Tissue-specific gene activation by MyoD: determination of specificity by cis-acting repression elements

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MyoD is a muscle-specific transcriptional activator, E12 is a B-cell activator. An IgH enhancer is activated almost 100-fold by E12 but not at all by MyoD; an MCK enhancer is activated almost 1000-fold by MyoD and not at all by E12. MyoD and E12 are both basic helix–loop–helix proteins that bind to similar E-box sequences (CANNTG); the IgH enhancer contains the same E boxes as the MCK enhancer, yet each retains exclusive specificity for either E12 or MyoD, respectively. We show that the IgH enhancer contains a cis-acting negative element that is directed at MyoD, but not at E12. This repression requires the μE5 E box within the IgH enhancer; however, the specificity for repression, as opposed to activation, is associated with 2 bp flanking each side of the μE5 E box. The target for repression of MyoD in the IgH enhancer is the bHLH region of MyoD. Our results suggest that MyoD only activates myogenic genes because nonmuscle enhancers that contain E boxes also contain negative elements that prevent MyoD activity.

[Key Words: MyoD, transcriptional repression, E box, IgH enhancer]

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Figure 1. Specificity of MyoD for the MCK enhancer and E12 for the IgH enhancer. (A) NIH-3T3 cells were cotransfected with either a MyoD or E12 expression vector and the entire MCK enhancer (−3300) MCK-CAT (Jaynes et al. 1988), or the IgH enhancer (μE3,2,5) as described previously (Kadesch et al. 1986). In a typical experiment, cells were plated at about one-fourth confluency and transfected using calcium phosphate the next day. Usually, the template was at 8 μg and the activator at 6 μg per plate. Cells were rinsed the next day and grown for 1 day in DME with 10% calf serum and then 2 days in DME with 10 μg/ml of transferrin and 10 μg/ml of insulin. The IgH enhancer is oligomerized (4x) and drives a single TATA box CAT construct (Ruezinsky et al. 1991). All of the experiments in Figures 1–5 have been repeated 3–10 times each, with the same results. Entries are averages normalized to the highest value in each series. (B) A cis-acting negative control element (μE5) directed at repression of MyoD in the IgH enhancer. Point mutations (Ruezinsky et al. 1991) in the various E boxes of the IgH enhancer (indicated by the vertical black bars) were assayed with the indicated trans-activators as described in A.

μE5 determines a repression mechanism for MyoD but not E12

To investigate why MyoD fails to activate the IgH enhancer, we initially mutated each of the E boxes in the μE5,2,3 enhancer (Fig. 1B). Mutation of μE5(ΔμE5) resulted in a higher basal level of activity (expression vector alone), ΔμE5 also retained the ability to be activated by E12 but lost the capacity to repress MyoD. The increased basal level of activity seen with ΔμE5 suggests that μE5 is associated with a cis-acting negative activity directed at one or more endogenous transcription factors present in most cells; for example, USF, TFE3, Myc, Max, etc. (Ruezinsky et al. 1991). As shown below (Fig. 2), the complication associated with a high basal level of activity in the ΔμE5 mutant can be eliminated by using a series of templates missing μE2. In contrast to the results with ΔμE5, mutation of μE3 or μE2 resulted in templates that retained both MyoD repression and the specificity for E12 activation. These results suggest that μE5 is a cis-acting negative element that prevents MyoD activation but does not prevent E12 activation of the IgH enhancer.

To characterize further the MyoD inhibitory element [μE5] in the IgH enhancer, we used a series of simplified wild-type and mutant E-box enhancers coupled to μE3
cis-Acting negative control

| Vector | MyoD | E12 | E12(AT) |
|--------|------|-----|---------|
|        | 290  | 400 | 22,000  | 3,000 |
|        | 150  | 83,000 | 18,000 | 24,000 |
|        | 240  | 800  | 12,000  | 2,500 |
|        | 360  | 30,000 | 2,000  | 12,000 |

Figure 2. Mapping of μE5 repression to 4 bp flanking the 6-bp E box. The indicated enhancers were oligomerized (4×) and linked to the same TATA box CAT construct (Genetta et al. 1994) used and assayed as in Fig. 1. Note that in contrast to the templates used in Fig. 1, these constructs have been simplified as much as possible so that μE2 is not present. E12(AT) is E12 containing the basic region residues of MyoD[A114T115] substituted into the corresponding region of E12 for NN (Davis and Weintraub 1992).

