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Genetic regulation of β_2-adrenergic receptors in 3T3-L1 fibroblasts

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The β_2-adrenergic receptor from mouse 3T3-L1 cells is up-regulated through genetic mechanisms by glucocorticoids and butyrate. To study the genetic regulation of these receptors, we sequenced a 5 kb region of genomic DNA from 3T3-L1 cells, containing the β-adrenergic receptor gene and approx. 1.5 kb of both 5′ and 3′ flanking sequences. The sequence contained one copy of an 8 bp consensus sequence which can confer phorbol ester-responsiveness to genes. Phorbol esters attenuated the up-regulation of β_2-adrenergic receptors by glucocorticoids but not by butyrate. This effect was probably due to a phorbol ester-induced decrease in glucocorticoid receptor number. Using methylation-sensitive restriction enzymes, we examined the methylation of a CG-rich region occurring 5′ to the gene and did not detect any changes in methylation of this region upon dexamethasone or butyrate treatment. A total of 16 putative glucocorticoid response elements were found which may mediate the glucocorticoid-induced increase in β_2-adrenergic receptors. A comparison of the regulatory sequences of the two β-adrenergic receptor subtypes from human and mouse confirms the observed physiological controls of receptor subtype expression and offers an explanation as to why the subtypes differ in genetic regulation.

INTRODUCTION

The β-adrenergic receptor has proved to be a useful model system with which to study the processes of receptor regulation. β-Adrenergic receptors have been divided into two subtypes based on their responses to the agonists adrenaline and noradrenaline (Lands *et al.*, 1967). At β_1-adrenergic receptors, adrenaline and noradrenaline are equally potent, whereas at β_2-adrenergic receptors adrenaline is much more potent than noradrenaline. The development of β-adrenergic receptor subtype-selective antagonists has helped to characterize the receptor subtypes. The genes for each of the β-adrenergic receptor subtypes have recently been cloned showing that they are indeed the products of separate genes (Dixon *et al.*, 1986; Frielle *et al.*, 1987; Kobilka *et al.*, 1987; Allen *et al.*, 1988). Both β-adrenergic receptor subtypes can be regulated by epigenetic and genetic mechanisms. The most extensively studied mechanism of β-adrenergic receptor regulation is desensitization, which occurs upon prolonged exposure of certain tissues or cultured cells to β-adrenergic agonists or phorbol esters. These treatments result in decreased responsiveness to subsequent challenge by β-adrenergic agonists. The attenuated responsiveness is associated with phosphorylation of the β-adrenergic receptor by intracellular protein kinases (Benovic *et al.*, 1987).

Regulation of β-adrenergic receptors by genetic mechanisms has only recently been investigated. Gene-activating hormones such as glucocorticoids and thyroid hormone have been shown to modulate adrenergic responsiveness and β-adrenergic receptor number in tissues and cultured cells (O'Donnell & Wanstall, 1987). In addition, butyrate, another gene-activating compound, induces β-adrenergic receptor up-regulation in a number of cell types (Nambi *et al.*, 1986; Stadel *et al.*, 1987). The 3T3-L1 cell line expresses both β-adrenergic receptor subtypes and therefore provides a system with which to study the regulation of the two subtypes in a clonal cell line. Glucocorticoids, as well as butyrate, promote an increase in β_2-adrenergic receptor expression in 3T3-L1 cells without causing a concomitant increase in β_1-adrenergic receptors (Lai *et al.*, 1982; Nakada *et al.*, 1987; Stadel *et al.*, 1987). These results are consistent with previous observations in tissues indicating that β-adrenergic receptor subtypes are independently regulated.

