The MyoD family of transcription factors regulates muscle-specific gene expression in vertebrates. In the adult rat, MyoD mRNA accumulates predominately in fast-twitch muscle, in particular type IIb and/or IIx fibers, whereas Myogenin mRNA is restricted to slow-twitch type I muscle fibers. Transgenic mice expressing the avian v-ski oncogene from the murine sarcoma virus (MSV) promoter-enhancer display preferential hypertrophy of type IIb fast-twitch muscle apparently because of the restricted expression of the transgene. We tested the hypothesis that preferential interactions of MyoD, as a heterodimer with E12, with the MSV enhancer, which has six E-box targets for MyoD family proteins, could contribute to v-ski gene expression in IIb muscle fibers. A series of quantitative binding studies was performed using an electrophoretic mobility shift assay to test MyoD-E12 versus Myogenin-E12 binding to the MSV enhancer. Our results indicate that MyoD-E12 binds the MSV enhancer with higher affinity and higher cooperativity than Myogenin-E12. Interestingly, MyoD-E12 bound all of the individual E-boxes tested with positive cooperativity indicating DNA-mediated dimerization of the protein subunits.

Activation of muscle-specific gene expression in vertebrates is controlled by the MyoD family of transcription factors, MyoD, Myogenin, MRF4, and Myf-5, which are exclusively expressed in skeletal muscle (1–5). Ectopic expression of any one member of this family in non-muscle cells, such as 10T1/2 or NIH3T3 fibroblasts, results in phenotypic conversion of that cell to muscle, indicating the trans-dominant nature of these proteins (3, 6). The four family members share 80% homology in a 60-amino acid region known as the basic helix-loop-helix (bHLH) domain (7). The basic region is responsible for DNA binding activity (8) and is involved in transactivation of regulated genes, whereas the helix-loop-helix motif constitutes an interface for dimerization with other bHLH proteins (9–11). MyoD family proteins are not thought to form efficient homodimers; rather they readily heterodimerize with other HLH proteins, including the E12 and E47 products of the E2A gene (12). Heterodimers are capable of activating transcription of muscle-specific genes as a result of binding to the E-box consensus sequence (CANNTG) in muscle-specific enhancers, presumably facilitating the formation of transcription-competent RNA polymerase-promoter complexes. The roles of these transcription factors in myogenesis has been reviewed recently (13).

Distinctions between these seemingly redundant transcription factors have been elucidated by developmental expression profiles and null mutant studies from which a general model has been proposed (14). Whereas myoD or myf-5 null mice have fairly normal muscle development, the double mutant is lethal, as are myogenin null mice (15–19). There is a temporal pattern of expression for the MyoD family members in the mouse. Myf-5 is expressed first, followed shortly after by Myogenin, MyoD, and MRF4. In the neonate, all four proteins are expressed at a time when the fetal isoforms of myosin heavy chain (MyHC), rather than the adult isoforms predominate. Following birth and maturation of innervation, Myf-5 expression is reduced to an undetectable level, whereas MRF4 expression remains relatively high. Interestingly, both MyoD and Myogenin expression is also reduced, however, to different extents in different fiber types. MyoD expression predominates over that of Myogenin in fast-twitch type IIb/Ix muscle fibers, whereas Myogenin is preferentially expressed in slow-twitch type I fibers (20, 21). During this time, the fetal isoform of MyHC is replaced by the adult isoforms. The fiber type-specific expression of MyoD and Myogenin is responsive to hormonal and neural signals. Denervation of either fiber type results in increased expression of both MyoD and Myogenin, as if the cells are reinnervating to the fetal form; adult MyHC is reduced, and fetal MyHC is reexpressed. Cross-reinnervation of a slow muscle (soleus) with a fast motor neuron or thyroid hormone treatment, both of which induce transformation to fast-twitch fiber types, increased the level of MyoD expression and lowered Myogenin expression in the affected myofibers (20). These results suggest that the bHLH factors regulate different sets of genes in fetal and adult muscle. In the adult, they may be involved in controlling fiber type-specific gene expression in response to external signals.

