2 and 78, maximum iterations: 10, convergence: 0.001, low and high density surface numbers: 2 and 10. Ligand solvation energy was subtracted from TS interaction energy, recalculated with a Delphi map that used the Hydren radius, a dielectric constant, and van der Waal parameters. The 67 sphere centers were treated as uncharged atoms of radius 1.4 Å in the Delphi calculation, which reduced the dielectric in the binding site to two.

For the secondary and tertiary searches, the crystallographic phosphate was included in the Distmap and Delphi calculations. Ligands were docked with the positions of the SB atom in each of its two TS complexes acting as spheres centers. The SB atoms were treated as protein atoms of radius 1.4 Å in the Delphi calculation.

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Deficiency in Rhabdomyosarcomas of a Factor Required for MyoD Activity and Myogenesis

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Rhabdomyosarcoma cells express the myogenic helix-loop-helix proteins of the MyoD family but do not differentiate into skeletal muscle cells. Gel shift and transient transfection assays revealed that MyoD in the rhabdomyosarcoma cells was capable of binding DNA but was relatively nonfunctional as a transcriptional activator. Heterokaryon formation with fibroblasts resulted in the restoration of transcriptional activation by MyoD and the differentiation of the rhabdomyosarcoma cells into skeletal muscle cells. These results suggest that rhabdomyosarcomas are deficient in a factor required for MyoD activity.

Rhabdomyosarcomas are one of the most common solid tumors of childhood. The expression of the myogenic determination gene *MyoD* has been shown as the most sensitive marker for the classification of sarcomas as rhabdomyosarcomas (*1*, *2*). This relation is paradoxical because MyoD, a member of the helix-loop-helix (HLH) family of transcription factors (*3*), has been shown to induce muscle differentiation in a wide variety of primary cells and transformed cell lines (*4*). However, rhabdomyosarcomas have a low propensity to differentiate into myotubes. Therefore, we analyzed several rhabdomyosarcoma cell lines for MyoD expression and function.

Three rhabdomyosarcoma cell lines were used to assess HLH gene expression: RD, Rh18, and Rh30 (*5*). Northern (RNA) analysis (Fig. 1A) demonstrated that these cells contained normal amounts of *myogenin* and *E2A* (*6*) transcripts and either normal or reduced amounts of *Id* (*7*) transcripts compared with the amounts detected in primary cultured human muscle cells. The RD and Rh30 cells, but not the Rh18 cells expressed *MyoD* RNA. All three rhabdomyosarcoma lines expressed *Myf5* (*8*). Indirect immunofluorescence experiments with antibodies to MyoD (Fig. 2), myogenin, and E2A (*8*) revealed a normal nuclear localization for each protein in the RD and Rh30 cells. The rhabdomyosarcoma cells exhibited a similar MyoD immunoreactivity as primary human muscle cells (*8*). Because the RD and Rh30 cells had no obvious abnormalities in expression of the HLH genes that are thought to be critical for regulating myogenesis, these two cell lines were chosen for further analysis.

To investigate the DNA binding properties of MyoD in the RD and Rh30 cells, we prepared nuclear extracts from the cells and used a gel shift assay to look for complexes that bound to a ^{32}P-labeled probe that contained a MyoD binding site. Similar to the positive control (nuclear extracts from C2C12 mouse myoblasts), the RD and Rh30 extracts produced two major species (bands 1 and 2) that were sensitive to competition with low amounts of unlabeled probe but insensitive to competition with an unlabeled probe that contained a mutation in the MyoD binding site (Fig. 1B). Antibodies against MyoD and E2A were used in double-shift assays to show that the slower of the two major species (band 1) contained MyoD and E2A in both C2C12 and rhabdomyosarcoma cells (*8*).

To determine whether an increase in the amount of MyoD could induce differentiation in RD and Rh30 cells, we transfected the cells with a *MyoD* expression vector (pLTR-*MyoD*) (*9*) and examined expression of MyoD and myosin heavy chain (MyHC) by immunohistochemistry. The transfected cells expressed high amounts of MyoD in the nucleus (Fig. 2, C and E). As in nontransfected cultures (Fig. 2B), about 1% of all cells underwent spontaneous differentiation to a muscle phenotype, which we determined by their MyHC expression,

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Table 1. MyoD, E12, and MyoD-VP16 (MDVP16) activity in fibroblasts and rhabdomyosarcomas. Cells were transfected (*19*) with the reporter construct (5 μg) and the indicated expression vector (5 μg). The *MyoD* expression vector uses the Moloney sarcoma virus LTR (MSV-LTR) to drive the *MyoD* cDNA. Therefore, to quantitate expression from the MSV-LTR after transfection into different cell types, we transfected cells in parallel with a construct that contained the CAT gene driven by the MSV-LTR, and the data were normalized to CAT activity from this construct.