The regulation of β-adrenergic receptor gene expression could involve a variety of mechanisms. Glucocorticoids and phorbol esters are known to associate with specific DNA sequences termed glucocorticoid-response elements (GRE) (Cato *et al.*, 1984) and TPA-responsive elements (TRE) (Angel *et al.*, 1987; Chiu *et al.*, 1987) respectively. These consensus sequences, which bind steroid receptors or *trans*-acting proteins, have been identified by mutational analyses of genes regulated by these agents. Butyrate is postulated to bring about its effect through histone hyperacetylation (Boffa *et al.*, 1981) or hypomethylation of DNA (Christman *et al.*, 1980; Szyf *et al.*, 1985). Methylation of DNA changes its ability to interact with proteins (Doerfler, 1983). In eukaryotic cells, methylation occurs primarily at cytosine residues which are followed by

Abbreviations used: GRE, glucocorticoid-response elements; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TPA-responsive elements; SSC, 0.15 M-NaCl/0.015 M-sodium citrate; ^{125}ICYP, [^{125}I]iodocyanopindolol.

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guanosines. This CG sequence is statistically under-represented in total DNA, but can be found over-represented in the promoter regions of certain genes. Restriction enzymes which contain the CG dinucleotide within their recognition sequence can be used to compare the methylation state of the promoter regions of specific genes. Using this technique, a correlation between hypomethylation of GC-rich promoter regions and the activation of certain genes has been reported (Doerfler, 1983; Saluz *et al.*, 1986).

To begin to understand the genetic regulation of β-adrenergic receptors, we cloned and sequenced the β_2-adrenergic receptor gene from 3T3-L1 cells and have examined the structure of the gene and its flanking regulatory elements. We have compared the functional regulation of the two β-adrenergic receptor subtypes and their genomic sequences to help in understanding how and why the regulation of the two receptor subtypes diverged.

MATERIALS AND METHODS

Cell culture

3T3-L1 cells, kindly provided by Dr. Charles Rubin (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) were cultured at 37 °C in 10% CO_2, in complete media consisting of Dulbecco's modification of Eagle's medium with 4.5 g of glucose/litre (Gibco, Grand Island, NY, U.S.A.), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.) and nonessential amino acids (Gibco). Immediately upon reaching confluence, cells were treated with the appropriate compound in complete media. Phorbol dibutyrate, dexamethasone and sodium butyrate used to treat the cells were obtained from Sigma (St. Louis, MO, U.S.A.).

Cloning and sequencing

A λgt10 library was constructed using size-selected *Eco*R1-restricted 3T3-L1 DNA. Phage were lifted on to duplicate nitrocellulose fiters which were baked at 80 °C under a vacuum and prehybridized overnight. Hybridization and prehybridization buffer consisted of 5×SSC, 50 mM-Na_2HPO_4, 10×Denhardt's, 0.1% SDS, and 20 μg of denatured salmon sperm DNA/ml. The filters were hybridized for at least 24 h with a ^{32}P-labelled probe consisting of the reading frame of a hamster β_2-adrenergic receptor cDNA clone which was kindly provided by Dr. R. A. Dixon (Merck, Sharp and Dohme, West Point, PA, U.S.A.). The probe was ^{32}P-labelled with random primers using the Pharmacia Prime Time System (Stockholm, Sweden). After hybridization, the blots were washed at 68 °C with 5×SSC/0.1% SDS for 15 min; 2×SSC/0.1% SDS for 2×15 min; and 1×SSC/0.1% SDS for 2×15 min, and exposed overnight at −70 °C. Two positive clones were isolated from 200000 plaques. Sequencing of single-stranded DNA was performed by the dideoxy chain termination method of Sanger (Sanger *et al.*, 1977) using ^{35}S-labelled dCTP (New England Nuclear, Boston, MA, U.S.A.).

Southern blotting

DNA in agarose gels was transferred on to nitrocellulose filters which were prehybridized and hybridized as described in 'Cloning and sequencing' except that a 1.4 kb *Kpn*l fragment of the 3T3-L1 β_2-adrenergic receptor genomic clone which began 850 bp 5′ to the gene and ended 500 bp into the coding region was used as probe. After washing, Southern blots were exposed at −70 °C for 3 days.

[^{125}I]Iodocyanopindolol (^{125}ICYP) binding

^{125}ICYP (New England Nuclear) binding to cell membranes was performed as described in Nakada *et al.* (1987). Incubations were carried out at 37 °C for 60 min. Receptor subtype was determined by computer modelling of competition binding data using the β_2-adrenergic receptor-selective antagonist ICI 118.551 (a gift of Imperial Chemical Industries, Macclesfield, U.K.) to inhibit ^{125}ICYP binding.

