such as DOX, with an established clinical profile, may offer a safety advantage over more potent but less defined agents. The toxic effects of DOX are dose-related and it is likely that increasing the intratumoral concentration of DOX will produce a significant increase in antitumor activity (*31*, *32*). Although studies on human tumors growing in immunocompromised animals are not ideal to predict anticancer activity in humans, our findings support the clinical evaluation of BR96-DOX.

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33. We thank W. Scott, B. Seizinger, P. Wallace, M. Jure, W. Rose, D. Vyas, J. Brown, and P. Senter for helpful comments and suggestions, and C. Bull and K. Tebolt for technical assistance.

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Separable Regulatory Elements Governing *myogenin* Transcription in Mouse Embryogenesis

Tse-Chang Cheng, Mia C. Wallace, John P. Merlie, Eric N. Olson*

Expression of the myogenic helix-loop-helix (HLH) protein myogenin in muscle cell precursors within somites and limb buds is among the earliest events associated with myogenic lineage determination in vertebrates. Mutations in the *myogenin* promoter that abolish binding sites for myogenic HLH proteins or myocyte enhancer factor–2 (MEF-2) suppressed transcription of a linked *lacZ* transgene in subsets of myogenic precursors in mouse embryos. These results suggest that myogenic HLH proteins and MEF-2 participate in separable regulatory circuits leading to *myogenin* transcription and provide evidence for positional regulation of myogenic regulators in the embryo.

The formation of skeletal muscle during development involves a series of events in which multipotential mesodermal stem cells give rise to myoblasts, which ultimately undergo terminal differentiation in response to external cues. Analysis of muscle determination and differentiation in tissue culture has revealed a family of muscle-specific HLH proteins including MyoD, myogenin, Myf-5, and MRF-4, each of which can activate myogenesis in a variety of cell types in vitro (*1*). These myogenic regulators are expressed only in skeletal muscle and are first detected during embryogenesis within myogenic precursor cells in the myotomal compartment of the somites and in the limb buds, localizations consistent with their involvement in myogenic lineage specification (*2–4*).

Activation of muscle-specific transcription by myogenic HLH proteins is mediated by their direct binding to the E box consensus DNA sequence CANNTG in the control regions of most muscle-specific genes (*1*). These regulatory factors also positively auto- and cross-regulate their own transcription in transfected cells (*5*, *6*). Whether autoregulatory interactions among these genes contribute to their expression during embryogenesis or whether this is simply a tissue culture phenomenon is unknown.

An indirect pathway for muscle gene activation has also been described in which myogenin and MyoD induce myocyte enhancer factor–2 (MEF-2) (*7*, *8*), which binds an AT-rich DNA sequence associated with numerous muscle-specific genes (*9*). MEF-2 can activate muscle transcription in the absence of the E box consensus sequence (*10–12*) and is up-regulated when myoblasts enter into the differentiation pathway (*9*). Paradoxically, activation of *myogenin* gene transcription in cultured muscle cells requires binding of MEF-2 to the *myogenin* promoter (*6*). These results suggest that myogenin and MEF-2 participate in a complex regulatory circuit involving positive feedback loops that amplify their expression and stabilize the myogenic program.

Whereas much has been learned about the mechanisms through which myogenic HLH proteins activate muscle-specific transcription in cultured cells, little is known of the mechanisms that regulate muscle gene expression during embryogenesis or of the regulatory circuits that control expression of the myogenic regulators themselves. Because myogenin is the only myogenic HLH protein expressed in all skeletal muscle lines (*13*, *14*), analysis of the mechanisms that control its expression should reveal upstream regulators of myogenic lineage specification. Here, we used *lacZ* transgenes linked to wild-type and mutant *myogenin* promoters to begin to define the regulatory networks that direct *myogenin* transcription in the mouse embryo.

The reporter gene Myo1565lacZ, which contains *lacZ* linked to the region extending from +18 to −1565 base pairs (bp) relative to the *myogenin* transcription initiation site (Fig. 1), was expressed in the same embryonic cells as the endogenous gene (Fig. 2, A and D) and therefore serves as a marker for activation of *myogenin* transcription in individual cells (*4*). Expression of *lacZ* from this transgene can be detected in rostral somites by day 9.0 after coitus (p.c.); expression progresses caudally over the next several days concomitant with somite maturation (*2*, *4*, *13*, *15*). In em-

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bryos from day 10.5 p.c., *lacZ* was expressed in the myotomal region of the 30 rostral-most somites and in the first visceral arch (Fig. 2A). By day 11.5 p.c., the transgene was expressed in nearly all somites as well as in myogenic cells within the fore- and hind-limb buds and the visceral arches (Fig. 2D). A 202-bp *myogenin* promoter fragment (from −184 to +18 bp) linked to *lacZ* also showed muscle-specific expression, but the level of expression directed by the truncated promoter was lower (*16*). We therefore used the segment from −1565 to +18 bp for further analysis of the *myogenin* control region.

