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DNA binding and transcriptional regulatory activity of mammalian achaete-scute homologous (MASH) proteins revealed by interaction with a muscle-specific enhancer

(basic helix–loop–helix proteins/MyoD/achaete-scute homologues/determination genes)

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ABSTRACT **The *MASH* genes are vertebrate homologues of achaete-scute, genes required for neuronal determination in *Drosophila*. The sequence of MASH1 and MASH2 contains a basic helix–loop–helix (bHLH) motif that is present in other transcriptional regulators such as MyoD and E12. In the absence of an authentic target for the MASH proteins, we examined their DNA binding and transcriptional regulatory activity by using a binding site (the E box) from the muscle creatine kinase (MCK) gene, a target of MyoD. Like myogenic bHLH proteins, the MASH proteins form heterooligomers with E12 that bind the MCK E box with high affinity *in vitro*. Unexpectedly, however, MASH1 and MASH2 also activate transcription of both exogenous and endogenous MCK in transfected C3H/10T½ fibroblasts. However, they do not induce myogenesis. Myogenic activity is not exclusively a property of the MyoD basic region, as substitution of this domain fails to confer myogenic activity on MASH1. These data suggest that different bHLH proteins may activate overlapping but distinct sets of target genes in the same cell type.**

The *MASH* genes are mammalian homologues of the neuronal determination genes of the *Drosophila* achaete-scute complex (1). Like its *Drosophila* counterparts, *MASH1* is specifically expressed in the developing nervous system (2). The *MASH* gene products are members of the basic helix–loop–helix (bHLH) family of transcription factors, whose members include the *myc* gene products (3) and MyoD (for review see ref. 4) and which appear to be involved in the control of proliferation and cell type determination. Members of the bHLH family share a common basic region required for DNA binding and a helix–loop–helix domain required for homo- and heterodimer formation (5, 6).

Heterodimers between E12/E47 and MyoD (or other myogenic bHLH proteins) bind a core consensus site, CANNTG, termed the E box (6–9). The E box is present in the IgH enhancer-like element of the muscle-specific creatine kinase (MCK) enhancer (10), as well as in the E2 element of the IgK enhancer (5), which appear to be downstream targets of E12/E47 and myogenic bHLH proteins, respectively. Heterodimers of E12 and the *Drosophila* achaete-scute protein T3 will bind the E-box sequences in the IgK enhancer *in vitro* (7).

We wished to determine whether, as predicted by their amino acid sequences, the *MASH* genes encode DNA-binding and transcriptional regulatory proteins. In the absence of an authentic target for the MASH proteins, we used the MCK E box, which has been shown to bind heterodimers of *Drosophila* scute (T4) and E12 *in vitro* (6). We found that both MASH1 and MASH2 form high-affinity E-box-binding complexes as heterooligomers with E12, confirming that they are DNA-binding proteins. Since DNA binding is a necessary but not sufficient condition for transcriptional regulation (6, 11), we also tested the ability of the *MASH* genes to activate transcription of the MCK enhancer. Unlike *Drosophila* scute and E12, which bind the MCK E box but do not activate transcription from this site (6, 11), the *MASH* genes activate transcription of both exogenous and endogenous MCK in C3H/10T½ cells. However, in contrast to MyoD, these genes do not activate the full myogenic program. Substitution of the MyoD basic region in MASH1 fails to convert MASH1 to a myogenic protein.

MATERIALS AND METHODS

In vitro transcription, translation, immunoprecipitation, and electrophoretic mobility shift assay (EMSA) assays were performed by using a reticulocyte lysate system as described (6, 12). Wild-type and mutant E-box oligonucleotides from the IgH enhancer-like sequence in the MCK enhancer (right E box) are as follows: wild-type top strand, GATCCCCCCAACACCTGCTGCCTGA; mutant top strand, GATCCCCCCAACACGGTAACCCTGA (10, 13).

C3H/10T½ mouse embryo fibroblasts were maintained and transfected essentially as described (12). β-Galactosidase staining was performed as described (14). Endogenous myosin and MCK expression were detected by using MF20 hybridoma supernatant (15) and MCK polyclonal antibody (16), respectively, and a Vectastain ABC kit. Cultures were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity as described (17).

Plasmids that have been described previously are RSVL (18), 3300MCKCAT (13), and SVC11S (19). Plasmid pMaori3 is a cytomegalovirus-promoted *lacZ* gene that includes a nuclear localization signal (M. Weber, personal communication). (2E)80MCKCAT (J. Buskin, personal communication) contains two wild-type E-box oligonucleotides (same as those used in the EMSA) oriented 5′/3′:3′/5′ upstream of 80MCKCAT (13). (2mE)80MCKCAT (J. Buskin, personal communication) contains two mutant E-box oligonucleotides (same as those used in the EMSA) oriented 3′/5′:3′/5′ also upstream of 80MCKCAT. pRSVMASH1 is pRSVSV40 (20) containing a 1.4-kilobase (kb) *MASH1* cDNA (1), pRSVMASH1E contains a truncated *MASH1* cDNA (20), pRSVMASH2 contains a 1.4-kb *MASH2* cDNA, pRSVMyoD contains the 1.8-kb *MyoD* cDNA (21). Oligonucleotide site-directed mutagenesis (Amersham kit) of *MASH1* and *MyoD* was used to generate the basic region deletion and substitution constructs (see Table 1).

