Rhabdomyosarcomas Do Not Contain Mutations in the DNA Binding Domains of Myogenic Transcription Factors

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Abstract

Skeletal myogenesis is regulated by a group of transcription factors (MyoD, myogenin, myf5, and myf6) that are "basic helix-loop-helix" proteins that bind to the promoters of muscle-specific genes and promote their expression. We have previously shown that after a mutation of Leu_{122} to Arg the DNA binding basic domain of MyoD confers c-*myc*-like functional characteristics to the protein. In this study we used single-strand conformation polymorphism analysis to determine whether such mutations occur naturally in rhabdomyosarcomas. We have found that the basic domains of all the myogenic factors remain unaltered in rhabdomyosarcomas. Selection against such mutations may be the result of functional redundancy of these myogenic transcription factors. (*J. Clin. Invest.* 1994. 93:5–9.) Key words: myogenic proteins • myoD • SSCP • c-*myc* • muscle differentiation

Introduction

MyoD, myogenin, myf5, and myf6 are a group of muscle-specific, "basic helix-loop-helix" (bHLH)[1] transcription factors that regulate skeletal myogenesis (1–3). These proteins bind to the promoters of many muscle-specific genes at sites containing the canonical "E-box" sequence CANNTG. After binding, other domains within these myogenic factors activate transcription of the adjacent gene (4–7). Although some differences have been noted in the various properties of these factors and in their expression during embryogenesis (8–10), each one by itself is fully capable of inducing myogenic differentiation when expressed ectopically in nonmyogenic cells such as fibroblasts (11). This suggests that MyoD, myogenin, myf5, and myf6 are components of a redundant cellular mechanism for ensuring the proper timing and progression of skeletal muscle morphogenesis. This concept has gained support from recent experiments demonstrating that skeletal muscle development in mice lacking functional copies of MyoD or myf5 proceeds relatively normally (12, 13).

DNA binding by the myogenic bHLH proteins requires that they first dimerize. However, such DNA binding by homodimers, while specific, is relatively weak (14). Binding is significantly enhanced by heterodimeric association with the ubiquitously expressed products of the E2A gene product (E12 and E47) and, indeed, the active transcriptional complexes in myogenic cells appear to consist of such heterodimers (14). In the case of MyoD, for example, each component of the heterodimer is thought to contribute a DNA half-site recognition specificity such that the optimal binding site (CACCTG) is a composite of the preferred binding sites of the individual components in homodimeric form (15).

myc family proteins are also members of the bHLH family and bind the E-box motif CACGTG in association with the recently described protein max (16, 17). Myogenic differentiation is accompanied by c-*myc* downregulation and enforced c-*myc* expression inhibits the differentiation process (18, 19). The myogenic proteins and c-*myc* can thus be viewed as components of opposing regulatory pathways in which MyoD and its homologues drive the differentiation process and exert an antiproliferative effect (20) whereas c-*myc* inhibits myogenic differentiation and drives proliferation (21).

The myogenic and myc family member bHLH proteins contain related DNA binding basic domains (Fig. 1). In particular, MyoD and c-*myc* are identical at 5 of the 14 amino acids that comprise this motif. Recently, we reported that substitution of the myogenically highly conserved Leu_{122} in MyoD with the similarly conserved Arg residue present in myc and max family members imparts novel properties to MyoD (22). Although the mutant protein retained the ability to bind to a consensus MyoD site, it was unable to transactivate a promoter containing this site and actually competed with wild-type MyoD in this regard. Furthermore, the mutant MyoD protein gained the capacity to recognize a c-*myc*/*max* binding site and, like c-*myc*, correctly downregulated a gene containing such a site. The acquisition of these unexpected properties by the mutant MyoD protein suggested a novel means by which skeletal muscle tumors (rhabdomyosarcomas) might arise. Point mutation of the highly conserved Leu_{122} codon (CTG) of MyoD to that encoding an arginine (CGG) found at the analogous position of the c-*myc* basic domain would result, on the one hand, in a dominant-negative form of the protein that would compete with wild-type myogenic factors and block their transactivation of target genes in myoblasts. On the other hand mutant MyoD could substitute for authentic c-*myc* protein, which would be downregulated in cells undergoing terminal myogenic commitment. c-*myc* target genes might therefore continue to be regulated normally resulting in a sustained prolifera-

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1. *Abbreviations used in this paper:* bHLH, basic helix-loop-helix; SSCP, single-strand conformation polymorphism.