To determine whether auto- or cross-regulatory interactions among myogenic HLH proteins were important for transcriptional activation of *myogenin* in somites or limb buds, we analyzed the function of an E box, called E1, in the *myogenin* promoter

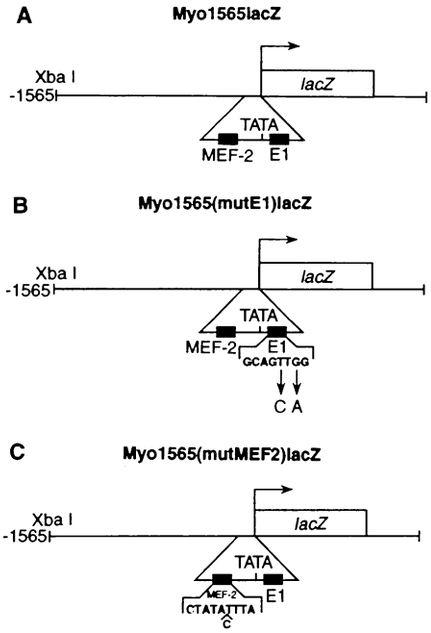

Fig. 1. Expression vectors for creation of transgenic mice. (**A**) The parental vector Myo1565-lacZ contains an Xba I–Hae III restriction fragment extending from −1565 to +18 bp, relative to the *myogenin* transcription start site, inserted into the polylinker sites of the promoterless pAUGLacZ vector (*4, 6*). (**B**) Myo1565(mutE1)-lacZ and (**C**) Myo1565(mutMEF2)lacZ were created by site-directed mutagenesis of Myo1565lacZ (*30*). The E1 E box and the MEF-2 site are located at −16 to −11 and −68 to −59 bp, respectively, relative to the transcription start site (*6*). Specific nucleotide changes in mutant promoters are indicated. Myo1565(mutE1)lacZ contains two nucleotide substitutions in the E box motif, which abolish binding of myogenic HLH proteins. Myo1565-(mutMEF2)lacZ contains one nucleotide insertion in the center of the MEF-2 site, which abolishes binding of MEF-2. Myo1565(mutE1/mutMEF2)lacZ contains both of the above mutations in the E1 and MEF-2 sites.

that binds myogenic HLH proteins with high affinity (6). Within the region extending to −184 bp relative to the transcription initiation site, which is sufficient to direct *myogenin* transcription in transgenic mice, E1 is the only E box that is conserved in the mouse (6), human (*17*), and rat (*18*) *myogenin* promoters. Mutation of E1 in the transgene Myo1565(mutE1)lacZ (Fig. 1) had little or no effect on *lacZ* expression in somites but greatly diminished expression in the limb buds and reduced expression in the visceral arches at day 11.5 p.c. (Figs. 2E and 3B) (*19*). Myo-1565(mutE1)lacZ was expressed, however, in differentiated muscle fibers in the limbs beginning about 1 day later, which suggests that the spectrum of *myogenin* activators changes during development.

To determine whether MEF-2 regulated *myogenin* transcription in the embryo as it does in tissue culture (6), we mutated the MEF-2 site in the context of the −1565- to +18-bp 5′ flanking region [Myo1565(mut-MEF2)lacZ] (Fig. 1). At day 10.5 p.c., this mutant transgene was expressed in the rostral-most somites in a manner similar to the expression of the wild-type transgene (Fig. 2C). However, in somites posterior to somite 7, there was a decreasing gradient of *lacZ* expression, especially in the dorsal regions of the somites posterior to somite 10. There was also a complete loss of expression in the central portions of these somites (Figs. 2, C and F, and 3D). Myo1565(mutMEF2)lacZ also failed to be expressed in limb buds at day 11.5 p.c., when the wild-type transgene was highly active (Fig. 2F). Expression in the visceral arches was also reduced at this time. Thus, the MEF-2 site is required for temporal regulation of *myogenin* transcription in the limb buds and in a subset of cells in the somite myotome. By day 12.5 p.c., Myo1565(mutMEF2)lacZ began to be expressed in the limb buds and back muscles, but the level of expression was substantially lower than that of the wild-type transgene or Myo1565(mutE1)lacZ (*20*).