[^{3}H]Dexamethasone binding to intact cells

Saturation binding of [^{3}H]dexamethasone (New England Nuclear) was carried out using concentrations of [^{3}H]dexamethasone from 0.1 nM–10 nM using the method described in Nakada *et al.* (1987). Incubations were at 37 °C for 2 h.

RESULTS

To study the mechanisms of β_2-adrenergic receptor regulation in 3T3-L1 cells, we cloned and sequenced the gene from these cells. Because the genes for the β-adrenergic receptors are intronless, we were able to clone the gene using a cDNA probe. Southern analysis of *Eco*R1-digested 3T3-L1 DNA using a full length hamster β_2-adrenergic receptor cDNA clone as probe revealed a single 6.3 kb band. Nuclear 3T3-L1 genomic DNA was cut with *Eco*R1 and fractionated on an 0.8% agarose gel. DNA ranging in size from 6.0–6.5 kb was cut out and eluted from the gel. This enriched DNA was used to construct a λgt10 library. Two positive clones were identified out of 2×10^5 recombinants. These clones had identical restriction maps. Fig. 1 shows the sequence of the 3T3-L1 β_2-adrenergic receptor gene with 2212 bp of 5′ flanking sequence and 1463 bp of 3′ flanking sequence. The sequence contains sites which are necessary for the proper regulation of a gene, such as a Kozak consensus sequence for efficient initiaton, a CAAT box, a TATA box, an SP1 transcription factor binding site, and polyadenylation sites. These sites are conserved in the other β_2-adrenergic receptor sequences reported (Emorine *et al.*, 1987; Kobilka *et al.*, 1987; Allen *et al.*, 1988). The reading frame and flanking regions of the β_2-adrenergic receptor gene from mouse 3T3-L1 cells were 99% similar to the mouse β_2-adrenergic receptor sequence previously published (Allen *et al.*, 1988).

The sequencing of the 3T3-L1 mouse β_2-adrenergic receptor gene allowed a comparison with the corresponding coding and flanking regions of the human β_2-adrenergic receptor gene. The University of Wisconsin computer program BESTFIT was used to optimally align pairs of sequences and calculate similarity values. These calculations (Smith & Waterman, 1981) exclude gaps which were assigned a penalty of 5. Fig. 2(*a*) is a dot-matrix comparison of the mouse 3T3-L1 and human β_2-adrenergic receptor sequences. The line of identity in the coding region indicates that the 3T3-L1 β_2-adrenergic receptor gene is homologous to the human β_2-adrenergic receptor gene previously reported (Emorine *et al.*, 1987).The degree of similarity in this region was 85%. Moreover, the 5′ and 3′ flanking sequences from mouse and human genes [shown in Fig. 2(*a*) to the left and right,

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TTCCAGGACAGCTGGGGCTCCACGAGAGAAACCCTGCCTCCCCCCCAAAAATGGCCCAAAGTTCACTGACAAACTCACATTTGTCTGATATTTAGCTATGTAACAGCACTTACG
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ACCCCCTAGTTACATATGCAGACACT
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Fig. 1. Genomic sequence of the 3T3-L1 β_2-adrenergic receptor

The reading frame of the gene is translated and shown below the sequence. Glucocorticoid response elements, defined by a consensus sequence, are marked above the sequence by circles. The filled circles indicate glucocorticoid response elements whose orientation and position are conserved in the human β_2-adrenergic receptor gene. An exact match to the reverse of an 8 bp TRE consensus sequence is underlined.