Cell line	Reporter	CAT activity (10^{-3} cpm)						
		LTR	LTR-MyoD	LTR-E12	LTR-E12 + LTR-MyoD	LTR-MDVP16	Gal-MyoD	Gal1-147
10T1/2	4RTK-CAT	1	87	18	826	553		
RD	4RTK-CAT	2	6	27	29	161		
Rh30	4RTK-CAT	6	6	8	20	65		
Rh28	4RTK-CAT	3	4	4	4	35		
RhJT	4RTK-CAT	4	4	4	4	77		
10T1/2	GM4TK-CAT		47				53	0
RD	GM4TK-CAT		2				209	0
Rh30	GM4TK-CAT		8				132	0

but differentiation was not increased in the transfected cells that expressed large amounts of MyoD (Fig. 2, D and F). Hiti *et al.* (*10*) concluded that stable transfection of the RD cell line with a *MyoD* expression vector increased the percentage of colonies with myotubes and the number of MyHC-expressing cells. However, as in our observations, the effect of MyoD on differentiation in the RD cells was small.

To establish whether MyoD can function as a transcriptional activator in rhabdomyosarcoma cells, we performed transient transfection assays with a reporter construct (p4RTK-CAT) that contained the chloramphenicol acetyltransferase (CAT) gene driven by four multimerized MyoD binding sites that were cloned upstream of the herpes simplex thymidine kinase (TK) promoter. Induction of CAT in 10T1/2 cells that had been cotransfected with the p4RTK-CAT reporter construct and the pLTR-*MyoD* expression vector was almost 100 times greater than in the cells transfected with the reporter alone. In contrast, no significant CAT induction occurred in any of the four rhabdomyosarcoma cell lines transfected with pLTR-*MyoD* (Table 1). Similar results were obtained with a reporter construct driven by the muscle creatinine kinase (MCK) enhancer (pMCK-CAT) (*8*). The low basal CAT activity in the rhabdomyosarcoma cells that were transfected with the reporter construct alone is probably due to endogenous MyoD or another endogenous HLH protein because this activity was inhibited by cotransfection with an *Id* expression vector (*8*).

In vivo MyoD is thought to bind DNA as a heterodimer with an E2A protein, such as E12 or E2-5 (*11*). Cotransfection of pLTR-*MyoD* and pLTR-*E12* expression vectors acted synergistically on p4RTK-CAT in 10T1/2 cells, but in rhabdomyosarcoma cells E12 did not rescue MyoD activity (compare LTR-*E12* with LTR-*E12* + LTR-*MyoD* in Table 1). Similar results were obtained with an expression vector for *E2-5*. Therefore, it is unlikely that deficiency of MyoD, E12, or E2-5 limits the activation of MyoD-dependent reporter constructs in rhabdomyosarcoma cells.

The inability of MyoD to trans-activate p4RTK-CAT in rhabdomyosarcoma cells might have resulted from a failure of MyoD to bind the appropriate DNA site or from a failure of the bound complex to function. To test whether the transfected MyoD is capable of binding, we used a chimeric protein that consisted of MyoD and the transcriptional activation domain of the HSV VP16 protein (pLTR-*MDVP16*) (*12*). CAT activity was ten times greater in rhabdomyosarcoma cells that expressed the chimeric protein than in cells that expressed native MyoD (Table 1). These data suggest that the MyoD-VP16 chimeric protein and presumably the native MyoD protein can bind to MyoD binding sites in the rhabdomyosarcoma cells.