To further assess the importance of the E1 and MEF-2 sites to *myogenin* gene activation, we mutated both sites within the −1565- to +18-bp 5′ flanking region,

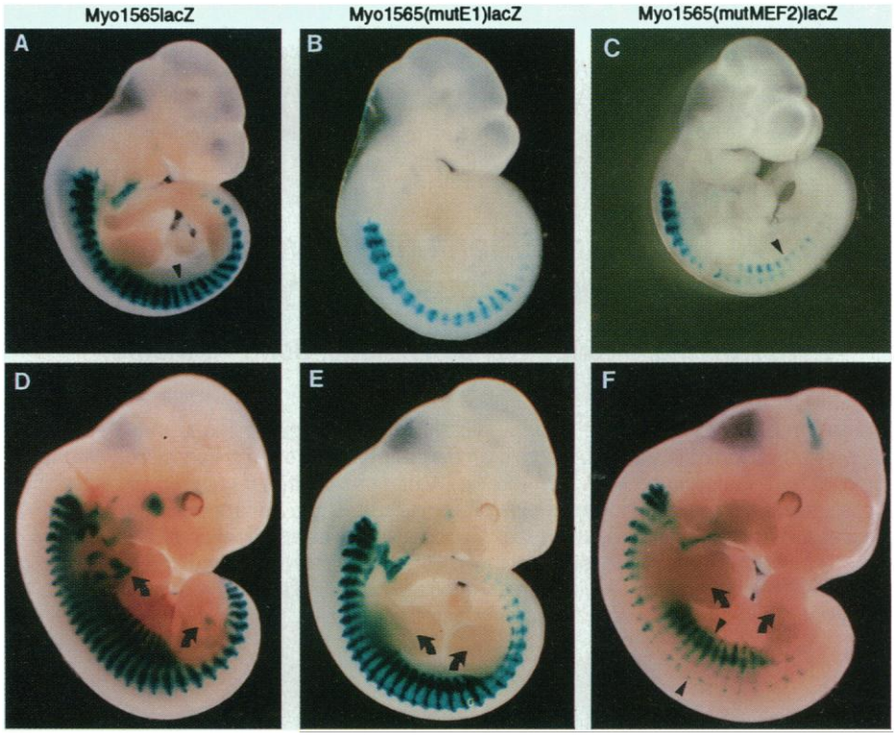

Fig. 2. Whole-mount staining of embryos with wild-type and mutant *myogenin-lacZ* transgenes. Transgenic mice were produced by injection of the indicated reporter genes (*31*), and embryos were stained for *lacZ* activity (*32*). (**A** to **C**) Day 10.5 p.c. embryos. Myo1565lacZ is expressed in the 30 rostral-most somites at day 10.5 p.c. Myo1565(mutE1)lacZ showed a pattern of expression in the somites similar to that of the wild-type transgene. The arrowheads in (A), (C), and (F) denote somite 18. Somites in this region show homogeneous *lacZ* staining in embryos with Myo1565lacZ, but staining is confined to the ventral region in embryos with Myo1565(mutMEF2)lacZ. (**D** to **F**) Day 11.5 p.c. embryos. Myo1565lacZ was expressed in the tail somites, the forelimb buds, and the visceral arches. Expression in the hindlimb bud is just beginning at this stage. Myo1565(mutE1)lacZ showed little expression in the limb buds. Expression of Myo1565(mutMEF2)lacZ was absent from limb buds and was primarily confined to the ventral portions of the thoracic somites. Muscle-forming regions in the limb buds are indicated with arrows.

yielding the transgene Myo1565(mutE1/mutMEF2)lacZ. This mutant promoter failed to direct *lacZ* expression in transgenic embryos at day 10.5 or 12.5 p.c. (*21*). The lack of expression of this transgene in cells where single-site mutations are active suggests that the E1 and MEF-2 sites may be functionally redundant in those cells.