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tive signal. This combination of inhibited differentiation and enhanced proliferation, mediated either by mutant MyoD protein or other similarly mutant myogenic factors, might initiate or contribute to the neoplastic phenotype. To test this idea, we have surveyed 19 primary rhabdomyosarcoma tumors and 14 rhabdomyosarcoma cell lines for evidence of mutation in the basic domains of any of the four known myogenic proteins. We used PCR to amplify the DNA binding domains of each of the four known myogenic factors and subjected these to single-strand conformation polymorphism (SSCP) analysis to detect point mutations. SSCP has proven to be a highly sensitive and reliable means of detecting such mutations in a variety of genes (23–25). In our survey we have not detected any differences in the electrophoretic mobility of the fragments obtained from the various tumors or cell lines. These results suggest that the generation of rhabdomyosarcomas is not commonly associated with the acquisition of mutations in the DNA binding domains of any of the known myogenic factors. Other molecular events are more likely to contribute to the sustained proliferative capacity and loss of differentiated phenotype of these highly aggressive childhood tumors.

Methods

Tumor tissue samples. 19 primary tumors (13 embryonal, 6 alveolar) and the following 14 cell lines (7 embryonal, 6 alveolar, 1 undifferentiated) were studied: Rh1, Rh3, Rh4, Rh5, Rh18, Rh30, SA2, AD2, Rh28, NIH, A673, A204, TC206C, and MCRB-1 (26–29). Histopathological classification was in accord with the guidelines proposed by Patton and Horn (30). Tumor tissue samples were collected from surgical specimens and snap frozen and stored under liquid nitrogen until further processing. Peripheral leukocytes were obtained from 10 healthy volunteers and separated from other blood components by Ficoll-Hypaque sedimentation. Genomic DNA was isolated from these tissues by standard procedures (31).

Primers for PCR amplification. The forward primers used to specifically amplify MyoD, myogenin, myf5, and myf6, respectively, and the lengths of the fragments amplified are as follows: Myo D, ^{283}CTGTGGGCCTGCAAGGCGTGCAAG^{306}: 167 bp; myogenin, GCCGGATCC^{274}TGTAAGAGGAAGTCGGTGTCCGTG^{291}: 154 bp; Myf5, GCCGGATCC^{269}AAGAGGAAGTCCACCACCATG^{288}: 157 bp; Myf6, GCCGGATCC^{309}AAGAGAAAATCTGCCCCCACTG^{330}: 156 bp.

In the case of myogenin, myf5, and myf6, BamHI sites and "G-C" clamps were added to the forward primers to facilitate cloning. The following reverse PCR primer was chosen for all four myogenic genes that bear identical sequences at this site: 5′ *GCCAAGCTT* CACCTTGGGCAACCGCTGGTTTGG. A HindIII site and "G-C" clamp were added to the reverse primer.

The following primers were used to amplify a 159-bp fragment containing the murine MyoD basic domain from plasmid DNAs: Fwd: ^{441}CTGTGGGCCRFCAAGGCGTGCAAG; Rev: CCGAACCAGCGGCTACCCAAGGTG^{600}.

PCR amplification and fragment isolation. For plasmid DNA amplification, we chose several MyoD cDNAs in which we had previously introduced specific point mutations in the DNA binding basic domains (22). These were used to establish that the conditions chosen for SSCP analysis could distinguish among these various mutants and the wild-type MyoD cDNA sequence. The specific MyoD plasmid DNAs chosen contained the following mutations: mutant 3: $Ala_{113} \rightarrow$ Thr; Mutant 6: $Met_{116} \rightarrow$ Val; Mutant 7: $Arg_{117} \rightarrow$ Leu; Mutant 9: $Leu_{122} \rightarrow$ Arg.