respectively, of the region bounded by the arrows] were also very similar. The 1263 bp of the 5′ region which were analysed were 73% similar and the 921 bp of the 3′ region were 79% similar. Because of the differences in pharmacology between β_1- and β_2-adrenergic receptors, and because the similarity at the protein level is only 54%, it was not surprising that there was less similarity (36%) in the coding regions of the mouse β_2-adrenergic receptor and the human β_1-adrenergic receptor genes. This is represented in the dot-matrix comparison of the 3T3-L1 mouse β_2-adrenergic receptor sequence with the human β_1-adrenergic receptor sequence (Fig. 2*b*). Sequence similarity in the coding regions between these two receptors can only be seen in the putative transmembrane spanning regions as previously reported (Frielle *et al.*, 1987). There is little similarity between the β_2- and β_1-adrenergic receptor genes in any of the flanking sequences (Fig. 2*b*). Although we have only compared the 87 bp of 3′ and 206 bp of 5′ flanking sequence which were reported with the β_1-adrenergic receptor sequence, a line of identity in

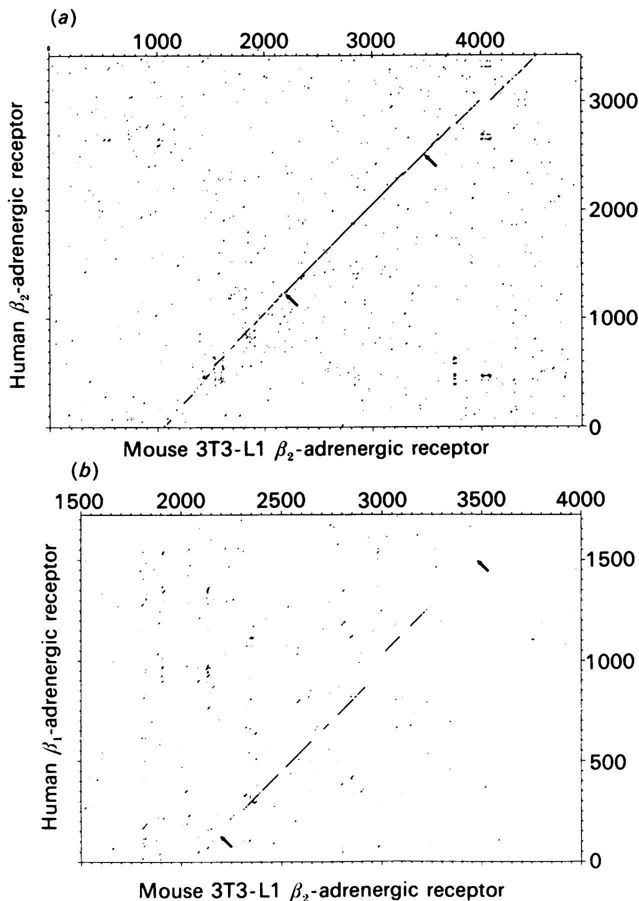

Fig. 2. Dot-matrix comparison of the mouse 3T3-L1 β_2-adrenergic receptor gene and human β_1- and β_2-adrenergic receptor genes

Sequences were compared using the University of Wisconsin program COMPARE. This program compares 21 bp at a time from the *x* and *y* axes and generates a dot on the matrix when 14 of the 21 bases are identical in both sequences. (*a*) The 3T3-L1 β_2-adrenergic receptor gene is compared with the human β_2-adrenergic receptor gene (Emorine *et al.*, 1987). (*b*) The 3T3-L1 β_2-adrenergic receptor gene is compared with the human β_1-adrenergic receptor gene (Frielle *et al.*, 1987). The reading frames of the genes are located between the arrows.

the corresponding short stretch of sequence can be seen in the dot-matrix comparison of β_2-adrenergic receptor sequences shown in Fig. 2(*a*). Computer-assisted optimal alignment did not reveal any similarity in the 5′ and 3′ flanking regions of the mouse 3T3-L1 β_2-adrenergic receptor and human β_1-adrenergic receptor genes. Comparison of the flanking regions of the human β_2- and β_1-adrenergic receptor genes also did not reveal any similarity in the 5′ and 3′ regions.