Our results suggest that activation of *myogenin* transcription during myogenic lineage specification in the mouse embryo is mediated by an interplay between myogenic HLH proteins and MEF-2, which bind conserved sites in the *myogenin* promoter. The importance of the E1 and MEF-2 sites for *myogenin* transcription in subsets of myogenic precursors was not anticipated by mutational analyses of the *myogenin* promoter in muscle cells in culture, in which the MEF-2 site is essential for efficient transcription and E1 does not substantially affect *myogenin* expression (*6*). That promoters containing E1 or MEF-2 site mutations ultimately become expressed in differentiated muscle fibers (*20*) indicates that activation and maintenance of *myogenin* transcription are controlled by different combinations of regulators.

The delayed expression of *myogenin-lacZ* transgenes lacking E1 in the early limb bud in mouse embryos suggests that the *myogenin* gene is a target for cross-activation by myogenic HLH proteins in the embryo. The Myf-5 protein is the only myogenic HLH protein known to be expressed in the limb bud before myogenin (*2*), which makes it a likely candidate to act as a regulator through the E1 site. Alternatively, activation through the E1 site could reflect positive autoregulation by the myogenin protein acting on its own promoter (*22*).

Given the ability of myogenic HLH proteins to induce MEF-2 activity in tissue culture (*7, 8, 11*), the apparent dependence of *myogenin* transcription on MEF-2 seems paradoxical. Activation of *myogenin* transcription through the MEF-2 site may reflect an indirect autoregulatory loop in which Myf-5 or myogenin induce MEF-2, which feeds back on the *myogenin* promoter, or it could indicate that MEF-2 is initially expressed in the somites or limb buds independently of myogenic HLH proteins. Consistent with the latter possibility, recent experiments indicate that at least two of the four mouse MEF-2 genes are expressed in the somites (*23*).

The differential activation of mutant *myogenin-lacZ* transgenes in the somites and limb buds of mouse embryos provides evidence for positional regulation of myogenic regulators in the embryo. Whether this position dependence is an intrinsic property of specific myogenic lineages or is extrinsically imposed by environmental cues remains to be determined. It is known, however, that limb and back muscle progenitors are derived from different lineages in the somite that segregate before gastrulation (*24, 25*). The MEF-2 and E1 mutations in the *myogenin* promoter may therefore be revealing steps in the myogenic determination process that differ between these lineages.