For genomic DNA amplification, the PCR mixture contained 500 ng of genomic DNA, 1 μg of each primer, 3.75 mM $MgCl_2$ (for myogenin and MyoD) or 1.5 mM $MgCl_2$ (for myf5 and myf6), 1:10 reaction volume of magnesium-free 10× PCR buffer (Promega Biotec, Madison, WI), 4 mM each of dNTP, 5 μCi of α-[^{32}P]dCTP (sp act, > 3,000 Ci/mmol; Amersham, Arlington, IL), and 0.5 U of Taq polymerase in a 25-μl reaction volume. Amplification was performed for 40 cycles with denaturation at 94°C, annealing at 58°C, and extension at 72°C for 1 min each. Similar conditions were used to amplify MyoD cDNA sequences except that the PCRs contained 100 pg of the appropriate linearized plasmid DNA. Specific bands were excised from a 5% acrylamide gel, incubated in an elution buffer containing 0.5 M ammonium acetate, 0.01 M magnesium acetate, 1 mM EDTA, and 0.1% SDS overnight, extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and resuspended in 20 μl of distilled water. The eluted fragments migrated as single bands upon reelectrophoresis in nondenaturing polyacrylamide or agarose gels (not shown). That the PCR product obtained derived from the appropriate gene was confirmed by restriction mapping and, in select cases, DNA sequencing (not shown).

SSCP analysis. SSCP analysis was performed essentially as described (23). Approximately 10,000 cpm of the purified PCR fragment (1 μl) was mixed with 9 μl of a loading dye (20 mM EDTA, 0.1% SDS, 95% formamide, 0.04% xylene cyanol, 0.04% bromophenol blue), boiled for 5 min, and quick chilled on ice. 2.5 μl was loaded on a 5% acrylamide gel containing 6.25% glycerol and electrophoresis was carried out at 4°C for 4 h at 35 mA. The gels were dried under vacuum and exposed to x-ray film for 16 h at − 80°C in the presence of an intensifying screen.

Results and Discussion

To first establish that the conditions chosen for SSCP analysis could distinguish a mutant gene from the wild type, we used several plasmids that encoded either a wild-type murine MyoD cDNA or the same cDNA with individual point mutations deliberately engineered into the DNA binding basic domain (22) (Fig. 2). In all cases tested, DNA fragments containing point mutations were readily distinguishable from the wild-type sequence and from one another after SSCP analysis. We conclude from these experiments that SSCP analysis provides a sensitive and reliable means of detecting point mutation in the MyoD basic domain.

We next analyzed genomic DNA samples obtained from 33 rhabdomyosarcomas (19 primary tumors and 14 cell lines) and from the peripheral leukocytes of 10 normal individuals.

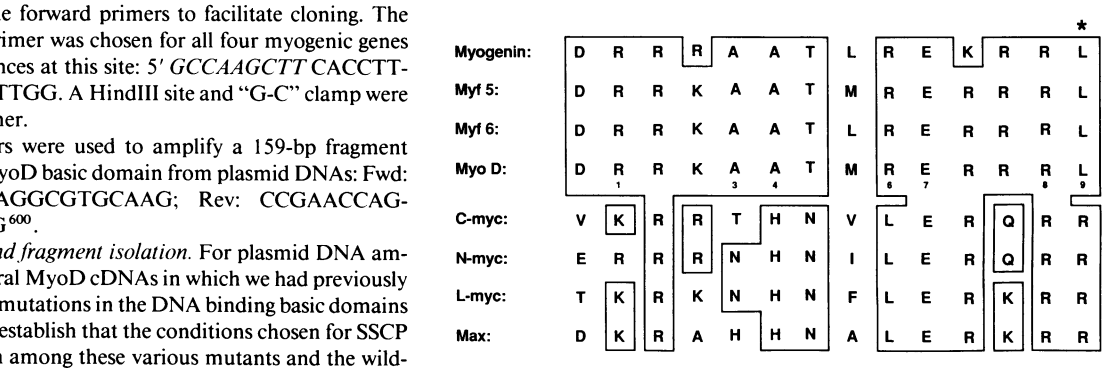

Figure 1. Amino acid (aa) sequence comparison of the basic domain. The sequences compared are the following: myogenin (aa 81–94), MyoD (aa 109–122), myf5 (aa 83–96), myf6 (aa 93–106). The amino acid residues in the basic region that were changed to those found in the analogous position in c-*myc* are numbered from 1 through 9. Mutant 9 where Leu_{122} was changed to Arg is indicated by an astrix.