Analysis of the 3T3-L1 β_2-adrenergic receptor gene sequence revealed 16 GREs using the consensus sequence $^{T}_{A}GT^{T}_{C}CT$ (Cato *et al.*, 1984). GRE sites are marked by open and filled circles in Fig. 1. Some of these sites are in the reverse orientation. Previous studies have determined that GREs can function in both possible orientations (Ponta *et al.*, 1985). Five of the GRE sites are conserved in the same location (within 50 bp) and orientation in the human β_2-adrenergic receptor sequence. These sites are identified by filled circles in Fig. 1. One or more of these conserved GRE sites may be necessary for the regulation of these receptors by glucocorticoids. Our studies with the mouse 3T3-L1 cell line show that β_1-adrenergic receptors are not dramatically affected by glucocorticoid treatment (Nakada *et al.*, 1987). This is reflected by the absence of numerous GREs in the β_1-adrenergic receptor sequence reported (Frielle *et al.*, 1987). In the reported sequence for the β_1-adrenergic receptor gene, which includes the reading frame and 87 bp upstream and 206 bp downstream, only one exact match with the GRE consensus sequence was found, approximately halfway into the reading frame. In contrast, in the comparable sequence for the 3T3-L1 mouse β_2-adrenergic receptor sequence which is regulated by glucocorticoids, 5 GRE sites are present, none of which correspond in location to the site found in the β_1-adrenergic receptor sequence.

We searched the β_2-adrenergic receptor gene for TREs, which mediate the enhancement of gene expression by phorbol esters. Previous studies have shown that phorbol esters can regulate receptor activity epigenetically through the activation of protein kinase C (Kelleher *et al.*, 1984; Sibley *et al.*, 1984), but there have been no studies on the effects of phorbol esters on β-adrenergic receptors at the genetic level. An identical match to the reverse of an 8 bp TRE consensus sequence (TGAGTCAG) (Angel *et al.*, 1987; Chiu *et al.*, 1987) was found in the 3T3-L1 sequence (Fig. 1) 1 kb 3′ to the coding region of the gene.

Radioligand binding studies were used to determine what effect phorbol esters might have on β-adrenergic receptor number and subtype and on the regulation by dexamethasone and butyrate. Cells were treated for 48 h with or without dexamethasone, which caused an increase in β_2-adrenergic receptor expression. At this time phorbol ester was added to the cultures. After 48 h, membranes were prepared from the cells and assayed for β-adrenergic receptor subtype and number by radioligand binding. These treatments had no effect on cell viability as assessed by Trypan Blue dye exclusion by the cells. Control levels of β_2-adrenergic receptors were not affected ($P < 0.25$) by phorbol dibutyrate treatment. However, phorbol esters prevented the level of β_2-adrenergic receptors from remaining high in the continued presence of dexamethasone (Fig. 3*a*). Cells which were exposed only to dexamethasone continued to express high levels of β_2-adrenergic receptors, but cells which were treated with dexamethasone and subsequently with phorbol ester had reduced levels ($P < 0.0025$) of β_2-adrenergic receptors compared with those treated with dexamethasone alone. When the same experiment was performed using butyrate to induce β_2-adrenergic receptor expression, phorbol ester had no effect ($P < 0.25$) on the regulation of receptor number or subtype.

Dexamethasone treatment also caused a small but significant ($P < 0.05$) reduction in β_1-adrenergic receptors (Fig. 3*b*). This effect on β_1-adrenergic receptors was not observed with butyrate treatment. Phorbol ester did not change β_1-adrenergic receptor expression in control cells ($P < 0.25$) or in butyrate-treated cells ($P < 0.10$), but did inhibit the dexamethasone-mediated decrease in β_1-adrenergic receptors ($P < 0.025$).

Phorbol ester affected two glucocorticoid-mediated processes, the increase in β_2-adrenergic receptors and the decrease in β_1-adrenergic receptors, but had no effect

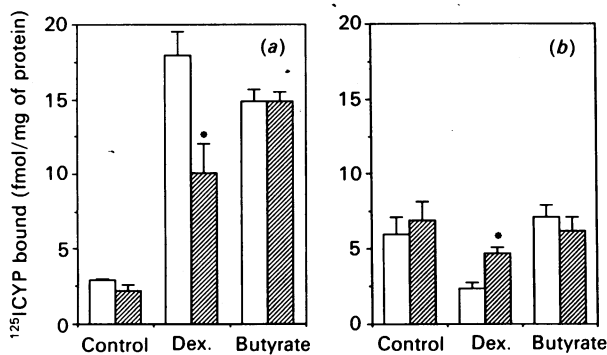

Fig. 3. Effect of phorbol dibutyrate on the regulation of β-adrenergic receptors by dexamethasone or butyrate