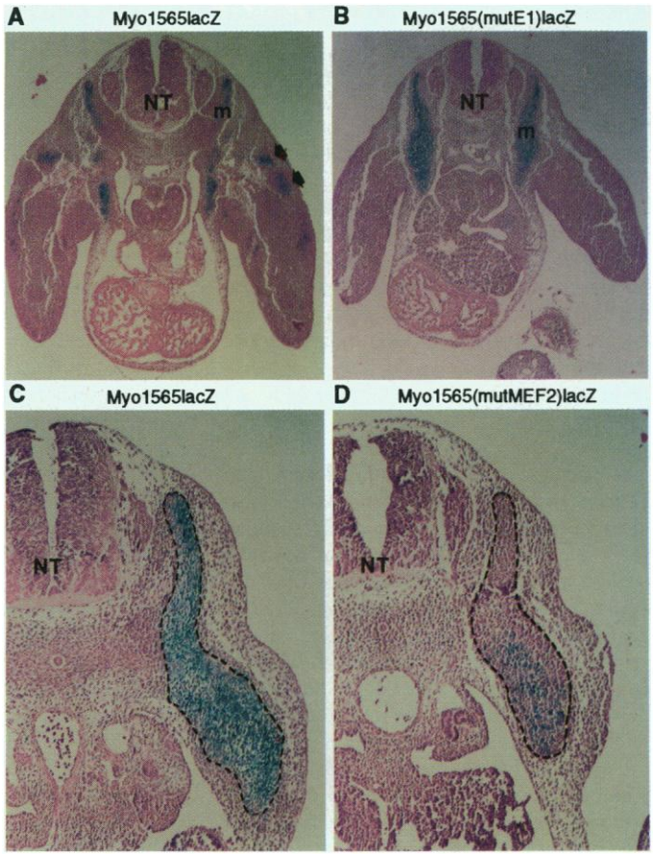

Fig. 3. Transverse sections of embryos with wild-type and mutant *myogenin-lacZ* transgenes. Transverse sections (*33*) were cut through the forelimb bud (A and B) and the thoracic somites (approximately somite 13) (C and D). The different staining patterns in the somites in (A) and (B) reflect slightly different planes of section. All sections were derived from the day 11.5 p.c. embryos shown in Fig. 2. (**A** and **B**) Transgene expression in limb buds. Cells positive for *lacZ* were localized to muscle-forming regions within the forelimb buds of embryos harboring Myo1565lacZ (A). Little or no staining was observed in the forelimb buds of embryos harboring Myo1565(mutE1)-lacZ (B). The arrows point to clusters of *lacZ*-positive cells in the limb bud. NT, neural tube; m, myotome. (**C** and **D**) Transgene expression in somites. Expression of *lacZ* was evident throughout the myotome of the thoracic somites of embryos harboring Myo1565lacZ (C), whereas *lacZ* expression was limited to the ventral musculature of embryos harboring Myo1565(mutMEF2)lacZ (D). Dashed lines demarcate the myotome. All sections were cut to a thickness of 5 μm.

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15. Expression of *lacZ* enzyme activity in the caudal somites appears to lag about a half day behind expression of endogenous myogenin mRNA measured by in situ hybridization (*13*). This may reflect differences between the sensitivity of the enzyme reaction and that of the in situ hybridization.
16. T.-C. Cheng and E. N. Olson, unpublished results. The region from −184 to +18 bp contains two E boxes, but only E1 shows appreciable affinity for myogenic HLH proteins (*6*). The region from −1565 to +18 bp contains eight E boxes.
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20. T.-C. Cheng, J. P. Merlie, E. N. Olson, unpublished results.
21. The presence of the transgene in mice harboring Myo1565(mutE1/mutMEF2)lacZ was confirmed by polymerase chain reaction amplification of yolk sac DNA from F_0 embryos (*26*).
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31 DNA for microinjection was purified by two cycles of CsCl centrifugation and then linearized by Hind III and Eco RI digestion, and the fragments were isolated from a SeaPlague agarose gel (FMC, Rockland, ME) and passed through NACS columns (BRL, Gaithersburg, MD). Microinjections and transfer of fertilized eggs were performed as described (*28*). (C57BL6 × CBA)F_1 mice (Jackson Laboratory, Bar Harbor, ME) were used as stud males, embryo donors, and mature females for breeding. Outbred ICR mice (Harlan Sprague-Dawley, Indianapolis, IL) were used for vasectomized males and pseudopregnant females. Expression of each reporter was observed in three to eight founder animals with similar results. Expression of Myo1565lacZ has been analyzed in F_0, F_1, and F_2 generations with identical results (*20*). Noon of the day that vaginal plugs were detected was counted as day 0.5 p.c., with the diurnal cycle of dark extending from 7:00 p.m. to 5:00 a.m. Embryonic development was also monitored according to Theiler (*29*).
32. Embryos were stored for 30 to 90 min in ice-cold phosphate-buffered saline (PBS) containing 2% paraformaldehyde and 0.2% glutaraldehyde, rinsed twice in PBS, and then stained with X-gal (1 mg/ml), 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$ in PBS at room temperature for 12 to 16 hours. They were cleared in PBS for more than 10 hours, fixed, and stored in 4% formaldehyde (*4*).
33. Whole-mount embryos were dehydrated, cleared, and embedded in paraffin. The embryos were oriented such that the limb buds or the thoracic somites were parallel to the sections. Serial sections were cut to a thickness of 5 μm and counterstained with hematoxylin and eosin.
34 Supported by grants from NIH and the Muscular Dystrophy Association to E.N.O. and J.P.M. E.N.O. also obtained support from The Robert A. Welch Foundation. We are grateful to P. Rigby for sharing results before publication.

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Inhibition of Adenylyl Cyclase by $G_{i\alpha}$

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Evidence suggests that both α and βγ subunits of heterotrimeric guanine nucleotide–binding regulatory proteins (G proteins) inhibit adenylyl cyclase. Although type I adenylyl cyclase is inhibited directly by exogenous βγ, inhibition of adenylyl cyclase by $G_{i\alpha}$ has not been convincingly demonstrated in vitro. Concentration-dependent inhibition of adenylyl cyclases by purified $G_{i\alpha}$ subunits is described. Activated $G_{i\alpha}$ but not $G_{o\alpha}$ was effective, and myristoylation of $G_{i\alpha}$ was required. The characteristics of the inhibitory effect were dependent on the type of adenylyl cyclase and the nature of the activator of the enzyme. The concentrations of $G_{i\alpha}$ required to inhibit adenylyl cyclase were substantially higher than those normally thought to be relevant physiologically. However, analysis indicates that these concentrations may be relevant and reasonable.