Comparing the two sequences [μE5+μE3] vs. [κE2+μE3] reveals that MyoD-directed inhibitory activity is correlated with 4 bp (circled) that flank the μE-5 E box. An additional comparison of the two plasmids [μE5+μE3] vs. [μE5 plus mutant μE2 plus μE3] suggests that the μE5 negative element (containing the core 6-bp E box plus 3 bp upstream and 3 bp downstream) can function when inverted and when placed almost one-half of a helical turn away from μE3. The same features of MyoD repression (Fig. 1 and 2) were also obtained using Myf-5 and myogenin, and identical results are also obtained after transfection of the various IgH mutants into C2 myoblasts, which provide MyoD and myogenin endogenously (data not shown). Our main conclusion is that a very few base pairs that flank the canonical 6 bp μE5 element determine whether or not μE5 is positive or negative for MyoD activation. These flanking base pairs have little effect on the ability of E12 to activate. In addition, the integrity of the μE5 E-box core sequence (CACCTG) is also needed for MyoD repression (Fig. 1B), because a mutation to [CAAATG] prevents repression. Recently, Simon and Burden (1993) have described an E box in the δ-acetylcholine receptor gene that is responsible for negative control of this muscle gene in fibroblasts. Because their sequence differs from μE5 in the flanking base pairs, it may interact with a different repressor.

The marked differences in expression between the various E-box-binding site combinations shown in Figure 2 are not easily explainable in terms of simple differences in binding affinities. Using MyoD–E47 heterodimers made in Escherichia coli, no differences in binding were observed between the two extreme sequences [κE2–μE3] and [μE5–μE3], either as pure proteins or in cell extracts (data not shown). In addition, a comparison of the capacity of MyoD–E47 heterodimers versus E47 homodimers to displace a possible zinc finger repressor, Zeb (see below), have also led to no differences manifest by the two sequences.

A striking result suggesting that the μE5 repressor can work at some distance and in an entirely different context is shown in Figure 3. The IgH enhancer was inserted either upstream or downstream from a 48-bp minimal MyoD-dependent, MCK enhancer, spanning the R and L E boxes. In both cases, MyoD-dependent activation of the chimeric enhancer was markedly inhibited while E12-dependent activation was, if anything, enhanced. In contrast to the full MCK enhancer (Fig. 1), the minimal MCK enhancer described in Figure 3 is activated by E12 — suggesting that cis-acting negative elements (located either upstream or downstream of the R and L E boxes) control E12 negatively but not MyoD at the full MCK enhancer. Identification of the sequence responsible for this putative E12-directed repression is currently in progress.

To test whether the μE5 site is inhibitory to MyoD in the context of the intact IgH enhancer (i.e., a larger enhancer element that is not oligomerized), a MyoD expression vector was cotransfected into 3T3 cells with the intact enhancer or the same enhancer mutated in just the μE5 site (Fig. 4). As a result of the μE5 mutation,
transcription increases 10–30-fold. Similar results are obtained using C2 myoblasts [data not shown]. These results again indicate that \( \mu E5 \) mediates cis-acting negative control on adjacent sites potentially activatable by MyoD.

\[ \text{\( \mu \text{E5 repression is targeted to the MyoD bHLH} \)} \]

To identify the domain in MyoD that is the target for \( \mu \text{E5-mediated repression}, \) we analyzed deletions of MyoD that are still capable of activation [Tapscott et al. 1988], as well as chimeric proteins between MyoD and E12 [see Davis and Weintraub 1992]. Removal of either the amino terminus of MyoD (A3–56) or the carboxyl terminus (167–318) or the Cys–His-rich region (63–99) had little effect on MyoD activation of the MCK enhancer and repression still occurred on the \( \mu \text{E5,2,3 enhancer} \) (Fig. 4B). By elimination, this suggests that the target for repression by \( \mu \text{E5} \) is the bHLH region of MyoD. This is supported by the observation that the inability of MyoD to activate the IgH enhancer can be overcome by replacing MyoD bHLH with E12 bHLH (Fig. 4B). Figure 4B also shows that MyoD [E12bHLH] fails to activate the MCK enhancer. This suggests that as is the case for the IgH enhancer, the repression system for MCK is, at least in part, directed against the bHLH region, in this instance, of E12. The discrimination between MyoD and E12 is subtle. A mutant version of E12, in which 2-amino-acid

\[ \text{\( \Delta C/H \text{- MyoD (}\Delta \text{E3-99)} \)} \]