Cells were treated with (▨) or without (□) 250 nM-dexamethasone (Dex.) or 5 mM-butyrate for 48 h. At this time, 100 nM-phorbol dibutyrate was added to some of the cultures. The cells were harvested 48 h later and membranes were prepared for radioligand binding using ^{125}ICYP as described in the Materials and methods section. (*a*) Regulation of β_2-adrenergic receptors; (*b*) regulation of β_1-adrenergic receptors. The values represent the means ± S.E.M. of four experiments assayed in duplicate. Asterisks signify $P < 0.05$.

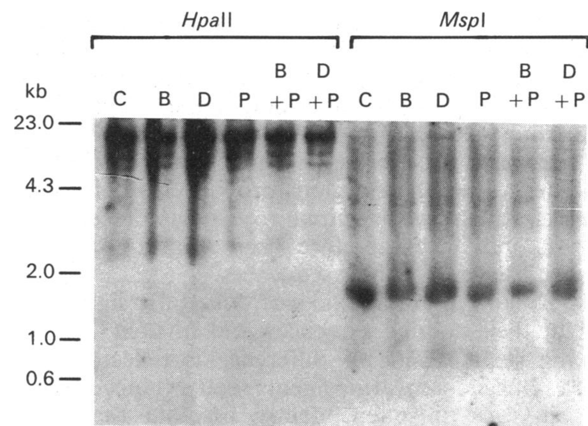

Fig. 4. Effects of dexamethasone and butyrate treatment of 3T3-L1 cells on DNA methylation as determined by restriction enzyme digestion

DNA was isolated from cells after treatment with medium alone (C), 5 mM-butyrate (B), 250 nM-dexamethasone (D), 1 µM-phorbol dibutyrate (P), or a combination for 24 h. DNA was digested with *Hpa*II (left) or *Msp*I (right) and 25 µg was loaded per lane in an 0.8% agarose gel. After electrophoresis, Southern blotting was performed as described in the Materials and methods section. The numbers on the left mark the positions of DNA standards, whose sizes are given in kb.

on control or butyrate-treated cells. We examined the possibility that phorbol esters reduced the number of glucocorticoid receptors, since this phenomenon has previously been observed (Davidson & Slaga, 1982; Salo & Oikarinen, 1985). Saturation binding of [^{3}H]dexamethasone to whole cells revealed that phorbol ester treatment of cells for 48 h caused a 43% decrease in the total number of [^{3}H]dexamethasone-binding sites from 61000 ± 8400 sites per cell in controls to 34800 ± 8400 sites per cell in phorbol ester-treated cells ($n = 4$). This difference was significant, with $P < 0.05$.

We investigated a possible mechanism by which glucocorticoids and butyrate might act to regulate β_2-adrenergic receptor gene expression. A CG-rich region exists 5′ to the coding region of the β_2-adrenergic receptor gene, beginning 700 bp upstream from the start of the gene and ending approx. 200 bp into the gene. We used methylation-sensitive restriction enzymes to determine if changes in methylation of this promoter region occurred upon treatment with dexamethasone or butyrate. Genomic 3T3-L1 DNA was isolated from control cells or cells treated for 24 h with 250 nM-dexamethasone, 5 mM-sodium butyrate, 1 µM-phorbol dibutyrate, or a combination. This DNA was digested with *Hpa*II or *Msp*I, separated on an agarose gel, blotted on to nitrocellulose and probed with a radioactively labelled 1.4 kb *Kpn*I fragment from the 3T3-L1 β_2-adrenergic receptor gene which began 850 bp 5′ to the gene and ended 500 bp into the coding region. These Southern blots revealed bands which contained a portion of the CG-rich 5′ region of the β_2-adrenergic receptor gene (Fig. 4).