The viral ski gene (v-ski) encodes a nuclear oncoprotein capable of causing morphological transformation, allowing cell growth in soft agar and inducing myogenesis in cultured avian cell lines (22–24). The avian c-ski proto-oncogene encodes a nuclear protein of unknown function which can bind DNA in the presence of other nuclear proteins (25). However, when expressed at high levels, similar to that of v-ski during infection, c-ski activates the oncogenic and myogenic activities of v-ski (26). This result led Calmenares et al. (26) to conclude that differences in the level of expression between v-ski and c-ski rather than differences in the encoded proteins per se was sufficient to account for the disparate characteristics of the two evolution-
Binding of MyoD-E12 and Myogenin-E12 to the MSV Enhancer

MATERIALS AND METHODS

Preparation of DNA Fragments—The MSV enhancer region contains six E-boxes that we refer to as E1, E2, etc. from 5' to 3' (Fig. 1A and B). Four overlapping MSV enhancer fragments were amplified using Vent DNA polymerase (New England Biolabs) with MSVski plasmid as a template and synthetic primers. MSVski was the generous gift of Charles P. Emerson, Jr. (University of Pennsylvania School of Medicine). Purified Myogenin was the generous gift of S. Maleki (University of Arkansas Medical Sciences). The expression and purification of MyoD, Myogenin, and E12 will be described elsewhere. The three proteins were judged to be approximately 90-95% pure following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining. Small aliquots of purified protein were stored at -20 °C and thawed freshly for each experiment. The concentrations of MyoD, Myogenin, and E12 were determined by absorbance at 280 nm using extinction coefficients of 17,750, 21,980, and 17,900 M⁻¹ cm⁻¹, respectively.

EMSA—Proteins for heterodimer experiments were diluted from frozen stocks to 800 nM on ice in MCK buffer (10 mM Hepes/KOH, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mMKCl, 0.1% Triton X-100, 5% glycerol). Equal volumes of MyoD or Myogenin were mixed with E12, and the resulting solutions were equilibrated for 20 min at room temperature. Protein samples were prepared in 2-fold dilutions from 400 nM to 50 pM and mixed with equal volumes of DNA substrates diluted to 20 pM in MCK buffer. Thus, binding reactions contained 10 pM DNA and proteins in concentrations ranging from 25 pM to 200 nM, with respect to the heterodimer. Binding reactions were allowed to equilibrate for at least 30 min prior to electrophoresis. Native, 1-mm thick, 6% acrylamide gels (50:1 acrylamide:bisacrylamide) in 1 x TBE (89 mM Tris borate, 2 mM EDTA) were prepared in Protean IIxi (Bio-Rad) cooled apparatus at least 1 h prior to electrophoresis and prerun at 200 V constant voltage to reach a constant current of approximately 18 mA. Gel temperature was maintained at 17°C with a bath circulator at all times. Binding reactions were loaded on running gels, and then the voltage was raised to 250 V. Resolved gels were fixed in 10% acetic acid, 7% methanol for 15 min and dried. Gels were exposed to Reflection x-ray film (DuPont) for 12 h and 72 h at -70 °C with intensifying screens. During development of the EMSA we tested several different concentrations of acrylamide and various temperatures. Higher concentrations of acrylamide decreased the absolute value and compressed the range of the apparent binding constants presumably because of "caging effects." We also tested temperatures ranging from 17 to 24 °C for EMSA. DNA complexes with MyoD-E12 were much more stable at the elevated temperatures than Myogenin-E12; however, both complexes were quite stable in the EMSA at 17°C (data not shown).

Analysis of Data—Undersaturated films from the 12-h exposure were developed, scanned by an Image Densitometer (Bio-Rad), and the relative optical densities of free DNA bands were measured. Apparent amounts of bound DNA were calculated and plotted against respective protein concentrations. The resulting data were fitted with a sigmoidal (power logistic) function using Origin (MicroCal).

\[
\frac{A_1}{1 + \left(\frac{x}{x_p}\right)^4 + A_2}
\]

(Eq. 1)

In this function, A1 is the initial y axis level, A2 is the final y axis level, \(x_p\) is the center point of the curve, and p is the rate of increase or slope of the curve. Our analysis of theoretical binding curves for single and multiple protein/DNA interactions showed that the variable p exponent existing in the sigmoidal fitting function can be used as a relative measure of binding cooperativity. A p value of 1.0 indicates no cooperativity; p > 1 or p < 1 indicates positive or negative cooperativity, respectively; and the magnitude of p is indicative of the degree of cooperativity. It is important to note that the p value is a relative value describing the slope of the fitted curve and is not directly related to the Hill coefficient.

