (between −369 and −371) to disrupt the Sp1 binding site without affecting the Egr-1 binding site (Fig. 4B). Conversely, by mutagenizing the nucleotides between −380 and −378, the Egr-1 binding site was disrupted without affecting the Sp1 binding site. These oligomers were used in competition EMSA assays between nuclear extract from PMA-treated cardiac myocytes and ^{32}P-labeled wild-type sequence between −385 and −366 (Fig. 4C). In these experiments the concentration of poly(dI-dC) was doubled to 2 μg/assay to disrupt the binding of the wild-type ^{32}P-oligomer to band 3 that was described earlier in Fig. 1B. Using this approach, three major bands were bound to the wild-type oligomer, with the more slowly migrating band corresponding to Sp1 factor binding (Fig. 4C, lane 1). The unlabeled wild-type oligomer effectively competed Sp1 binding at 10 × molar excess, while the oligomer mutagenized between −369/−371 (*mut*-Sp1) did not compete for Sp1 binding at 10 × molar excess (Fig. 4C, compare lanes 3 and 6). On the other hand, the *mut*-Sp1 oligomer was equally effective to the wild-type oligomer in competing with the ^{32}P-labeled probe for binding to the two faster migrating bands that comigrated

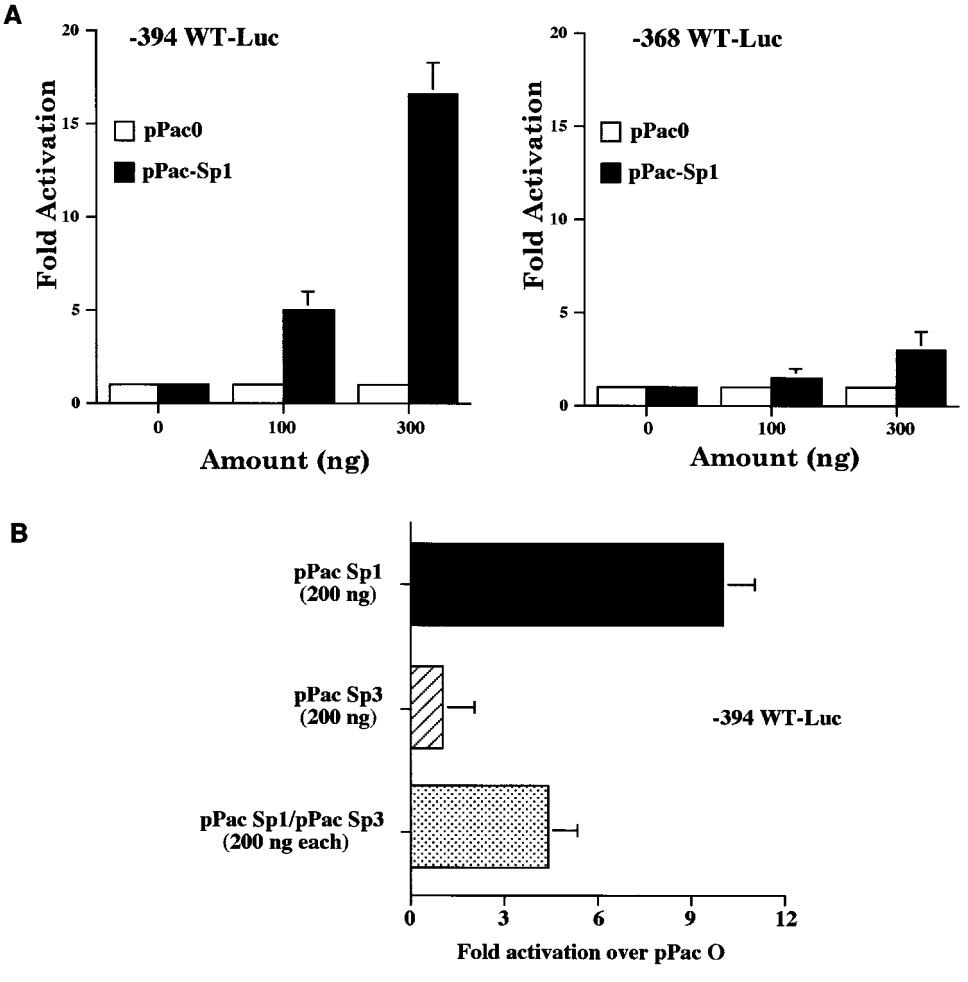

Fig. 3. Effect of transient expression of Sp family members in Schneider *D. melanogaster* SL2 cells on reporter gene expression by β_1-AR-luciferase constructs. A total of 1.5 μg DNA per 35-mm culture plate consisting of 1 μg β_1-AR-luciferase expression vector alone or with the indicated amounts of pPacSp1, pPacSp3 or pPac0 with 0.1 μg of pRL-CMV *R. reniformis* luciferase vector were transiently transfected into *D. melanogaster* SL2 cells. The expression of the β_1-AR construct in response to Sp1 or Sp3 relative to the expression of pPac0 are provided. The data represent the mean ± S.E. for the combined results of four transfections. Each transfection used triplicate samples.

with recombinant Egr-1. Therefore, it seems that selective disruption of the Sp1 binding site was achieved using this strategy. The oligomer in which the sequence between −380 and −378 was mutated (*mut*-Egr-1) was equally effective to the wild-type oligomer in competing Sp1-binding to the ^{32}P-labeled probe. However, this mutant oligomer was significantly weaker than the wild-type or the *mut*-Sp1 probes in competing for Egr-1 binding (Fig. 4C, compare lane 8 versus lanes 2 and 5). These data suggest that Sp1 and Egr-1 binding can be selectively inhibited by this approach.