The protein-protein interactions necessary for activation of adenylyl cyclase are well characterized and first involve the association of an appropriate agonist-receptor complex with the guanosine diphosphate (GDP)–bound form of the heterotrimeric G protein G_s. The receptor catalyzes exchange of GDP for guanosine triphosphate (GTP) on the G protein α subunit. Subsequently, $G_{s\alpha}$-GTP dissociates from a complex of the G protein β and γ subunits and is free to activate adenylyl cyclase (*1*). By contrast, the mechanisms that underlie hormonal inhibition of adenylyl cyclase are less well understood. Although interactions of G_i proteins with hormone-bound receptors also result in guanine nucleotide exchange and subunit dissociation, it is unclear whether the GTP-bound $G_{i\alpha}$ protein, the βγ complex, or both inhibit adenylyl cyclase and by what mechanisms they operate. Furthermore, the heterogeneity of adenylyl cyclases suggests that there may be several mechanisms by which both stimulation and inhibition can be accomplished, depending on the enzyme in question.

Exogenously added βγ inhibits adenylyl cyclase activity in platelet and S49 cell membranes, whereas activated $G_{i\alpha}$ has only a modest effect at what have been considered high concentrations (*2*). The researchers hypothesized that the mechanism of inhibition by βγ was indirect, resulting from the deactivation of stimulatory $G_{s\alpha}$. The βγ subunit complex also has a direct inhibitory effect on calmodulin- or $G_{s\alpha}$-activated type I adenylyl cyclase (*3*, *4*). (This form of adenylyl cyclase is not the type that is present in platelets or S49 cells.) However, this phenomenon is not general. For example, βγ activates type II and type IV adenylyl cyclases directly but only in the presence of activated $G_{s\alpha}$ (*5*, *6*).

Direct inhibition of adenylyl cyclase by activated $G_{i\alpha}$ proteins is the most obvious potential mechanism. However, biochemical evidence for this interaction is lacking. Brain $G_{i\alpha}$ inhibits adenylyl cyclase only modestly when this protein is tested at concentrations between 10 and 50 nM (*2*). By contrast, these proteins affect K^+ channels in the picomolar range (*7*). Recombinant $G_{i\alpha}$ proteins (from *Escherichia coli*) have no effect on adenylyl cyclase activity at 2.5 μM concentrations (*8*). Unlike their natural counterparts, these proteins are neither myristoylated nor palmitoylated (9). Nevertheless, the expression of constitutively activated $G_{i\alpha}$ proteins, but not $G_{o\alpha}$, does cause substantial inhibition of adenylyl cyclase activity (*10*). The methods used to reach this conclusion involved long-term overexpression of α, which meant that mechanisms could not be assessed and compensatory cellular reactions were difficult to exclude.

Given the availability of several newly discovered isoforms of adenylyl cyclase (including some that are expressed in nonneural tissues) and the capacity to produce myristoylated recombinant G_α proteins in *E. coli* (*11*), we have again assessed the possibility of direct interactions between $G_{i\alpha}$ and adenylyl cyclase. Type V adenylyl cyclase was expressed in Sf9 cells with recombinant baculovirus, and the enzyme in Sf9 cell membranes was activated with either half-maximally effective concentrations of recombinant GTP-γ-S–$G_{s\alpha}$ (Fig. 1) or forskolin (Fig. 2). Concentration-dependent inhibition of adenylyl cyclase activity was observed on addition of either activated $G_{i\alpha}$ from bovine brain (a mixture of isoforms, predominantly $G_{i\alpha1}$) (Fig. 1) or activated, myristoylated recombinant $G_{i\alpha1}$ (from *E. coli*) (Figs. 1 and 2). In the latter case the inhibition was saturable; the concentration required for 50% inhibition (IC_{50}) was approximately 100 nM. Boiled protein did not elicit the inhibitory response. Myristoylated recombinant $G_{o\alpha}$ and nonmyristoylated recombinant $G_{i\alpha1}$ were ineffective. The GDP-bound form of myristoylated recombinant $G_{i\alpha1}$ was similarly inactive (*12*). The inhibitory effects of myristoylated recombinant $G_{i\alpha2}$ and $G_{i\alpha3}$ were indistinguishable from those of myristoylated recombinant $G_{i\alpha1}$ (*12*). The inhibition by activated myristoylated $G_{i\alpha1}$ of both $G_{s\alpha}$- and forskolin-stimulated adenylyl cyclase indicates that this inhibition does not result only from competition with $G_{s\alpha}$

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