The enzymes *Hpa*II and *Msp*I are isoschizmers which recognize the same CCGG site but differ in their ability to cut when cytosine residues within their recognition site are methylated. *Hpa*II does not cut when the internal cytosine is methylated whereas *Msp*I cuts regardless of the methylation status of the internal cytosine (Razin & Szyf, 1984). Mapping of *Hpa*II/*Msp*I sites in the region of DNA which we cloned predicts fragments of the following sizes (from 5′ to 3′): > 1611 bp, 50 bp, 152 bp, 21 bp, 21 bp, 43 bp, 18 bp, 129 bp, 85 bp, 53 bp, 689 bp, > 3428 bp. The sizes of the first and last fragments cannot be accurately determined because, for each, one site lies beyond the region which was sequenced. Fragments smaller than 200 bp, under the conditions employed, were not detected. The fragment of > 3.4 kb does not overlap with the probe used. This leaves two fragments, > 1.6 kb and 0.7 kb, which were observed after digestion with *Msp*I (Fig. 4). This same digestion pattern was observed for all the cell treatments. If all *Hpa*II sites were unmethylated, then the banding pattern for *Hpa*II should appear identical to that of *Msp*I. Instead, these expected bands were not observed upon *Hpa*II digestion (Fig. 4), suggesting that most, or all, of the sites studied were methylated and remained methylated upon dexamethasone or butyrate treatment.

DISCUSSION

Sequencing of the 3T3-L1 mouse β_2-adrenergic receptor gene and its flanking regions allowed us to compare the regulatory regions of this gene with those of other β-adrenergic receptor sequences. A similar comparison of the flanking regions of the β_2-adrenergic receptor genes from hamster and human has previously been reported (Kobilka *et al.*, 1987). The high degree of conservation (approx. 75%) of large stretches (1 kb) of 3′ and 5′ flanking regions from species to species suggests that important regulatory elements are necessary for proper control of receptor expression, and have therefore been preserved. Humans and rodents diverged approximately 75 million years ago. Using the rate of accumulation of mutations during evolution (about 1%

per million years) to calculate the expected similarity of human and rodent sequences under no selective pressure, a value of 52% has been derived (Yaffe *et al.*, 1985). Since the similarities of the β_2-adrenergic receptor 5′ and 3′ flanking regions from mouse and human (73% and 79% respectively) are significantly greater than this calculated value, these regions probably contain genetic elements which are required to regulate receptor expression and have therefore been subject to selective pressure to preserve their sequences.

The β_2- and β_1-adrenergic receptors differ significantly both in their coding sequences and in their 3′ and 5′ flanking sequences. This difference is reflected in the abilities of gene-activating agents such as glucocorticoids and butyrate to independently regulate the two β-adrenergic receptor subtypes. It has been proposed that the β-adrenergic receptor evolved from an ancestral gene which also gave rise to a number of other G-protein-coupled receptors (Ohno, 1987). From the parental β-adrenergic receptor, at least two subtypes were generated. The β_1-adrenergic receptor may have lost the ability to be regulated by gene-regulating compounds such as glucocorticoids or butyrate, or alternatively, the β_2-adrenergic receptor subtype may have acquired this ability. The purpose for this difference may lie in the functions of the two receptors. The β_1-adrenergic receptor is generally considered to be a neurotransmitter receptor (Broadley *et al.*, 1986). An increase in the number of β_1-adrenergic receptors on a target cell might not dramatically change the sensitivity of the cell to the large quantities of neurotransmitter released at the synapse. On the other hand, β_2-adrenergic receptors respond to very minute amounts of circulating hormone. An increase in the number of receptors on a cell's surface would enhance the ability of the cell to detect circulating catecholamines. Genetic analyses of the regulatory sequences of the two subtypes confirm the observed physiological controls of receptor subtype expression and offer an explanation as to why the subtypes differ in genetic regulation.