For the purposes of this work, we define the binding constant (\(K_{bound}\)) as the concentration of protein required to bind 50% of the total concentration of DNA. We use the term \(K_{bound}\) rather than \(K_d\) because we are

arially related proteins. c-skI expression driven by the promoter-enhancer region of the murine sarcoma virus (MSV) long terminal repeat has unexpected effects in transgenic mice. In three independent lines of mice, hypertrophy of skeletal muscle was observed. One of these lines (B566) was studied in detail, and the hypertrophy of muscle was found to be the result of specific hypertrophy of type IIb fibers, the fastest of the fast-twitch fibers (27). c-skI expression and the hypertrophic phenotype became elevated at approximately 12 days postpartum, the time at which innervation matures, and the adult isoforms of contractile proteins accumulate. c-skI mRNA accumulation in MSV-ski transgenic mice was reduced in denervated hindlimb muscle or in response to chemically induced hypothyroidism (27).

The obvious correlation between the expression patterns of the endogenous myoD gene and the MSV-v-ski transgene suggests the possibility that the MSV promoter-enhancer may be responding to MyoD activity specifically in adult IIb muscle fibers. In this case, the MSV enhancer would be a useful tool, not only in studies of differences in MyoD and Myogenin activity, but also for analyses of changes in MyoD activity during development. The idea that MyoD in type IIb fibers may regulate the MSV enhancer is supported by the finding that the bHLH transcription factor E47 can bind and transactivate the murine leukemia virus promoter-enhancer in vivo (28). The murine leukemia virus and MSV enhancers, as well as those from all type C retroviruses, are highly homologous (29). Based on these facts, we postulated that the tissue-specific expression of v-ski in the transgenic mice may result from the action of muscle-specific regulators available in fast fibers but absent in slow fibers, of which MyoD is the most likely candidate. As a first test of this hypothesis, we asked if there were intrinsic differences in the ability of MyoD (fast-specific) and Myogenin (slow-specific) to bind the MSV enhancer. A quantitative, in vitro protein-DNA binding analysis of the MSV enhancer region with MyoD-E12 and Myogenin-E12 heterodimers was performed. Our results indicate that MyoD-E12 has higher affinity for the MSV enhancer and that it binds with higher cooperativity than Myogenin-E12. Taken together our results indicate that MyoD is likely to contribute to the fast fiber-specific expression of c-skI in transgenic mice.
Binding of MyoD-E12 and Myogenin-E12 to the MSV Enhancer 9143

Fig. 1. Sequence of the MSV enhancer region and DNA fragments used for EMSA. Panel A, the sequence of the MSV enhancer with the six E-boxes enclosed. Binding sites for other transcription factors are shown below the horizontal bars and labeled. MCREF-1, LVa; GR, glucocorticoid receptor; NF-1, nuclear factor 1; CAT, CCAAT box-binding protein. Panel B, the DNA fragments tested in EMSA.

considering multiple equilibria where either MyoD or Myogenin is interacting with another protein, E12, and binding to multiple, non-equivalent target sites. Considering a single binding site, there are at least two formal types of equilibrium which could be occurring. The MyoD family of proteins is commonly thought to bind as dimers, in which case, there is a monomer-dimer equilibrium, for example MyoX (MyoD or Myogenin) dimerizing with E12, described as follows.

\[
\text{MyoX} + \text{E12} \rightleftharpoons \text{MyoX-E12}
\]

REACTION 1.

where

\[
K_D = \frac{[\text{MyoX}[\text{E12}]]}{[\text{MyoX-E12}]}
\]

There is also a protein-DNA equilibrium, for example MyoX-E12 interacting with DNA, described as follows.

\[
\text{MyoX-E12} + \text{DNA} \rightleftharpoons \text{MyoX-E12-DNA}
\]

where

\[
K_D = \frac{[\text{MyoX-E12}][\text{DNA}]}{[\text{MyoX-E12-DNA}]}
\]

Since there are no data currently to describe the protein/protein interactions, we are unable to assign accurate values to the dissociation constant for the protein/DNA interaction. Alternatively, the proteins may bind the target DNA successively as monomers, as described.

\[
\text{MyoX} + \text{E12} + \text{DNA} \rightleftharpoons \text{MyoX-E12-DNA}
\]

REACTION 3.

where

\[
K_D = \frac{[\text{MyoX}[\text{E12}][\text{DNA}]]}{[\text{MyoX-E12-DNA}]}
\]

In this case, the units of \(K_D\) would be M² and cannot be derived directly from our data.