Next, we examined the promoters of β_1-AR genes from several mammalian species to determine whether a shared Sp1/Egr-1-binding element was present (Fig. 5). Our analyses revealed a strong conservation of the overlapping Sp1/Egr-1 binding site in mammalian β_1-AR genes both in terms of sequence and localization within the promoter.

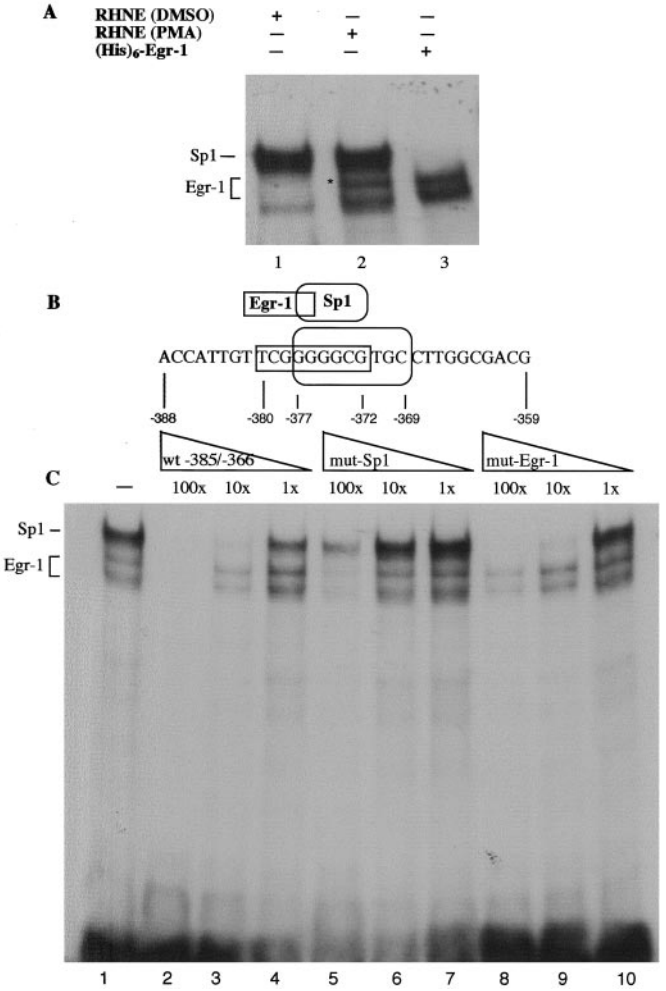

Fig. 4. Electromobility shift assay between nuclear extracts prepared from phorbol ester-treated ventricular myocytes and wild-type or point mutants of the sequence between −385 and −365. A, nuclear extracts were prepared from rat neonatal ventricular myocytes that were cultured in serum-free medium for 2 days and then exposed either to DMSO or 50 ng/ml PMA for 1 h. Nuclear extracts (1 μl) were combined with 20,000 cpm of ^{32}P-labeled oligomer representing the sequence between −385/−365. Hexa-histidine human Egr-1 purified from *Escherichia coli* was added in lane 3 to mark the migration of Egr-1. B, the sequences of oligonucleotides representing either the wild-type sequence between −365/−365 or those with a 3-bp mutation between −371 and −361 (*mut* Sp1) or with a 3-bp mutation between −380 and −378 (*mut*-Egr-1) are shown. C, represents competition between increasing amounts of unlabeled wild-type, *mut*-Sp1, and *mut*-Egr-1 oligonucleotides versus the ^{32}P-labeled −385/−365 oligomer in binding to nuclear extracts prepared from PMA-treated ventricular myocytes. As indicated above each lane, a 100-fold molar excess (100×), 10-fold molar excess (10×), or equal amounts of unlabeled oligomer (1×) was added to each binding reaction mixture.

Transcriptional Regulation of the Rat β_1-AR Gene by Transient Expression of Egr-1. The induction of Egr-1 mRNA in neonatal rat ventricular myocytes in response to PMA or other hypertrophic stimuli is transient in nature (Knowlton et al., 1993). As illustrated in Fig. 6A, Egr-1 mRNA was undetectable in neonatal rat ventricular myocytes that were cultured in serum-free medium. Exposing these cells to PMA, maximally induced Egr-1 mRNA within 30 min and this effect was attenuated in 3 h even though the concentration of PMA was sustained through out the incubation period. These data indicated to us that proper kinetics of induction and suppression of the Egr-1 gene are required to appropriately assess its transcriptional regulatory effects.

We adopted two strategies to achieve transient induction of Egr-1. The first was the classical approach that involves chemical treatment with PMA to transiently induce Egr-1 expression (Table 1). This approach has a drawback in that PMA is known to induce cardiac hypertrophy and induces a plethora of protooncogenes including *c-fos, c-jun,* and others (Chien et al., 1991; Knowlton et al., 1993). Nevertheless, in PMA-treated neonatal rat ventricular myocytes, the luciferase activity of the wild-type −494 to −126-pGL3 construct was reduced by 35 ± 11% (n = 4) compared with cells exposed to vehicle alone.