Figure 4. Repression of MyoD at the IgH locus is directed at the bHLH domain. (A) Repression of MyoD at the intact IgH enhancer. A single IgH enhancer [339 bp HindIII–EcoRI (Kadesch et al. 1986)] was linked to TK–CAT and assayed for activation by MyoD in 3T3 cells [Fig. 1]. A variant [Mut\( \mu \text{E5} \)] was also assayed [Kadesch et al. 1986]. (B) Localization of \( \mu \text{E5-mediated repression of MyoD to the MyoD bHLH region. The oligomerized IgH enhancer, \( \mu \text{E5,2,3, or the 3300 MCK enhancer were assayed as in Fig. 1 with various MyoD deletions [Tapscott et al. 1988], various MyoD and E12 chimeric proteins [Davis and Weintraub 1992], or MyoD–VP16 [Weintraub et al. 1991].} \)
residues present in the basic region of MyoD (Ala-114 and Thr-115) are swapped into the corresponding positions in E12 (Davis and Weintraub 1992), is a good activator of [κE2 + μE3], but a poor activator of [μE5 + μE3] (Fig. 2B). We interpret this to mean that as the basic region of E12 becomes more MyoD-like, the μE5 repressor becomes a more effective inhibitor of E12. In support of this, like MyoD and in contrast to E12, E12 ΔT can activate a reporter driven by only the μE3 sequence (Fig. 2; mutant μE5 + μE3).

MyoD–VP16 activates the IgH enhancer

Repression of MyoD at the IgH locus may function by “silencing” already bound MyoD. Some evidence for this comes from the observation that addition of the VP16 activation domain to MyoD (MyoD–VP16) activates the IgH enhancer (Fig. 4B). One interpretation of these results is that MyoD can bind to the IgH enhancer but it is normally silenced by the μE5 repressor; addition of the VP16 activation domain bypasses this silencing. Other interpretations are also possible. For example, perhaps the presence of the VP16 activation domain allows MyoD–VP16 to displace the μE5 repressor.

E12 bypasses the μE5 repressor

E12 fails to activate (mutant E5 + μE3) (Fig. 2). This is not surprising as E12 binds poorly to the μE3 sequence because the T immediately upstream of the E box is incompatible with binding (Blackwell and Weintraub 1990). However, E12 can activate the wild-type [μE5 + μE3] construct, suggesting that the μE5 site is used for E12 activation in this template. These results suggest that E12 can bypass (or displace) the μE5 repressor responsible for MyoD repression (see also Ruezinsky et al. 1991; Genetta et al. 1994). In contrast, MyoD can activate (mutant μE5 + μE3) but cannot activate (μE5 + μE3), suggesting that MyoD, although capable of binding to and activating through each of the core, 6-bp E boxes associated with μE5, μE2, and μE3 (data not shown), cannot bypass or displace the repression mechanism associated with the IgH μE5 E-box and flanking sequences.