RESULTS

EMSA Analysis of MSV Enhancer—The MSV enhancer region under study contains six E-box sequences, which were numbered from 1 to 6 starting from the 5’ end (E1, E2, etc; Fig. 1). DNA binding sites for six different nuclear factors were previously identified in the same region (Fig. 1A). To examine MyoD-E12 and Myogenin-E12 binding properties to DNA targets containing multiple E-box motifs, we created sets of nested deletion DNA fragments. The DNA fragment containing all six E-boxes (E123456) was too large for EMSA. Therefore, the first set contained DNA fragments where E-boxes were successively deleted from the 5’ end of the E23456 fragment. A second set of nested deletions was generated from the 5’ end of fragment E23456. Another 3’ set of deletions was analyzed on parent DNA fragment E123 to test the contributions of E1. Using the EMSA described in the previous section, we measured apparent equilibrium binding constants (\(K_{app}\)) for protein-DNA complexes formed with MyoD-E12 or Myogenin-E12 heterodimers and MSV enhancer fragments.

Analysis of 5’ Deletions of E23456—The results of our saturation experiments for 5’ deletions of fragment E23456 in which a limiting amount of DNA (10 pM) was incubated with increasing concentrations of MyoD-E12 or Myogenin-E12 (25 pM to 200 nM) are shown in Fig. 2, C and D. A representative autoradiogram for MyoD-E12 interactions with the E23456 DNA fragment is presented in Fig. 2A and for Myogenin-E12 interactions with the E3456 DNA fragment in Fig. 2B. The unbound DNA signal was quantitated by densitometry, and the percentage of bound DNA was plotted versus the concentration of protein used (Fig. 2, C and D). For all saturation experiments, the concentration of protein required to shift 50% of the DNA was taken as the \(K_{app}\).

The apparent binding constants for the parent E23456 fragment in this set were 0.2 nM and 0.3 nM for MyoD-E12 and Myogenin-E12, respectively. The \(K_{app}\) values for MyoD-E12 increased 16-fold from 0.2 nM for the E23456 fragment to 3.2 nM for the E6 fragment (Table I). There were only very slight changes in \(K_{app}\) when E3 or E5 was deleted from the fragments, indicating that these E-boxes do not contribute significantly to binding affinity by MyoD-E12. The changes in the \(p\) value in Table I for MyoD-E12 do not correlate with changes in affinity. Although removal of E2 from the target DNA increases the \(K_{app}\), 4-fold, there is no significant change in cooperativity. However, deletions of E3 and E4 reduce the positive cooperativity of binding.

The 15-fold increase in \(K_{app}\) for Myogenin-E12, as a result of deleting all but E2 from the parent E23456 fragment, is similar to that of MyoD-E12, and the absolute values for \(K_{app}\) are similar (Table I). However, the large increase in \(K_{app}\) (10-fold) caused by deleting E2 indicates that this E-box is perhaps the most important for Myogenin-E12 binding. The presence of E4 in the fragment does not enhance Myogenin-E12 binding as it did for MyoD-E12. The \(p\) values determined from the curves in Fig. 2D indicate that there is positive cooperativity in binding.
for all DNA fragments tested except E6, but less than that observed for MyoD-E12 binding to the same fragments. Analysis of 3' deletions of E23456—The results obtained for MyoD-E12 and Myogenin-E12 complexes with 5' deletion DNA fragments showed that the presence of E2 increases the affinity of Myogenin-E12 binding, whereas both E2 and E4 enhance formation of MyoD-E12-DNA complexes. Our experiments also indicated that cooperative binding is important in the formation of complexes with these proteins, but it is most pronounced for MyoD-E12. To test these ideas further, we analyzed MyoD-E12 and Myogenin-E12 binding with 3' deletion fragments of the MSV enhancer using assays performed as described above for the 5' deletion fragments. A representative autoradiogram for MyoD-E12 interactions with the E234 DNA fragment is shown in Fig. 3A and for Myogenin-E12 interactions with the E2345 DNA fragment in Fig. 3B. The data from densitometric analysis are presented in Fig. 3, C and D. The values for \( K_{app} \) and cooperativity are summarized in Table II. The parent E23456 fragment in this set was the same as in 5' deletion group. For MyoD-E12, deletion of E6 caused a 12-fold decrease in affinity, and binding was less cooperative. Removal of E5, generating the E234 fragment, almost completely restored binding to the level observed with E23456 (\( K_{app} = 0.3 \) nM), but without the high level of cooperativity observed in binding the E23456 fragment. Subsequent deletion of E4 had devastating effects on binding: the E23 and E2 fragments were bound by Myogenin-E12 with \( K_{app} \) values of 26 and 23 nM, respectively, and all positive cooperativity of binding was lost.