The other approach involved regulated expression of Egr-1 by the tetracycline responsive promoter of Bujard (Gossen and Bujard, 1992; Yin et al., 1996). This approach selectively induces a single gene in the absence of tetracycline and represses it in the presence of tetracycline or its more active analog doxycycline. To examine the effects of transient expression of Egr-1, the cDNAs for Egr-1 or of its inactive analog ETTL, which lacks two zinc fingers (Sukhatme et al., 1988), were stably expressed in a HeLa S3 cell line that contains a stably integrated copy of the Tet repressor plasmid. A double-stable HeLa S3 cell line with proper induction and suppression of Egr-1 mRNA (Eg-15) and another for ETTL (Et-32) were selected by the criteria outlined under *Experimental Procedures*. The expression of Egr-1 in Eg-15 cells was undetectable in the presence of doxycycline (Fig. 6B, lane 1) but was rapidly induced when doxycycline is removed (Fig. 6B, lane 2). The levels of Egr-1 mRNA in the S3 system were 3 ± 1-fold higher than those induced by PMA in neonatal cardiac myocytes. After 1.5 h, doxycycline was added and the levels of Egr-1 mRNA declined rapidly and

rat ß$_1$-AR	-380	TCG GGG GCG TGC CTT	-366
mouse ß$_1$-AR	-380	TCG GGG GCG TGC CTT	-366
human ß$_1$-AR	-396	ACG GGG GCG TGC CTT	-383
rhesus macaque ß$_1$-AR	-385	ACG GGG GCG TGC CTT	-371
porcine ß$_1$-AR	-380	TCG GGG GCG TGC TTC	-366
ß$_1$-AR consensus GC region		**TCG GGG GCG TGC** A	

Fig. 5. Comparison of the Sp1 and Egr-1 binding region in 5′-flanking region of mammalian β_1-AR. A sequence comparison of the Sp1 and Egr-1 binding region in promoters of the human, rat, mouse, monkey, and pig β_1-AR genes. The numbers are with respect to the translation initiation site. The accession numbers of the genes are as follows: X75540, rhesus; AF042454, porcine; D00634 and J05561, rat; X69168, human, and mouse (Cohen et al., 1993).

were undetectable after 4 h from the readdition of doxycycline (Fig. 6B, lanes 3–6). The kinetics of induction of Egr-1 in the Tet-Off system in HeLa S3 cells were comparable with those obtained by PMA treatment in heart cells because the half-life of Egr-1 mRNA is short (<15 min) allowing quick down-regulation after transcriptional silencing by doxycycline (Gossen and Bujard, 1992; Yin et al., 1996). The expression pattern of ETTL in response to doxycycline was similar to that shown in Fig. 6B for Egr-1 (data not shown).

In the next series of experiments, the Eg-15 and Et-32 cell lines were transfected with the β_1-AR-luciferase vector containing the putative Egr-1 site (−394, −126) or that without it (−368, −125). Transient induction of Egr-1 for 90 min, followed by silencing of Egr-1 expression for 24 h, reduced the luciferase activity of the −394, −126 construct to 58 ± 16% (Fig. 6C). The magnitude of Egr-1-mediated inhibition of reporter gene activity was ~42% that correlates favorably with the percentile of basal promoter activity imparted by the Sp-1 binding site. The luciferase activity of the −368, −126-pGL3 was not affected whether Egr-1 was induced or suppressed. In ETTL expressing Et-32 cells the luciferase activities of the −394, −126-pGL3 and −368, −126-pGL3 under