E12 can derepress μE5, allowing MyoD to activate

To test the idea that E12, but not MyoD, can displace or otherwise obviate the μE5 repressor, we attempted to show synergy between E12 and MyoD at the IgH enhancer. We use an E12 derivative (E12ΔN) lacking the amino-terminal activation domain. This fails to activate the IgH enhancer (Table 1). Similarly, MyoD fails to activate. However, the two together give a high level of activity. In contrast, E12ΔN is a weak inhibitor of MyoD using either the [κE2 + E3] template (Fig. 2) or the 4R template (Fig. 1B). These results are consistent with the notion that E12ΔN can displace the μE5 repressor of MyoD and, when cotransfected with MyoD, allow MyoD to activate the IgH enhancer. The level of synergy is restricted by a competing reaction in which E12ΔN also inhibits MyoD, presumably because it is a weaker activation partner for MyoD than full-length E12. Thus, it seems that E12ΔN can displace or otherwise bypass the inhibitory activity of the μE5 repressor on MyoD, but MyoD is not equipped to do this. An alternative explanation is that cotransfection of E12AN increases the concentration of a MyoD–E12AN heterodimer and that this species can bind more efficiently and activate because of its increased concentration. To test this, increasing concentrations of MyoD vector were transfected with saturating levels of E12 and the IgH expression vector. Addition of MyoD actually reduced activation by E12 (data not shown), suggesting that with more MyoD, the levels of E12 homodimers decrease because of formation of MyoD–E12 heterodimers, and the MyoD–E12 heterodimer is inhibited at the IgH locus even though it is presumably at a much higher concentration.

Table 1. Activation of MyoD at the IgH enhancer by a truncated E12

| Vector        | 4R | κE2 + E3 | IgH |
|---------------|----|---------|-----|
| E12ΔN         | 280| 350     | 470 |
| MyoD          | 98,000| 110,000| 160 |
| MyoD + E12ΔN | 47,000| 41,000 | 24,000 |
| E12           | 74,000| 93,000 | 110,000 |
| E12 + E12ΔN  | 64,000| 71,000 | 48,000 |

E12ΔN is missing the amino-terminal activation domain and is described in Davis and Weintraub (1992). All other procedures are described in Figs. 1 and 2. Templates were at 5 μg per dish, activators were at 7 μg per dish, and E12 were at 7 μg per dish. Values are the average of three separate transfections.

MyoD is unlikely to be the μE5 repressor

The experiments presented thus far do not rule out the possibility that MyoD itself, when bound to the μE5 sequence, acts as an inhibitor. To test this possibility we assayed a number of IgH derivative reporters [similar to those shown in Fig. 2] for those that would retain MyoD repression of an adjacent μE5 site but lose in vitro MyoD binding to μE5. Figure 5 shows the sequence of the parent μE5 site together with two additional templates. The [Mef-1 + μE3] template contains a sequence [Mef-1] that can bind MyoD, yet it fails to inhibit activation, presumably because of an A-T change at position +5; thus, MyoD binding per se to a site adjacent to μE3 is not sufficient to cause repression. In addition, the [mutant–Mef-1 + μE3] template has a sequence that fails to bind MyoD, but still retains repression of MyoD on the neighboring μE3 site. Thus, for these two templates, binding by MyoD and repression of MyoD can be dissociated, and consequently, we believe it unlikely that MyoD is the μE5 repressor; however, these results cannot rule out a more complex model wherein MyoD in conjunction
with another protein (i.e., a corepressor) acts as an inhibitor associated with μE5.

μE5 is not a general repressor

To examine whether the μE5 repressor could inhibit other trans-activators besides MyoD, we cloned five Gal4 DNA-binding sites adjacent to an intact IgH enhancer (Fig. 6). When challenged with either Gal–E1a or Gal–VP16 chimeric activators, no inhibition was observed even though this construct preserved its property of inhibiting MyoD while allowing E12 activation. Thus, the μE5 repressor seems to be rather specific in that it cannot inhibit an adjacent Gal–VP16 or Gal–E1a fusion protein. When challenged with Gal MyoD, there is a reproducible three- to fivefold inhibition of Gal–MyoD compared with Gal–VP16 or Gal–E1a. Although the degree of inhibition is significant, it is also much less than that of MyoD.

Discussion

cis-Acting negative sequences contribute to the tissue specificity of bHLH proteins