Deletion of E6 from the E23456 fragment reduced the affinity of Myogenin-E12 binding very much like it did MyoD-E12 binding with an increase in \( K_{app} \) of approximately 12-fold but without the concomitant reduction in cooperativity. There was a slight increase in affinity, approximately 2-fold, when E5 was removed, leaving E234. Removal of E4 had devastating effects on binding: the E23 and E2 fragments were bound by Myogenin-E12 with \( K_{app} \) values of 26 and 23 nM, respectively, and all positive cooperativity of binding was lost.

Analysis of 3' Deletions of E123—Since fragment E123456
was too large for EMSA we tested the contribution of E1 in enhancer binding using E123 as the parent fragment and removed E-boxes from the 3' end. A representative autoradiogram of MyoD-E12 and the E234 DNA fragment. Panel B, representative autoradiogram of Myogenin-E12 and the E234 DNA fragment. Panel C and D, the amount of unbound DNA in each lane was quantitated by densitometry and used to calculate the concentration of bound DNA. The percentage of bound DNA is plotted versus the concentration of protein (calculated as the heterodimer). Arrows indicate resolved protein-DNA complexes. Panel C, MyoD-E12; panel D, Myogenin-E12. □, E23456; ●, E234; ▲, E23; ●, E2.

**TABLE II**

| DNA fragment | MyoD-E12<sup>a</sup> | Myogenin-E12<sup>a</sup> |
|--------------|----------------------|--------------------------|
| 23456        | 0.2 nm (3.5)         | 0.3 nm (1.8)             |
| 2345         | 2.5 nm (2.0)         | 3.6 nm (2.1)             |
| 234          | 0.3 nm (2.2)         | 1.5 nm (1.6)             |
| 23           | 1.9 nm (2.2)         | 26.0 nm (0.8)            |
| 2            | 6.5 nm (1.4)         | 23.0 nm (1.0)            |

<sup>a</sup> Numbers in parentheses are cooperativity values, p values, from analysis of the binding curves.

**FIG. 3. EMSA for interaction of MyoD-E12 and Myogenin-E12 with 3' deletions of the E23456 DNA fragment.** MyoD-E12 or Myogenin-E12 and 32P-labeled DNA fragments (10 pm) were equilibrated and electrophoresed through native acrylamide gels as described under "Materials and Methods." Panel A, representative autoradiogram of MyoD-E12 and the E234 DNA fragment. Panel B, representative autoradiogram of Myogenin-E12 and the E234 DNA fragment. Panel C and D, the amount of unbound DNA in each lane was quantitated by densitometry and used to calculate the concentration of bound DNA. The percentage of bound DNA is plotted versus the concentration of protein (calculated as the heterodimer). Arrows indicate resolved protein-DNA complexes. Panel C, MyoD-E12; panel D, Myogenin-E12. □, E23456; ●, E234; ▲, E23; ●, E2.

**Analysis of Internal Mutations**—To clarify the contributions of the individual E-boxes in the MSV enhancer to binding by MyoD-E12 and Myogenin-E12, we tested internal mutations of E2, E3, E4, and E5 using the EMSA. A representative autoradiogram of MyoD-E12 interactions with the E13 DNA fragment is shown in Fig. 5A and of Myogenin-E12 interactions with the E13 DNA fragment in Fig. 5B. The binding profiles are shown in Fig. 5, C and D, and the derived values for K<sub>app</sub> and cooperativity are summarized in Table IV. Compared with the E123 fragment, the E13 fragment was bound with slightly higher affinity by MyoD-E12, but only 10% as well by Myogenin-E12. Thus, E2, in this context, has significant and opposite effects on MyoD-E12 and Myogenin-E12 interactions. Mutation of E3 within an E23456 parent fragment caused a small reduction in MyoD-E12 binding affinity and a much larger 15-fold loss in Myogenin-E12
binding. Compared with the E2345 fragment, the E235 fragment was bound with approximately 4-fold higher affinity by MyoD-E12 and with slightly increased affinity by Myogenin-E12. Removal of E5 from the E456 fragment had no significant effects on MyoD-E12 binding but increased Myogenin-E12 binding 4-fold. Taken together, the results from all of the EMSA experiments indicate that contributions of the various E-boxes within the MSV enhancer differ with respect to MyoD-E12 and Myogenin-E12 as well as with the context of the DNA fragment that encompasses them.