To test whether the MyoD activation domain can function in the rhabdomyosarcoma cells, we transfected the cells with a gene that encodes a chimeric protein composed of the DNA binding region of *Gal4* (p*Gal1-147*) and of the intact MyoD protein (p*Gal-MyoD*) (*12*). We cotransfected a reporter construct (pGM4TK-CAT) that contained the CAT gene driven by four MyoD sites that alternated with four Gal4 binding sites cloned upstream of the TK

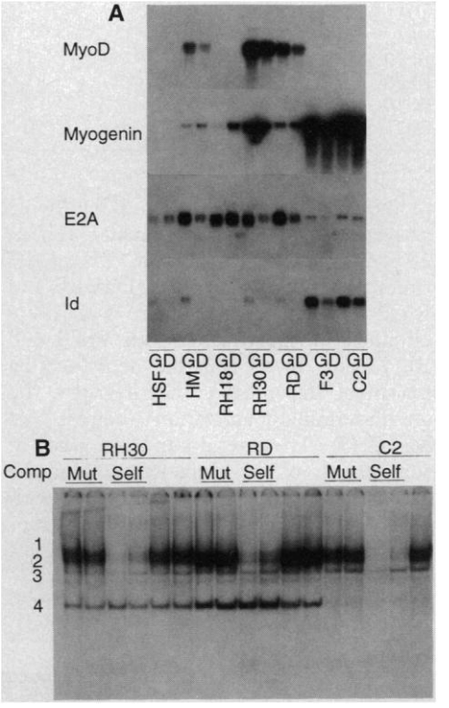

Fig. 1. Expression of HLH genes and DNA binding properties of HLH proteins in rhabdomyosarcoma cells. (**A**) RNA analyses were performed on human skin fibroblasts (HSF), primary human myoblasts (HM), three rhabdomyosarcomas (Rh18, Rh30, and RD), a myoblast cell line derived from mouse 10T1/2 fibroblasts (F3), and the C2C12 mouse myoblast line (C2) (*16*). Cells were cultured in high serum growth medium (G) or for 2 days in low serum [2% horse serum with insulin (10 μg ml^{-1}) and transferrin (10 μg ml^{-1})] differentiation medium (D) before RNA isolation. The RNA samples (5 μg) were subjected to electrophoresis on 1.2% agarose gels containing 6.7% formaldehyde. The *MyoD* probe was specific for human sequences and so did not label the RNA from the cells of lines F3 and C2; however, previous studies have shown that these cell types have abundant amounts of mouse *MyoD* (*10*). Quantitative cross-species comparisons cannot be made because of the differing cross-hybridization efficiencies of each probe. (**B**) Gel shift assays with nuclear extracts from rhabdomyosarcoma cells (RD and Rh30) or from C2C12 myoblasts (C2). The "right" MyoD binding site from the MCK enhancer (*17*) was used as a probe. The competition (Comp) experiments were performed with a 20-fold and a 100-fold molar excess of the unlabeled probe (Self) or the probe containing a mutation in the MyoD binding site (Mut). Nuclear extracts were prepared and gel shift assays performed as described in (*11*).

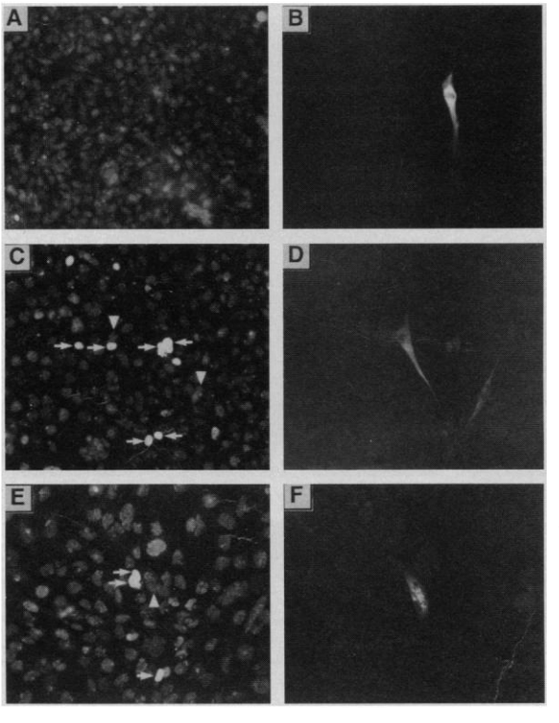

Fig. 2. RD and Rh30 rhabdomyosarcoma cells stained with antisera to (A, C, and E) MyoD and to (B, D, and F) MyHC. (**A** and **B**) RD cells show normal nuclear staining of (A) MyoD but rarely show staining of (B) MyHC. (**C** and **D**) RD cells transiently transfected with the pLTR-*MyoD* expression vector show intense nuclear staining of MyoD (arrows) but no staining of MyHC. The nuclei of the MyHC- positive cells in (D) are indicated by arrowheads in (C). (**E** and **F**) Rh30 cells transiently transfected with LTR-MyoD show elevated amounts of nuclear MyoD immunoreactivity (arrows). Again, the MyHC-positive cell in (F) does not display intense staining of MyoD [arrowhead in (E)].