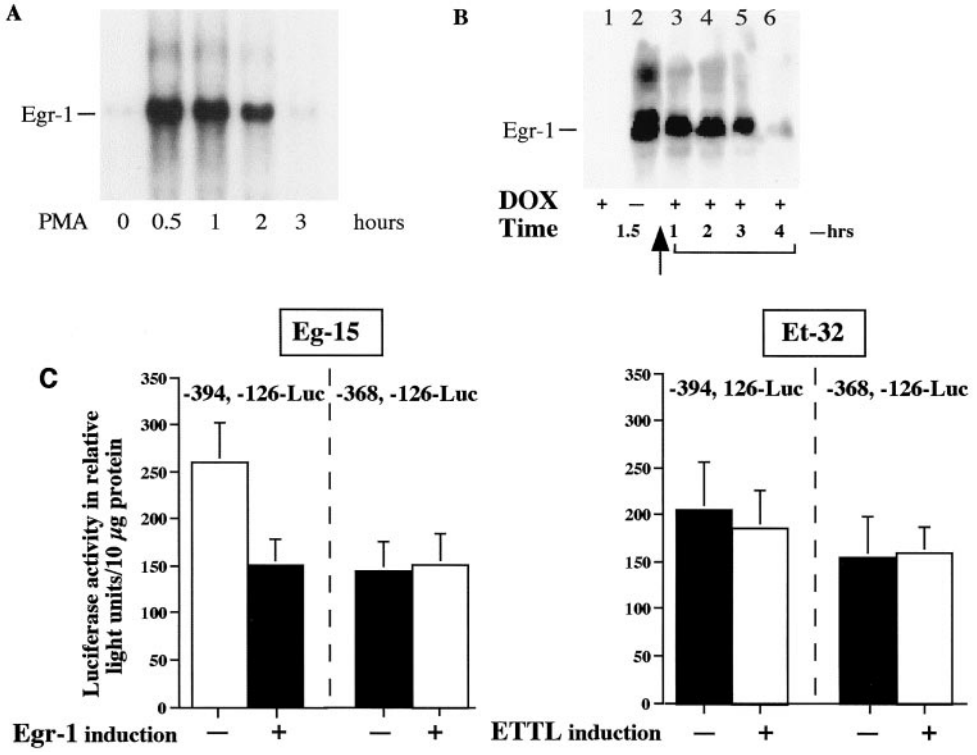

Fig. 6. Comparison between the kinetics of transient Egr-1 induction in ventricular myocytes by phorbol-12-myristate-13-acetate (PMA) and regulated Egr-1 expression in HeLa cells by the Tet-Off system. A, rat neonatal ventricular myocytes were cultured in DMEM/medium-199 (80:20) for 2 days and then exposed to 50 ng/ml of PMA. B, HeLa S3 cells expressing pTet-Off and human Egr-1 cDNA that were cultured for 2 days in medium containing 0.5% FBS and doxycycline to suppress Egr-1 expression. Then doxycycline was removed and Egr-1 was induced for 90 min, followed by the re-addition of 5 ng/ml doxycycline (arrow) to suppress Egr-1. RNA was prepared before Egr-1 induction, during Egr-1 induction, and 1, 2, 3, and 4 h after Egr-1 was suppressed. RNA were subjected to Northern blotting and probed with ^{32}P-labeled human Egr-1 cDNA probe. C, plasmid DNA (2 μg) consisting of 1.9 μg of β_1-AR–luciferase expression vector and 0.1 μg of pRL CMV *R. reniformis* luciferase vector were transiently transfected into 60-mm plates containing the double-mutant HeLa S3 cells Eg-15 or Et-32. Two vectors were used, −394, −126-Luc with the Egr-1 binding site and −368, −126 Luc without the Egr-1 binding site. After transfection, the cells were cultured overnight in the presence of 5 ng/ml doxycycline to suppress Egr-1 or ETTL. The next day, Egr-1 or ETTL were induced for 90 min, followed by the readdition of 5 ng/ml doxycycline. After 24 h, the cells were lysed for the measurement of luciferase activities. The corrected luciferase activity in light units/10 μg of protein ± S.E. for the combined results of four transfections, each in triplicate were calculated.

TABLE 1

Effect of PMA on the responsiveness of the wild-type and mutated β_1-AR promoter in neonatal ventricular myocytes

The wild-type and mutagenized β_1-AR promoters indicated below were ligated to luciferase in pGL3*basic*. A total of 5 μg of DNA per 60-mm culture plate consisting of 4.8 μg of β_1-AR-luciferase expression vector with carrier DNA and 0.2 μg of pRL-CMV *R. reniformis* luciferase vector were transiently transfected into neonatal rat ventricular myocytes. The next day, the cells were washed with phosphate-buffered saline and cultured for 48 h in 80:20 DMEM/medium-199 to suppress Egr-1 expression. PMA (50 ng/ml) was added and its effect on luciferase activity was determined after 24 h. The corrected luciferase activity in light units/10 μg of protein ± S.E. in the presence or absence of PMA were calculated and divided by the luciferase activity in the absence of PMA to determine the percentage change in activity in response to PMA. The data represent the combined results of four transfections where each transfection was in triplicate.

β_1-AR Luciferase Construct	Luciferase Activity		Luciferase Activity in Response to PMA
	−PMA	+PMA	
	relative light units/10 μg protein		%
Wild type, −494, −126-pGL3	311 ± 50	205 ± 40*	65
−494 [−380/−378], −126-pGL3	380 ± 60	350 ± 50	92
−494 [−371/−369], −126-pGL3	160 ± 30	145 ± 25	90

* $p < 0.05$.

conditions of transient ETTL induction or suppression were the same. These data reveal that suppression of β_1-AR transcription by Egr-1 is dependent upon the −394 to −368 region in the β_1-AR promoter.

Reciprocal Regulation of β_1-AR mRNA Expression by Sp1 and Egr-1 in SK-N-MC Cells. To test the functional relevance of Sp1 and Egr-1 in regulating the transcriptional activity of the intact β_1-AR gene, we determined the effect of transient expression of these transcription factors on β_1-AR mRNA levels in SK-N-MC cells. SK-N-MC cells are human neuroepithelioma cells that express moderate amounts of cell-surface β_1-AR (Esbenshade et al., 1992; Bahouth et al., 2001). Transient transfection of a CMV-driven Sp1 expression vector increased the levels of β_1-AR mRNA by 235 ± 50% compared with cells that were transfected with the control inactive vectors (Fig. 7). The expression of CMV-Egr-1 on the other hand reduced β_1-AR mRNA levels by 40%. Coexpression of equal amounts of Sp1 and Egr-1 vectors reduced Sp1-mediated induction of β_1-AR mRNA by 60%. Therefore, under these conditions the transcriptional activity of the β_1-AR gene was reciprocally regulated by Sp1 and Egr-1.

A Shared Overlapping Site Is Involved in Reciprocal Regulation of β_1-AR Expression by Sp1 and Egr-1. To determine the mechanism by which Sp1 and Egr-1 bind to their individual sites in their common binding region between −380 and −369, the ^{32}P −385/−365 oligonucleotide was incubated with increasing amounts of Egr-1 in the presence of a fixed amount of Sp1 (Fig. 8A). As the concentration of Egr-1 was raised, the amount of Egr-1 complexed to ^{32}P −385/−365 increased, whereas the Sp1 complexed to ^{32}P −383/−365 decreased. In the next set of experiments, we performed EMSA using the ^{32}P-labeled *mut*-Egr-1 oligomer to determine whether the Egr-1 binding site is necessary for the competitive interaction of Egr-1 with Sp1 (Fig. 8B). The binding of Sp1 to the ^{32}P-*mut*-Egr-1 was not affected by this mutation. However, the ability of Egr-1 to competitively displace Sp1 was severely compromised. These data indicate that Sp1 and Egr-1 do not simultaneously bind to −385/−365 and that these two transcription factors compete for binding.