**DISCUSSION**

We tested the hypothesis that MSV-v-ski transgene expression in fast-twitch muscle fibers was responsive to MyoD activity by examining the DNA binding characteristics of MyoD-E12 and Myogenin-E12 (slow fiber-specific) for the MSV enhancer in vitro. The results reported here are our initial analyses of this system and indicate complex interactions between these proteins bound to the six E-boxes in terms of affinity and cooperativity. Further analyses will be required to understand fully the molecular mechanisms involved in regulating expression of the MSV enhancer-promoter. Nevertheless, our results clearly indicate that MyoD-E12 bound nearly all of the DNA fragments tested with higher affinity and higher cooperativity than Myogenin-E12. Thus, we conclude that fiber type IIb-specific expression of MSV-ski in transgenic mice likely results in part from activation by MyoD, which preferentially accumulates in these fibers.

MyoD-E12 binding to all the DNA fragments containing single E-boxes (E1, E2, or E6) showed positive cooperativity, whereas only E1 was bound by Myogenin-E12 cooperatively (Tables I–III). In addition, MyoD-E12 bound all and Myogenin-E12 bound most of the DNA fragments containing multiple

**TABLE III**

Comparison of equilibrium binding constants of MyoD-E12 and Myogenin-E12 with DNA fragments with 5' deletions of E123

| DNA fragment | MyoD-E12 | Myogenin-E12 |
|--------------|----------|--------------|
| 123          | 3.7 nm (2.2) | 2.6 nm (1.4) |
| 12           | 1.0 nm (1.4) | 12.0 nm (1.0) |
| 1            | 1.1 nm (2.2) | 2.4 nm (1.8) |

* Numbers in parentheses are cooperativity values, p values, from analysis of binding curves.
E-boxes with cooperativity. Thus, there are multiple contributions of cooperativity to DNA binding for these transcription factors. These results are strikingly similar to the interactions of a repressor with $O_{R1}$, $O_{R2}$, and $O_{R3}$ (31). A repressor dimerization is enhanced by DNA binding, thus there is cooperativity observed in binding a single operator. Repressor also shows cooperativity in binding tandemly arranged target DNAs, via protein/protein interactions between repressor bound to $O_{R1}$ and $O_{R2}$. Binding to $O_{R3}$ is not cooperative, however, because repressor at $O_{R2}$ interacting with repressor at $O_{R1}$ cannot interact with repressor at $O_{R3}$. However, mutations in $O_{R1}$, which disallow repressor binding, promote repressor binding to $O_{R2}$ and $O_{R3}$ with cooperativity. Such high levels of cooperativity dramatically increase the regulatory response due to small changes in protein concentration or activation (31).

Cooperativity of dimeric protein interactions with a single DNA target site can arise from two distinct mechanisms. The stepwise binding of monomers can show cooperativity by increasing the affinity of binding for the second monomer in the presence of the first bound DNA. Alternatively, cooperativity can also arise for binding of dimeric proteins to a single binding site if protein dimerization is significantly enhanced by DNA binding. It should also be noted that these two mechanisms are not mutually exclusive; both can be occurring. Although our results show cooperativity in binding a single site, they do not help distinguish between whether these proteins bind DNA as dimers, stepwise as monomers, or both. We are currently conducting experiments to clarify the mode of binding utilized by MyoD-E12.