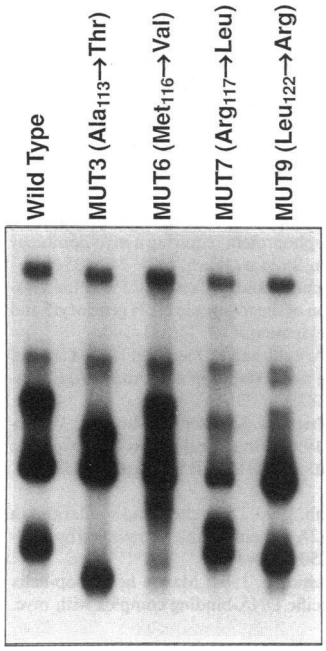

Figure 2. Mobility shift analysis of murine MyoD mutants. DNA fragments encoding the basic domain of MyoD from wild-type and mutants 3, 6, 7, and 9 (22) were analyzed for SSCP as described in Methods. Each mutant fragment migrated with a characteristic pattern different from that of the wild type.

PCR was performed using primers specific for MyoD, myogenein, myf5, and myf6. Specific amplified bands were obtained in all cases and were subsequently purified by polyacrylamide gel electrophoresis before being subjected to SSCP analysis. The myogenic gene of origin of the amplified fragment was confirmed by establishing the presence of predicted restriction endonuclease sites or by DNA sequencing of selected cloned fragments (not shown). Under our experimental conditions, the patterns of DNA strand migration were identical for fragments obtained from either normal or tumor DNAs, suggesting identical DNA sequences in the basic domains (Fig. 3). In the case of myogenin this was confirmed by cloning and sequencing several of the PCR-amplified DNA fragments obtained from tumor DNAs. In all cases, the DNA sequences of the basic domains corresponded to the previously published sequence (32–34). In particular, we found no evidence of mutation at the highly conserved Leu that corresponds to Leu_{122} of MyoD. Thus, we can conclude from this study that mutation within the DNA binding domain of none of the four myogenic factors discussed here is commonly associated with the occurrence of rhabdomyosarcomas.

We and others have provided evidence that Arg_{367} of c-*myc* is a critical determinant of inner dinucleotide (CG) binding specificity in the c-*myc* E-box recognition site, CACGTG (35, 36). This amino acid, highly conserved among myc/max family members, corresponds to the equally well-conserved leucine residue of the four myogenic factors (Leu_{122} in MyoD), which, by analogy, would be important in the recognition of the CC inner dinucleotide sequence in the MyoD binding site (CACCTG) by the MyoD-E12/E47 heterodimer. Given the importance of these basic domain residues to DNA binding and, in particular, to the profound functional consequences of mutation at these sites (35), how might we explain why they are not mutated in rhabdomyosarcomas? Perhaps the simplest explanation lies in the redundancy of the myogenic pathway. Assuming that a mutation such as the MyoD $Leu_{122} \rightarrow$ Arg did occur, it is quite conceivable that its effects would be negligible due to its competition for binding sites, not only with wild-type MyoD encoded by the unmutated allele but with the other myogenic factors as well. Similarly, despite the progressive decline of c-*myc* protein levels in differentiating cells, c-*myc* binding sites might continue to be occupied by max homodimers that bind with quite high affinity to these sites (37, 38). Other bHLH proteins, such as the upstream stimulatory factor (39), and other more recently described max dimerization partners, such as mad and mxi (40, 41), would also be expected to compete with mutant MyoD proteins for these sites as well. Under such conditions basic region mutations of any individual myogenic protein might not be subject to the selective pressures necessary for the genesis of a rhabdomyosarcoma. Thus, one additional beneficial consequence of bHLH family redundancy might be to guard against the full consequences of such deleterious mutations.