Table 2. Differentiation in heterokaryons that were formed between rhabdomyosarcoma cells and fibroblasts. Numbers are the percentage of multinucleated cells that stained positive with antiserum to MCK. In the fusions with 10T1/2 cells, only heterokaryons were counted; in the fusions between different rhabdomyosarcoma lines, multinucleated cells with three or more nuclei were counted. Abbreviations: emb, embryonal; alv, alveolar.

Cell line	Cell line					
	10T1/2	RD (emb)	Rh30 (alv)	Rh28 (alv)	Rh18 (emb)	RhJT (alv)
10T1/2	0					
RD	94	4				
Rh30	30	3	1			
Rh28	70	8	1	1		
Rh18	0	0	0	0	0	
RhJT	85	12	0	6	1	7

promoter. Although MyoD and Gal-MyoD activated pGM4TK-CAT equally well in 10T1/2 cells, Gal-MyoD was 15 to 100 times more effective than MyoD in RD and Rh30 cells (Table 1). Similar results were obtained in experiments with Rh28 and RhJT cells. In all cells the activation by Gal-MyoD depended on the integrity of the amino-terminal activation domain of MyoD (8). These results indicate that, in the context of the chimeric protein, the activation domain of MyoD can work with other factors to establish an active transcription complex in the rhabdomyosarcoma cells.

The inactivity of MyoD in rhabdomyosarcomas could be due to a factor that suppresses MyoD function or to the absence of a factor that is required for MyoD function. To test these possibilities, we generated heterokaryons between 10T1/2 cells and five rhabdomyosarcoma cell lines by polyethylene glycol–mediated cell fusion. After fusion, the cells were stained with antisera to MCK and to MyHC. The DNA stain 4′,6-diamidino-2-phenyl indole (DAPI, Sigma) was used to distinguish between the rhabdomyosarcoma nuclei and the fibroblast nuclei. For four of the five rhabdomyosarcoma lines, heterokaryon formation produced a high proportion of cells that expressed MCK (Table 2) and MyHC (Fig. 3, B and D) (8). In addition, when these rhabdomyosarcoma cells were fused to a line of 10T1/2 cells that had been transfected with the pMCK-CAT reporter construct, the heterokaryons expressed CAT activity (Fig. 3, A and C). When RD cells were transfected with a reporter construct consisting of multimerized MyoD binding sites, the TK promoter, and the β-galactosidase gene (p4RTK–β-gal), heterokaryon formation produced a high proportion of cells that expressed β-galactosidase (8). These results demonstrate that 10T1/2 fibroblasts complemented the differentiation defect in these four rhabdomyosarcoma cell lines and that the 10T1/2 cells provide a factor to the rhabdomyosarcoma cells that permits MyoD to function as a transcriptional activator.

We also generated heterokaryons between the five different rhabdomyosarcoma cell lines to see if they would complement each other. On the basis of MCK immunohistochemistry, none of the rhabdomyosarcoma cell lines complemented each other (Table 2). Rhabdomyosarcomas are grouped by histologic and cytogenetic criteria as either embryonal or alveolar: A balanced translocation, t(2:13)(q35;q14), is associated with alveolar rhabdomyosarcomas, whereas a loss of heterozygosity that encompasses chromosome 11p15.5 is associated with embryonal rhabdomyosarcomas (*1*, *13*). Cell lines from both embryonal and alveolar rhabdomyosarcomas (Table 2) failed to complement each other in the heterokaryon assay, which suggests that these distinct tumor types share a common genetic deficiency. Fusion with Rh18 cells suppressed spontaneous differentiation, suggesting that this line, which is not complemented by 10T1/2 cells, may contain dominant negative suppressors of differentiation.