Only one GRE site was found in the reported β_1-adrenergic receptor sequence (Frielle *et al.*, 1987), while five GRE sites were found in a comparable region of 3T3-L1 β_2-adrenergic receptor sequence. This reflects the receptor regulation observed with glucocorticoid treatment of 3T3-L1 cells. β_2-Adrenergic receptors doubled in number whereas there was only a small, but significant, decrease in β_1-adrenergic receptors (Nakada *et al.*, 1987). It is possible that the GRE site which was found in the β_1-adrenergic receptor sequence may act to mediate this inhibition. GRE sites are known to mediate both gene activation (Evans *et al.*, 1982) and suppression (Adler *et al.*, 1988).

An exact match to the reverse of an 8 bp TRE consensus sequence was found 1 kb 3′ to the mouse β_2-adrenergic receptor gene. Phorbol ester induction of genes has been proposed to occur through genetic elements analogous to those used by glucocorticoids (Angel *et al.*, 1987). Enhancer sequences similar to GREs can exist in both orientations and be located either 5′ or 3′ to the gene. A specific 8 bp sequence will occur randomly only once in every 65 kb. The close proximity of this site to the β_2-adrenergic receptor gene, the proposed similarity of action of TRE and GRE sites, and the discovery of an identical sequence located at a similar distance from the human β_2-adrenergic receptor gene, led us to investigate whether phorbol esters may play a role in the genetic regulation of β_2-adrenergic receptors. We found that phorbol esters partially inhibited the increase in β_2-adrenergic receptors elicited by dexamethasone treatment, but did not affect the increase in β_2-adrenergic receptors caused by butyrate (Fig. 3*a*). The effects of phorbol esters were specific to dexamethasone-mediated changes in receptor expression.

Phorbol esters did not affect control levels of β_1- or β_2-adrenergic receptors, suggesting that they did not play a direct role in the activation of the receptor genes. A possible mechanism for the actions of phorbol esters on glucocorticoid-mediated effects was inactivation of the glucocorticoid receptor. [^{3}H]Dexamethasone binding sites were reduced in cells treated with phorbol esters. It has been previously demonstrated that glucocorticoid receptor levels can be reduced by prolonged treatment with phorbol esters (Davidson & Slaga, 1982; Salo & Oikarinen, 1985). Phorbol esters do not seem to regulate β_2-adrenergic receptor expression through a TRE site, but instead down-regulate glucocorticoid receptor levels through an unknown mechanism.

The studies addressing the methylation status of the CG-rich promoter region of the 3T3-L1 β_2-adrenergic receptor gene do not support a proposed role for methylation in the regulation of β_2-adrenergic receptor gene expression by dexamethasone or butyrate. Because *Hpa*II digestion did not generate any of the smaller fragments seen with digestion by *Msp*I, we conclude that the sites which generated the *Msp*I fragments were methylated, and remained methylated while the cells were treated with dexamethasone or butyrate. The inability of *Hpa*II to cut the DNA was not due to inactivation of the restriction enzymes by contaminants in the genomic DNA, since plasmid DNA containing the β_2-adrenergic receptor gene was completely cut by the enzymes in the presence of 3T3-L1 DNA (results not shown).

Previous studies using a similar methodology have been able to detect changes in methylation (Compere & Palmiter, 1981). Because $< 15\%$ of the total CG dinucleotides in the DNA are recognized with this technique, it is possible that specific sites at which methylation plays an important role in gene activation were not detected in this study.

We have previously characterized the effects of two gene-regulating agents, glucocorticoids and butyrate, on β-adrenergic receptor expression in 3T3-L1 cells. Sequencing of the 3T3-L1 β_2-adrenergic receptor revealed GRE sites which may function to mediate glucocorticoid-induced increases in receptor number. A phorbol ester-responsive element was also found which does not appear to play a role in receptor regulation at the genetic level. Surrounding the promoter region of the gene lies a CG-rich region which is generally methylated. Regulation of β_2-adrenergic receptor expression does not appear to be correlated with changes in the methylation of this region, but may instead be correlated with other changes such as DNA unwinding or histone modification. The independent regulation of the two β-adrenergic receptor subtypes is parallelled by the difference in their flanking sequences.

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