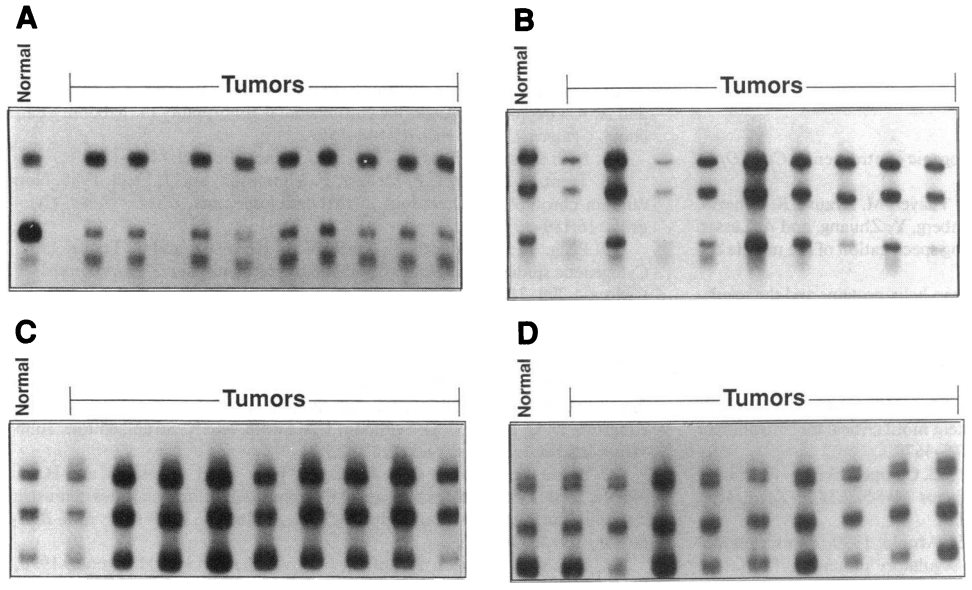

Figure 3. Representative SSCP analysis of myogenin, MyoD, myf5, and myf6 DNA fragments. Genomic DNA from either normal or tumor tissue were amplified by PCR with primers specific for each of the myogenic genes and subjected to SSCP analysis as described in Methods. The various panels represent samples amplified with primers specific for the following order of genes: (*A*) myogenin, (*B*) MyoD, (*C*) myf5, and (*D*) myf6. There appears to be no difference in the migratory pattern between the normal and tumor DNA for all the genes analyzed.

At least two additional factors might conspire against the selection of "activating" mutations within the basic regions of myogenic genes. One of these is the apparent absence in rhabdomyosarcomas of one or more accessory molecules necessary for the function of the MyoD transactivation domain (42). The lack of a myogenically conducive environment might explain why neither endogenous myogenic proteins, commonly expressed in rhabdomyosarcomas, nor those driven by transfected expression vectors, significantly alter the transformed phenotype of such tumors (43). The second factor that might select against the generation of activating mutations is the nature of the transactivating domains of the myogenic proteins themselves. Although the MyoD $Leu_{122} \rightarrow Arg$ mutation does indeed mimic c-*myc* in terms of its ability to regulate a c-*myc*-responsive gene, this effect is unlikely to be universal. Other accessory proteins that interact specifically with the c-*myc* transactivation domain may be essential for the proper regulation of target genes involved in the transformation pathway. Mutant myogenic proteins with the capacity to bind c-*myc* sites might not necessarily interact with the accessory factors required for subsequent transcriptional control of all c-*myc* regulated genes. Consistent with this notion is the inability of the mutant MyoD protein to transform susceptible rat embryo cells in conjunction with an activated *ras* oncogene (our unpublished observations).

In summary, we have demonstrated that mutations in the DNA binding domains of four myogenic proteins do not occur with any appreciable frequency in rhabdomyosarcomas. Such mutations may be selected against as a result of the redundancy of the myogenic and c-*myc* pathways, an intracellular environment that prevents the proper function of the transcriptional activation domains of the myogenic proteins, or lack of binding of accessory proteins necessary for the proper regulation of c-*myc*-responsive genes.

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