3 J. Hill and C. A. Royer, personal communication.
Studies on DNA recognition by bHLH muscle transcription factors showed that the **CANNTG** E-box sequence establishes a minimal DNA binding site for these proteins. Consensus DNA recognition sequences for MyoD-E12 heterodimer (32) and Myogenin homodimer (and heterodimer) (4th E12 as proposed in Ref. 33) derived in affinity selection experiments show a high level of similarity. The MSV enhancer contains six E-box sequences. Four of the E-boxes (E1, E4, E5, E6) have the sequence **CAGCTG**, which is different from the consensus sequences determined in selection experiments for either MyoD or Myogenin, but is present in enhancers of muscle-specific promoters (34–36). Two of the E-boxes (E1, E3) have the sequence **CAGCTG**, matching the consensus binding site from selection experiments with Myogenin. The crystal structure of the MyoD bHLH domain-DNA complex (8) revealed protein contacts with the bases of the primary E-box determinants, CA and TG, and some contacts to 5’- and 3’-flanking residues in the double-stranded TCAACAGCTGTGGTA DNA, indicating that specific bases outside an E-box may be involved in recognition and binding. It has also been postulated that the central two bases of the E-box motif could contribute to distinct MyoD and Myogenin-DNA binding preferences. Binding site selection experiments showed that Myogenin has a preference for a symmetrical binding site, whereas MyoD binding sequences showed a nonpalindromic distribution of bases in central NN bases of an E-box. In general, our results show only slight differences in affinity for both proteins in combination with E12 binding to single E-box containing DNA fragments (except for E2) and that binding occurs with low to moderate affinity. Thus, MyoD and Myogenin prefer similar DNA targets and bind them with similar affinity.

On the other hand, numerous in vivo experiments have shown that MyoD and Myogenin have distinct functions in the regulation of muscle-specific gene expression. One proposed solution to this apparent contradiction is that heterodimerization, rather than homodimerization, with other bHLH proteins such as ubiquitous E12 and E47 or Id, leads to MyoD- or Myogenin-specific gene activation or inactivation. It has also been shown that other regulatory DNA elements in combination with E-box sequences can differentially contribute to muscle-specific gene activation (37). These regulatory models, although attractive and well documented, share one weakness: namely, E-box sequences are widespread in genomes (average of one E-box in 256 bases) and are present in the promoters of genes not specifically expressed in muscle. Nevertheless, it has been shown that under certain experimental conditions a single E-box motif can be responsible for Myogenin-dependent gene activation (38). In our studies, we showed that MyoD-E12 and Myogenin-E12 DNA binding affinity to targets containing multiple E-box sequences is much higher than interactions of these proteins with single DNA fragments containing a single E-box. This indicates that multiple E-box motifs, when present in promoter regions, could contribute to high affinity DNA binding for these proteins and simultaneously provide an additional level to MyoD and Myogenin DNA recognition specificity. Cooperative binding of MyoD-E47 to DNA targets containing multiple E-box motifs (39) and E-box cooperation in the promoter of the rat acetylcholine receptor subunit gene (40, 41) enhance transcriptional activity. We demonstrated that mutations of certain E-box sequences within MSV enhancer significantly reduce affinity of MyoD-E12 and Myogenin-E12 in cooperative DNA binding presumably through lack of communication between proteins. Conversely, the same situation could arise in vivo when two or more transcriptionally active proteins compete for overlapping DNA recognition sites. This competition could result in a delicate balance of repression and activation. Such a situation can be easily predicted in the case of MSV enhancer, where E2 and E4 constitute internal parts of the glucocorticoid response element. Binding of the glucocorticoid receptor in response to steroid hormone treatment could inhibit MyoD-E12 or Myogenin-E12 binding. In fact, clinical application of glucocorticoids in cancer therapy leads to repression of muscle-specific gene expression and a loss of muscle mass (42). There are similar sequence relationships in other muscle-specific promoters where, in addition to glucocorticoid motif/E-box overlapping motifs, other transcription factor binding sites (e.g. LVA, Ets-1) consistently share the same stretch of bases with E-box sequences.

We have reported the results from an initial probe into the interactions of transcription factor binding with the MSV enhancer. It is clear from our results that complex protein/protein interactions and protein/DNA interactions are likely to contribute to enhancer activity. Specifically, cooperativity of binding plays a much larger role than previously expected. Currently, we are pursuing experiments that will further address the mechanisms contributing to cooperative binding, the biological relevance of MyoD-E12 and Myogenin-E12 DNA binding within MSV enhancer, and for possible modulation of MSV enhancer activity through glucocorticoid receptor/MyoD, Myogenin competition for their DNA binding sites.

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