The inhibition of differentiation in four rhabdomyosarcoma cell lines is recessive, consistent with the loss or inactivity of a gene that is required for myogenic differentiation. The data suggest that MyoD can bind DNA in the rhabdomyosarcoma cells but cannot effectively enhance transcription. The 10T1/2 cells may supply a gene product or products necessary for MyoD function that is deficient in rhabdomyosarcoma cells. For example, the rhabdomyosarcoma cells may contain an inhibitor of MyoD function and 10T1/2 cells may supply an inhibitor of that inhibitor. This possibility seems less probable because many heterokaryons that display complementation contain four or more rhabdomyosarcoma nuclei to one fibroblast nucleus.

Many rhabdomyosarcomas develop in tissues not known to contain muscle stem cells. Microcell transfer experiments have demonstrated that, in primary fibroblasts, chromosomes 4, 8, or both repress *MyoD*

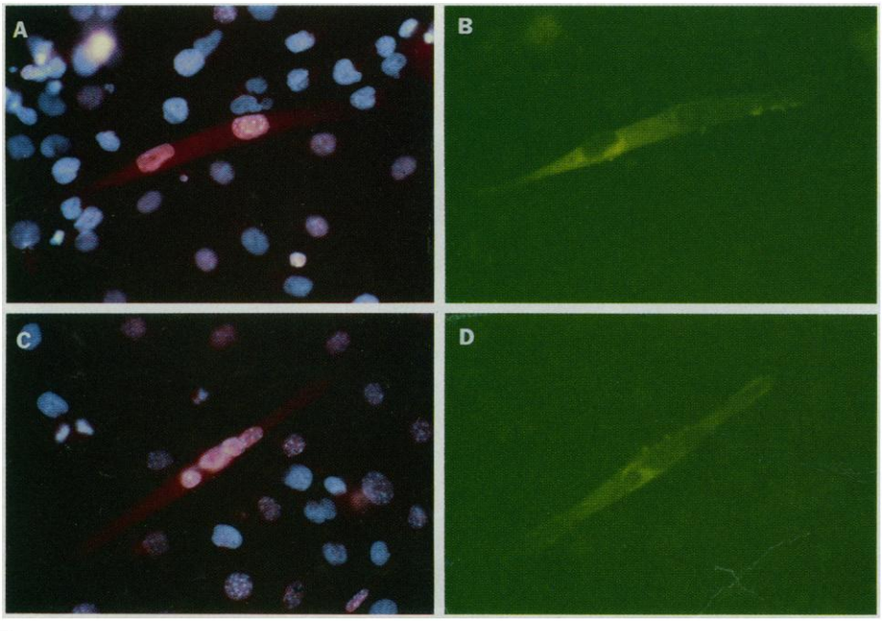

Fig. 3. Heterokaryons generated between 10T1/2 mouse fibroblasts and RD or Rh30 rhabdomyosarcoma cells after staining with antisera to CAT and to MyHC (*18*). (**A**) Double exposure showing rhodamine staining of an antibody to CAT (red) and DAPI staining of DNA (blue). The human nuclei from the rhabdomyosarcoma cells stain homogeneously with DAPI, whereas the mouse nuclei from the fibroblasts stain in a reticulated pattern. The cell expressing CAT contains one nucleus from a mouse and one from a human. (**B**) The same heterokaryon as in (A), showing localization of antibody to MyHC with a fluorescein-conjugated secondary antibody (green). (**C** and **D**) Stained as in (A) and (B), respectively, but the rhabdomyosarcoma is the Rh30 line and in this case there are three mouse nuclei to one human nucleus in the heterokaryon.

transcription (*14*). To generate a muscle cell from nonmuscle tissue, fibrosarcoma cells may repress (or lose) these loci, resulting in the activation of *MyoD* transcription. Because MyoD can induce growth arrest (*15*), its expression in a fibrosarcoma may serve as a "fail-safe" mechanism to prevent cell growth. Additional genetic defects that prevent MyoD function, such as the recessive defects described here, would prevent differentiation and result in the generation of a rhabdomyosarcoma. A similar mechanism may apply to other types of tumors that express full or partial differentiation programs.