Functional Relevance of the Egr-1 Binding Site in the β_1-AR Promoter. To determine whether reciprocal regulation of the β_1-AR gene described earlier was caused by the interaction of Sp1 or Egr-1 with their respective binding sites, the nonoverlapping Sp1 and Egr-1 binding sites were mutated by site directed mutagenesis. Two rat genomic β1-AR constructs were mutagenized, a long β_1-AR promoter construct extending from −3311 and −126 and a shorter version extending from −494 and −126. To disrupt Egr-1 binding, the sequences between −380/−378 that are involved in Egr-1 binding were mutated. To disrupt Sp1 binding, the sequences between −371/−369 that are involved in Sp1 bind-

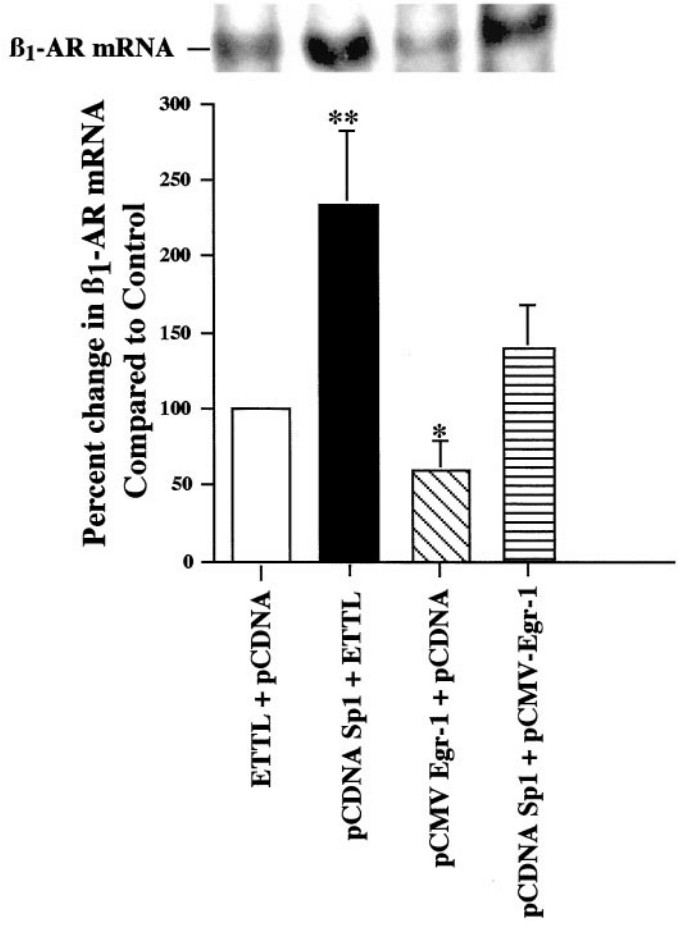

Fig. 7. Effect of transient expression of Sp1 and Egr-1 on β_1-AR mRNA levels in SK-N-MC cells. SK-N-MC cells in DMEM were transiently transfected for 6 h with mammalian expression vectors harboring pcDNA, Sp1, Egr-1, or its inactive derivative ETTL by the LipofectAMINE method. After 6 h, the medium was aspirated and the cells were cultured in DMEM + 10% FBS for 48 h. RNA was extracted and β_1-AR mRNA levels were determined by Northern blotting as described under *Materials and Methods*. The values of β_1-AR mRNA are the means ± S.E.M. of 3 separate determinations. The percentage of β_1-AR mRNA in control (ETTL + pCDNA) was 100%; in Sp1, 235% ± 50; in Egr-1 alone, 60% ± 20; in Egr-1 + Sp1, 140% ± 30. $^{**}p < 0.001$ for β_1-AR mRNA in control versus Sp1, $^{*}p < 0.01$ for β_1-AR mRNA in control versus Egr-1.

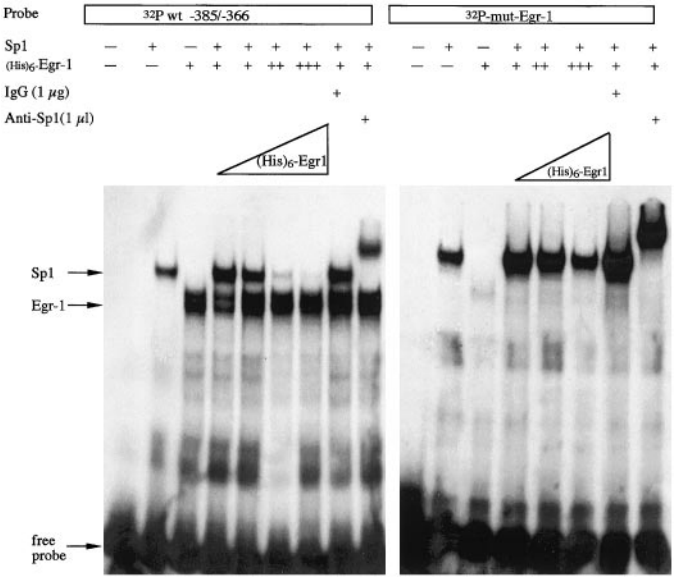

Fig. 8. Competitive displacement of Sp1 binding of the −385 and −365 G+C-rich region in the β_1-AR promoter by Egr-1. EMSA was performed on ^{32}P-labeled wild-type −385/−365 oligonucleotide (left) or ^{32}P-*mut*-Egr-1–385/−365 oligonucleotide (right), in which the Egr-1 binding site was mutated. The oligomers were incubate with a constant amount of Sp1 (0.05 footprinting unit/incubation) and increasing amounts of $(His)_6$-Egr-1 fusion protein (2–50 ng/lane) for 20 min at room temperature. The resulting complexes were the resolved by electrophoresis and visualized by autoradiography.