Our results demonstrate that the complex IgH enhancer has evolved in such a way that it contains negative control sequences that inhibit MyoD but do not inhibit E12. Cis-acting inhibitory sequences are common control elements found in association with a large number of genes. What is unusual to the present situation is (1) the degree of specificity—the μE5 repression system is almost absolute for inhibiting MyoD as compared with the related bHLH protein, E12; (2) the functional similarity of the two activators—substitution of only 3 amino acid residues from MyoD into E12 converts E12 into a myogenic protein (Davis and Weintraub 1992) and substitution of only 2 amino acids from the MyoD basic region into the corresponding positions in E12 converts E12 into a protein more like MyoD when assayed at the IgH
locus (Fig. 2); (3) the fact that the “target” for repression on MyoD (the IgH region) is also the DNA-binding domain as well as an important component of the transcriptional activation process (Davis et al. 1990); and (4) the fact that only very few base pairs flanking μE5 determine whether it is an inhibitor or activator sequence for MyoD. An analysis of the MCK enhancer similarly suggests that cis-acting negative sequences inhibit the activity of E12 but fail to inhibit MyoD (cf. Figs. 1A and 3). As is the case for MyoD and the μE5 repressor, the cis-acting negative sequences in the MCK enhancer also seem to be directed, at least in part, at the bHLH region of E12 (Fig. 4B, MyoD [E12b|H1]). Cis-acting negative elements such as the μE5 repressor are likely to contribute to the exquisite tissue specificity of E-box-binding proteins. Together with our previous results showing that only 3 amino acid residues from MyoD can convert E12 to a myogenic protein [E12 [A, T, and K]], the present studies raise the possibility that this myogenic conversion occurs not because E12 [A, T, and K] binds a positive coactivator, but because E12 [A, T, and K] avoids negative regulation normally directed at a crucial endogenous myogenic control gene, for example, at endogenous MyoD or myogenin.

The mechanism of μE5 silencing

Our experiments with the μE5 silencer raise two overlapping issues. One is the ability of this silencer to specifically inhibit MyoD bound to rather distantly located sites. The second is the capacity of E12, but not MyoD, to either displace or otherwise inactivate or bypass the μE5 silencer. Previous work also identified μE5 as an inhibitory element for the constitutively expressed bHLH zipper protein TFE3 (Ruezinsky et al. 1991; Kadesch 1992, 1993; Genetta et al. 1994). The identity of the μE5 repressor(s)—either for TFE3 or for MyoD—is not known nor is the repression mechanism; however, a zinc finger protein [ZEB] that binds to μE5 recently has been cloned (Genetta et al. 1994) and attempts to determine whether Zeb is the MyoD specific μE5 repressor are in progress. Because the DNA-binding domains of Zeb can bind in vitro equally well to both KE2 and μE5 (Genetta et al. 1994), it is at first difficult to see how Zeb could be the μE5 MyoD repressor, however, other factors could contribute in the cell. Related work (Gonzalez-Crespo and Levine 1993) has shown that the zinc finger repressor, snail, can compete for binding with the bHLH protein activator, twist, and that depending on relative concentrations, an on/off threshold of activation can be seen in vivo. In contrast, the μE5 repressor can act from a distinct site at some distance and is demonstrated to distinguish between different bHLH proteins.

As our assays are largely in both 3T3 and C2 cells, we presume that the negative factors associated with μE5 are not muscle specific. Given the way this system of negative regulation seems to have evolved, where the specificity for inhibition is directed at target domains in tissue-specific bHLH activator proteins, there need not necessarily be any tissue specificity at all to the expression of the negative regulator[s]. It will be interesting to know whether the μE5 silencer can also inhibit the nerve cell-specific achaete-scute bHLH protein, or whether another cis-acting repression system elsewhere in the IgH locus is responsible for supposed repression directed at achaete-scute. Less specific mechanisms for repression, for example, those mediated by histones, should also be considered. Our experiments also fail to rule out a mechanism wherein the μE5 sequences force MyoD itself into a “negative conformation” and, hence, MyoD may be the μE5 repressor (e.g., see Saver and Jäckle 1993). Some evidence against this possibility is presented in Figure 6, where we describe one site that fails to bind to MyoD but maintains repression and another site that maintains MyoD binding but fails to mediate repression; however, these results do not rule out a more complex model where a coinhibitor functions in conjunction with MyoD, both for binding and for repression.

It is also not known whether in myoblasts the same MyoD-directed μE5 repressor that functions at the IgH locus is used to repress the potential activity of MyoD at other nonmuscle, E-box-containing tissue-specific enhancers. Similarly, in nonmuscle cell types [e.g., B cells] that use different bHLH proteins [e.g., E12], it is not known whether the same μE5 repressor described here is used to repress the activity of myogenic genes [e.g., MCK].