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6. The *E2A* gene codes for *E12*, *E47*, and *E2-5* RNA (*3*).
7. Id is a negative regulator of MyoD [R. Benezra, R. L. Davis, D. Lockshon, D. L. Turner, H. Weintraub, *Cell* **61**, 49 (1990); J. Yale, H. Weintraub, R. Benezra, *Genes Dev.* **6**, 1466 (1992)].
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18. Cells were plated together in 6-cm tissue culture dishes, allowed to grow for 24 hours, and then rinsed with Dulbecco's minimum essential medium (DMEM, Sigma) at 37°C. Sterile 50% polyethylene glycol in DMEM was added for 1 min, and the cells were then washed four times with DMEM. Cells were fed again, with DMEM supplemented with 15% calf serum (Hyclone) for 24 hours, and switched to low-serum medium for 48 hours. For fixation, cells were treated with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 5 min at room temperature. Antisera for MyoD [S. J. Tapscott *et al.*, *Science* **242**, 405 (1988)] and MyHC [D. Bader, T. Masaki, D. A. Fischman, *J. Cell Biol.* **95**, 763 (1982)] have been described. The antiserum to CAT was obtained from 5-Prime 3-Prime (Boulder, CO).
19. All cells were grown in DMEM containing penicillin and streptomycin and supplemented with 15% calf serum (Supplemented Calf Serum, Hyclone). Transient transfections and CAT assays were performed as described in (*12*).

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Antagonism of Catecholamine Receptor Signaling by Expression of Cytoplasmic Domains of the Receptors

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The actions of many hormones and neurotransmitters are mediated by the members of a superfamily of receptors coupled to heterotrimeric guanine nucleotide–binding proteins (G proteins). These receptors are characterized by a highly conserved topographical arrangement in which seven transmembrane domains are connected by intracellular and extracellular loops. The interaction between these receptors and G proteins is mediated in large part by the third intracellular loop of the receptor. Coexpression of the third intracellular loop of the α_{1B}-adrenergic receptor with its parent receptor inhibited receptor-mediated activation of phospholipase C. The inhibition extended to the closely related α_{1C}-adrenergic receptor subtype, but not the phospholipase C–coupled M1 muscarinic acetylcholine receptor nor the adenylate cyclase–coupled D_{1A} dopamine receptor. These results suggest that the receptor–G protein interface may represent a target for receptor antagonist drugs.

The binding of agonists to G protein–coupled receptors induces conformational changes in the receptor that promote formation of an agonist receptor–G protein ternary complex (*1*) that leads to G protein activation and productive coupling to the effector. The interaction between receptor and G protein is mediated by the intracellular loops of the receptor. Mutagenesis studies of the third intracellular (3i) loop of several G protein–coupled receptors indicate that this region is critical for productive coupling (*2*). Studies with chimeric adrenergic receptors (ARs) (*3*, *4*) and muscarinic cholinergic receptors (AChRs) (*5*) implicate the putative 3i loop as the major, but not the sole, determinant of selective coupling to their respective G proteins.

A large number of clinically important therapeutic agents are drugs that can occupy the ligand-binding pocket of G protein–coupled receptors without inducing receptor activation, also known as receptor antagonists. To date, virtually all known antagonists of such receptors share the common property of blocking access of the ligand to the receptor. We wondered whether an alternative strategy for designing receptor antagonists might be to block the interaction of the receptor itself with the G protein. Given the importance of the 3i loop of several G protein–coupled receptors in forming the site of contact between the receptor and G protein, we tested the feasibility of expressing this isolated region of the receptor in intact cells and its effects on receptor function. We report that coexpression of the 3i loop with its homologous receptor resulted in a specific antagonism of receptor-mediated signaling.

To determine the effects of the putative 3i loop of the α_{1B}-AR on basal and receptor-mediated second messenger production, we prepared minigene constructs (Fig. 1A) that encoded either the full 3i loop (residues 225 to 295) (α_{1B}-3i), its NH_2-terminal portion (residues 225 to 272) (α_{1B}-3iN), or its COOH-terminal portion (residues 273 to 295) (α_{1B}-3iC). The α_{1B}-3iN construct spans a 27–amino acid motif, residues 233 to 259, important in determining the selectivity of receptor–G protein coupling (*4*). The α_{1B}-3iC construct contains a 7–amino acid domain, residues 288 to 294, that influences the affinity of agonist binding and the potency of agonist-stimulated activation of phospholipase C (PLC) (*3*, *6*). Another minigene construct

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