ing were mutated. In addition we disrupted the Sp1 and Egr-1 binding sites simultaneously by mutagenizing the GCG sequence in the core Sp1/Egr-1 binding region between −374/−372 (Table 2). Transient transfection of the long β_1-AR promoter constructs into ventricular myocytes indicated that mutating the Egr-1 site between −380 and −378 did not inhibit the activity of the β_1-AR promoter (Table 2). However, mutating the Sp1-binding site between −371 and −369 or the core sequence between −374 and −372, caused in both instances a 38% drop in the activity of these constructs. Therefore, the Sp1-binding site seems to be the dominant site in stimulating the expression of the β_1-AR promoter from this region.

In follow-up experiments, we transfected the wild-type and mutagenized versions of the short promoter extending from −494 to −126 into *D. melanogaster* SL2 cells (Fig. 9) and into HeLa S3 cells (Fig. 10). It was necessary to use the short promoter in these studies because the luciferase activity of the long promoter was very low. The expression of the long-promoter is efficient in cells that express the β_1-AR endogenously and low in cells that do not express endogenous β_1-AR, such as SL2 and HeLa cells (Bahouth et al., 1997b). Coexpression of the wild-type −494 to −126 promoter with the Sp1 expression vector in SL2 cells caused a 10-fold stimulation of luciferase activity compared with the activity generated by the Sp0 vector (Fig. 9). Similarly, coexpression of Sp1 with the promoter in which the Egr-1-binding site was disrupted resulted in luciferase activity comparable with the wild-type vector. The luciferase activity of the β_1-AR promoter with a mutation in the Sp1-binding site was 20% of that attained by the wild-type vector, whereas no induction of luciferase activity by Sp1 was observed in the vector with a mutation in the core region.

To determine whether the region between −380 and −378 was functionally involved in Egr-1 mediated events, the activity of the wild-type and mutagenized promoters in HeLa cells in response to transient induction of Egr-1 or ETTL was determined (Fig. 10). Transient induction of Egr-1 in Eg-15 cells was associated with 45% decline in the luciferase activity, whereas the induction of ETTL had no effect on the luciferase activity of the wild-type −494 to −126 construct. Disruption of the Egr-1 biding site abolished the inhibitory effect of transient Egr-1 expression. Transient induction of Egr-1 or ETTL in cells expressing the −494-*mut* Sp1 construct was associated with 50% decline in luciferase activity compared with the wild-type construct. Moreover, the luciferase activity of the construct with the disrupted Sp1-binding site was not further diminished in response to transient Egr-1 expression. These experiments were repeated in PMA-treated heart cells (Table 1). Mutagenesis of the Sp1-binding was associated with 50% reduction in activity. Mutagenesis of Sp1 or the Egr-1 binding sites abolished PMA-mediated inhibition of β_1-AR promoter activity. These data demonstrate the requirement for intact Egr-1 and Sp1-binding sites in the proximal β_1-AR promoter sequence for the effects of Egr-1 on β_1-AR expression to be observed.

TABLE 2

Effect of disrupting the Sp1 or Egr-1 binding sites by site-directed mutagenesis on the activity of the β_1-AR promoter-luciferase chimera in neonatal rat ventricular myocytes

Vectors and carrier DNA (4.8 μg) were transiently transfected into neonatal ventricular myocytes with 0.2 μg pRL-CMV by calcium phosphate precipitation. After 2 days, the activity of firefly luciferase were measured and corrected for minor changes in transfection efficiency. The relative expression of luciferase by the mutated β_1-AR promoter constructs relative to the expression of luciferase by the intact β_1-AR promoter are provided. The data represent the mean ± S.E. for the combined results of four transfections. Each transfection utilized triplicate samples.

β_1-AR Luciferase construct	Luciferase Activity Relative to WT −3311, −126
	%
Wild-type, −3311, −126-pGL3	100%
−3311 [−380/−378], −126-pGL3	95 ± 7
−3311 [−371/−369], −126-pGL3	62 ± 6*
−3311 [−374/−372], −126-pGL3	59 ± 7*

WT, wild-type.
* $p < 0.05$, n = 4, each experiment in triplicate.

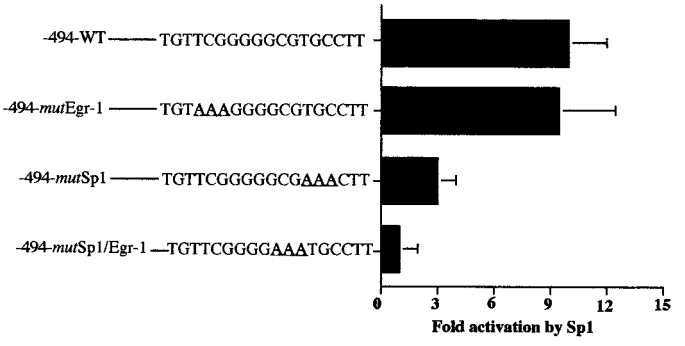

Fig. 9. Effect of transient expression of Sp1 in *D. melanogaster* SL2 cells on reporter gene expression by mutated β_1-AR genomic-luciferase constructs. 5′flanking sequences containing point mutations within the rat β_1-AR promoter were subcloned into the luciferase expression vector and transfected into SL2 cells as described in the legend of Fig. 3. The effect of Sp1 on the expression of the wild type β_1-AR construct and the constructs in which the Sp1-binding site (−494-*mut*-Sp1), the Egr-1-binding site (−494-*mut*-Egr-1) or the Sp1/Egr-1 binding sites (−494-mutSp1/Egr-1) were mutated are provided. The data represent the expression of each vector in response to Sp1 relative to its expression in response to pPacO. The data represent the mean ± S. E. for the combined results of three transfections. Each transfection used triplicate samples.