One mechanistic model that can explain our data is the following: The μE5 silencer inhibits MyoD bound to μE3 by targeting its bHLH domain. E12 escapes this repression, in part because it can bind to μE5 and displace or inactivate the silencer bound to μE5. How E12, but not MyoD would do this is not clear. The bHLH region of MyoD is not only required for DNA binding, but it is also involved in subsequent transcriptional activation because positive control mutations [e.g., at Ala-114 in the basic region] bind normally to DNA but fail to activate transcription (Davis et al. 1990, Bengal et al. 1994; Ma et al. 1994). There is also evidence that positive control may be mediated through a cofactor that interacts with the bHLH region of MyoD and is absent in rhabdomyosarcoma muscle tumor cells (Tappcott et al. 1993). The present study extends the involvement of the bHLH region to include not only DNA binding and transcriptional activation, but silencing as well. It also raises the possibility that the MyoD positive control mutants fail to activate transcription because they gain the activity of interacting with a MyoD-specific inhibitor, such as the μE5 repressor. This is somewhat less likely as there are many different types of amino acid substitutions that give rise to the positive control phenotype.

Why negative control?

The question arises why the system of tissue-specific bHLH proteins [MyoD, achaete-scute, and SCL] did not evolve so that each member binds a unique target DNA sequence. c-Myc is known to also bind quite well to several noncanonical sequences (Blackwell et al. 1993;
Ma et al. 1993) and the Drosophila bHLH protein, Enhancer of split, binds the non-E box sequence CACNAG (Tietze et al. 1992). Thus, some bHLH proteins can bind to non-E box DNA sequences. One explanation is that there is something very fundamental and unchangeable about the mechanism of activation by these bHLH proteins and this requires the same basic region that also determines DNA-binding specificity. As discussed above, there is now a large body of evidence that the basic region of MyoD not only mediates sequence-specific DNA binding but also an additional step in subsequent transcriptional activation (Tapscott 1988; Davis et al. 1990; Davis and Weintraub 1992; Bengal et al. 1994). This activation process may be too complex to tolerate alterations in the basic region that would have to coevolve with different and diverging DNA-binding sites. Contributing to the necessity of keeping binding sites constant may be the pressure to keep E12 as a partner for each of the tissue-specific bHLH proteins. It has been argued that this allows the cell to make mutually exclusive developmental commitments [Weintraub 1993]. Finally, conservation of a consensus binding site can also be explained if all tissue-specific bHLH proteins activate a common set of downstream genes (in addition to a unique set of cell type-specific genes) for each to orchestrate its unique cell-program. Thus, rather than change the primordial network of proteins that interact with the basic region, perhaps the alternate solution—to derive cis-acting bHLH specific negative control —was more expedient.

Materials and methods

Plasmids

IgH enhancer-containing plasmids and mutant derivatives have been described previously [Henthorn et al. 1990; Ruezinsky et al. 1991; Genetta et al. 1994]. Briefly, ΔΔE5 changes CACCTG to CAAATG; ΔΔE2 changes CAGCTGGC to CATCTAGA; and ΔΔE3 changes CATGTGGC to CATCTAGA. The MCK enhancer (Jaynes et al. 1988) and MyoD and E12 expression vectors and mutant derivatives [Davis and Weintraub 1992] have also been described. The chimeric IgH–MCK enhancer (Fig. 3) was made by cutting a TK–CAT plasmid that contains 48 bp encompassing the R and L sites of the MCK enhancer [Bengal et al. 1994] either upstream [HindIII] or downstream [XbaI] of the enhancer. This was ligated with the oligomerized IgH enhancer [Fig. 1] released by BamHI and BglII digestion.

Cells and transfections

NIH-3T3 cells and C2 myoblasts were maintained at low density in Dulbecco’s modified Eagle medium (DME) plus 10% calf serum (GIBCO). Cells were transfected with calcium phosphate precipitates, and the next day, rinsed with phosphate buffered saline and incubated for 1 day in DME plus 10% calf serum. MyoD was then activated by serum removal and growth in DME plus 10 μg/ml of insulin and 10 μg/ml of transferrin. Cells were collected and assayed for CAT expression [Weintraub et al. 1991].

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