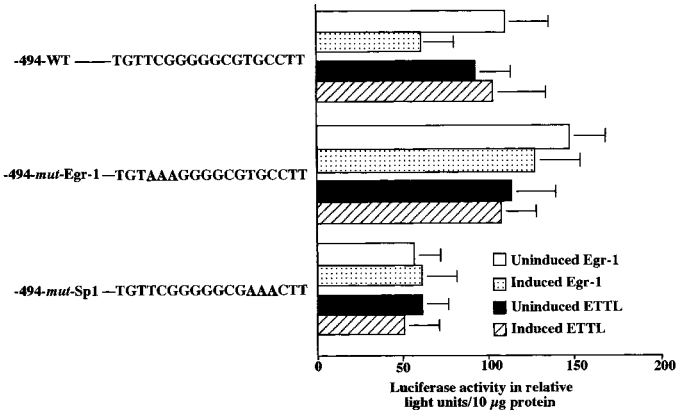

Fig. 10. Effect of transient expression of Egr-1 or ETTL on the expression of mutated β_1-AR genomic-luciferase constructs in HeLa cells. A total of 2 μg DNA per 60-mm culture plate consisting of 1.9 μg of the β_1-AR–luciferase expression vectors (described in the legend of Fig. 9) and 0.1 μg of pRL CMV *renilla* luciferase vectors were transiently transfected into the double-mutant HeLa S3 cells Eg-15 or Et-32 cells as described under *Materials and Methods*. After transfection, Egr-1 or ETTL were induced for 90 min and the cells were processed as described in the legend of Fig. 6. The corrected luciferase activity in light units/10 μg of protein ± S. E. for the combined results of three transfections each were calculated in triplicate.

Discussion

In this work, we identified the core promoter region of the rat β_1-AR gene and characterized a G+C-rich region that has a consensus Sp1-binding site. An intact Sp1 site was required for the full activity of the β_1-AR promoter in heart cells. The conservation of this binding site across mammalian β_1-AR genes suggests that this element is crucial to the regulated expression of the β_1-AR gene (Fig. 5). Sp1 is a potent activator of a wide variety of G protein-coupled receptor promoters, such as the α_{1B}- and α_{1D}-adrenergic receptor genes (Chen et al., 1997; Arai et al., 1999), the δ- and μ-opioid receptor genes (Ko et al., 1998; Liu et al., 1999), and the dopamine D_2-receptor gene (Yajima et al., 1998). The migration patterns of nuclear extracts from SH-SY5Y cells bound to a 40-mer fragment containing the Sp1 site of the μ-opioid receptor (Liu et al., 1999) were similar to those shown in Fig. 1B. In both instances, the more slowly migrating bands were supershifted by anti-Sp antibodies, whereas the faster migrating band was not affected. In this study, we inhibited the binding of nuclear factors to the faster migrating band by increasing the concentration of poly(dI-dC) in the EMSA. This result suggests that the binding of nuclear factors to the faster migrating band had a lower affinity than their binding to the more slowly migrating complex.

In the μ-opioid and the D_2 dopamine receptor genes, Sp1 and Sp3 were bound to the Sp-binding sites of these GPCR promoters. The effect of Sp3 on Sp1-mediated activation of transcription of these GPCR genes was promoter dependent. In the μ-opioid receptor gene, Sp3 *trans*-activated the promoter and its activity was additive with Sp1 (Liu et al., 1999). Whereas in the D_2 dopamine receptor gene, Sp3 alone failed to affect transcription and repressed Sp1-induced *trans*-activation of transcription. The mechanism by which Sp3 inhibits Sp1-mediated *trans*-activation of transcription is unknown. In the collagen 2A1 promoter, Sp3 did not influence gene transcription, but concomitant overexpression of Sp1 and Sp3 inhibited the transcription of the collagen 2A1 promoter via Sp3-mediated blockade of Sp1-mediated induction of the collagen 2A promoter activity (Ghayor et al., 2001). These results indicate that the behavior of Sp3 over the β_1-AR promoter resembles its activity over the promoters for the D_2 dopamine receptor and collagen 2A1.

The binding of Sp1 to G+C boxes is often critical to achieving significant levels of transcription from promoters that lack TATA and CCAAT elements, such as the β_1-AR gene (Araki et al., 1991; Boisclair et al., 1993). The activity of the basal promoter of the β_1-AR gene relative to the SV40-driven pGL3*control* vector was about 60%, indicating that the β_1-AR gene contained a strong minimal promoter. However, the expression of β_1-AR mRNA is very low in tissues and cell lines that endogenously express β_1-AR (Bahouth et al., 1997b, 2001; Searles et al., 1995). Promoter studies have shown that the complete 3.3-kb rat β_1-AR promoter has very low inherent ability to drive transcription compared with the minimal promoter (Searles et al., 1995; Bahouth et al., 1997b; Evanko et al., 1998). Therefore, it is likely that the activity of the minimal promoter is modulated by sequence specific enhancer and/or silencer binding proteins that produce low-level as well as tissue-specific expression. Progressive deletions and site-directed mutagenesis of the β_1-AR gene revealed positive and negative regulatory domains flanking both ends of the core promoter between −394 to −330 that played a critical role in regulating the expression of this gene. A strong negative regulatory domain between the sequences from −2870 to −2740 was found in the rat and human β_1-AR promoters (Bahouth et al., 1997b; Evanko et al., 1998). In addition, two hormonally responsive domains in the 5′-flanking region were identified. An element that inhibited the transcription of the β_1-AR gene in response to glucocorticoids was localized in the sequence between −950 and −926 and a cyclic AMP-responsive element that inhibited the transcription of the β_1-AR gene in C6 glioma cells in response to β-agonists was localized within the sequences between −1258 and −1250 (Bahouth et al., 1996; Fitzgerald et al., 1996). The mechanism of β-agonist-induced repression of β_1-AR gene transcription is interesting. β-Agonists rapidly stimulate the expression of the inducible cyclic AMP early repressor which is a member of the cyclic AMP response element modulator family of transcription factors (Fitzgerald et al., 1996). Increased expression of inducible cyclic AMP early repressor and cyclic AMP response element modulator inhibited the expression of β_1-AR–luciferase constructs that contained the cyclic AMP-responsive element.

Kirigiti et al. (2000) and Searles et al. (1995) identified two additional domains within the rat β_1-AR promoter that lie 5′ and 3′ to the TSS. The first domain situated upstream from the TSS at −389 was necessary for expression and possessed AP-2 like consensus elements which bound the recombinant AP-2 protein (Kirgiti et al., 2000). The domain 3′ to the TSS consisted of two clusters between −1 to −159 and −186 to −211 that when deleted increased or decreased transcription, respectively (Searles et al., 1995). In addition, Bahouth et al. (1997b) identified two additional domains that lie 3′ to the TSS. One domain was a thyroid hormone responsive element between nucleotides −101 and −117 that mediated transcriptional activation of the β_1-AR gene in response to thyroid hormones and the other was an adjacent domain between nucleotides −118 and −125 that suppressed the expression of β_1-AR-luciferase chimera in a tissue-specific manner (Bahouth et al., 1997a,b). These data suggest that low expression levels of the β_1-AR gene were not primarily caused by a weak promoter but more probably by inhibitory sequences in the promoter of the β_1-AR gene (Machida et al., 1990; Searles et al., 1995; Fitzgerald et al., 1996; Bahouth et al., 1997a,b; Evanko et al., 1998).

Our studies revealed that the protooncogene Egr-1 binds to a sequence that overlaps with the Sp1 binding site. The binding of Egr-1 to its consensus element can either activate or suppress the transcription of associated genes depending on the respective promoter (Cao et al., 1993; Khachigian et al., 1995; Cui et al., 1996; Thottassery et al., 1999). The data in Figs. 4 and 8 show that Sp1 and Egr-1 do not bind simultaneously to their respective binding sites and that elevated amounts of Egr-1 can displace prebound Sp1 from the Sp1-binding site. Functionally, Egr-1-mediated inhibition of basal transcription was dependent on the Egr-1 binding site as well as on the Sp1-binding site.

What is the physiological role of the Egr-1 site in the β_1-AR promoter? As described in Fig. 7, transient expression of Egr-1 alone reduced the transcription of the endogenous β_1-AR gene and reversed Sp1-mediated activation of β_1-AR gene transcription. Therefore, it is conceivable that the Egr-1 site might mediate a novel inhibitory feedback loop between

GPCR that induce transient Egr-1 expression and the β_1-AR. Egr-1 is induced in response to activation of phospholipase C-coupled receptors such as α_1-adrenergic receptors (Chien et al., 1991; Knowlton et al., 1993), angiotensin II type$_1$-receptors (Day et al., 1999), and muscarinic acetyl choline-receptors (von der Kammer et al., 1998). Activation of each of these GPCRs invariably diminishes the density or the response of membranous β_1-AR. For example, α_1-AR and β-AR are inversely cross-regulated and sustained activation of myocardial α_1-AR reduces the actions that are mediated via the β_1-AR (Kunos and Ishac, 1987; Molderings and Schummann, 1989; von der Kammer et al., 1998). Similarly, infusion of angiotensin II onto primary cultures of ventricular myocytes produced 38 and 55% decreases in the density of β_1-AR after 3 and 8 days, respectively (Henegar et al., 1998). Regulation of β_1-AR by angiotensin II might be physiologically relevant, because activation of β_1-AR increases the release of renin from the kidney, which ultimately increases the concentration of angiotensin II. In this regard, increased activation of the angiotensin II type$_1$-receptor by angiotensin II activates protein kinase C that in turn diminishes the density and responsiveness of β_1-AR (Schwartz and Naff, 1997). The antagonism between muscarinic cholinergic and β_1-AR is well documented. In primary cultures of rat neonatal myocytes, the muscarinic receptor agonist carbachol reduced by 30% the number of cell-surface β_1-AR after 20 h of exposure (Paraschos and Karliner, 1994). These data provide evidence for a physiologically relevant inhibitory feedback loop between phospholipase C-coupled receptors and the β_1-AR. Consequently, β_1-AR expression might be transcriptionally repressed through a novel receptor cross talk pathway involving transient expression of Egr-1.

Heterologous induction Egr-1 and its potential role in β_1-AR regulation provides another dimension to β_1-AR regulation in addition to the phenomena described previously for homologous and heterologous GPCR desensitization, phosphorylation, and mRNA destabilization that are intimately involved in long- and short-term receptor regulation.

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