RESULTS

MASH1 and MASH2 Are DNA-Binding Proteins. Neither the MASH1 nor MASH2 *in vitro* translation products were able to

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Abbreviations: MCK, muscle creatine kinase; bHLH, basic helix–loop–helix; EMSA, electrophoretic mobility shift assay(s); CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus.

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bind an oligonucleotide containing an MCK E box (see *Materials and Methods*), when assayed by an EMSA (data not shown). A similar result was obtained for *in vitro*-translated MyoD assayed in parallel (data not shown), consistent with previous observations (6). The affinity of MyoD for the E box is strongly increased by interaction with E12/E47 (5, 7) (Fig. 1, lane 1). Similarly, both MASH1 and MASH2 in combination with a rat E12 protein (S.J.B., unpublished data) formed E-box-binding complexes of similar size (Fig. 1, lanes 6 and 11). Binding was abolished by competition with excess wild-type oligonucleotide (Fig. 1, lanes 2 and 3, 7 and 8, and 12 and 13), but not by a mutant oligonucleotide unable to compete for MyoD/E12 binding to the E box (Fig. 1, lanes 4 and 5, 9 and 10, and 14 and 15). These data indicate that MASH1 and MASH2 like MyoD form high-affinity E-box-binding heterooligomeric complexes with E12. However, MyoD plus E12 ran as a doublet, suggestive of higher order oligomers, whereas MASH1 plus E12 formed a single band under our conditions. Studies using bacterially expressed MASH1 indicate that homooligomers will bind the MCK E box, but only at micromolar concentrations (data not shown).

To determine whether the interaction between MASH1 and E12 is dependent on the presence of DNA, we performed coimmunoprecipitation experiments with a specific anti-MASH1 monoclonal antibody (2). As shown in Fig. 2, when the E12R (5) and MASH1 proteins were mixed prior to immunoprecipitation, E12R was recovered in the immunoprecipitate (Fig. 2, lane 2), indicating an interaction with MASH1. The amount of E12R coprecipitated by anti-MASH1 was not changed by the presence of unlabeled wild-type or mutant E-box-containing oligonucleotide (Fig. 2, lanes 3 and 4). These data indicate a direct interaction between MASH1 and E12 that is not quantitatively influenced by the presence of their DNA-binding site. The efficiency of heterooligomerization appears low and may reflect an instability of the complexes under our conditions of immunoprecipitation and washing. We have been unable to

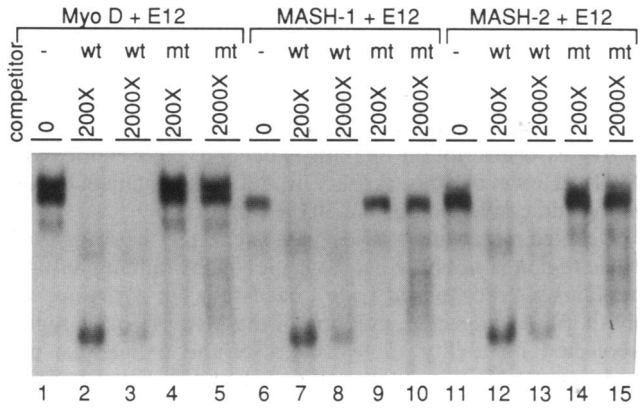

FIG. 1. DNA binding of MASH–E12 complexes with the MCK enhancer. EMSA were performed by using *in vitro*-translated proteins mixed with a ^{32}P-labeled oligonucleotide from the MCK enhancer in a final volume of 20 μl. Lanes 1–5, MyoD and rat E12 combined; lanes 6–10, MASH1 and rat E12 combined; lanes 11–15, MASH2 and rat E12 combined. The numbers in the lanes refer to the fold excess of unlabeled wild-type (wt) or mutant (mt) oligonucleotide added to the assay. The lower shifted band that appears with the excess wild-type oligonucleotide (lanes 2, 3, 7, 8, 12, and 13) was also seen in control reticulocyte lysates (data not shown) and may represent low-affinity binding of a bHLH protein present in the lysate; such a protein has been implicated by the studies of Blackwell and Weintraub (22). The region of the gel with the unbound oligonucleotide probe is not shown. Analysis of [^{35}S]methionine-labeled, *in vitro*-translated MyoD, E12, MASH1, and MASH2 by SDS/PAGE indicated that similar amounts of these proteins were synthesized and added to the gel shift reactions (data not shown).

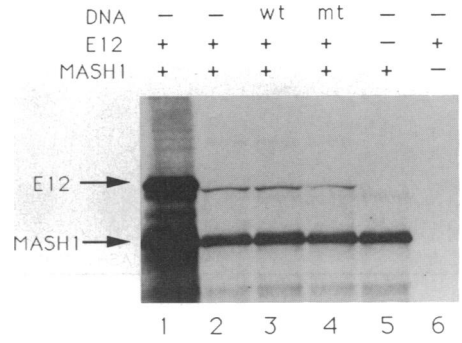

FIG. 2. Coimmunoprecipitation of MASH1 and E12. *In vitro*-translated [^{35}S]methionine-labeled MASH1 and E12 proteins were coimmunoprecipitated by a monoclonal antibody recognizing MASH1 (2). Lane 1, total reticulocyte lysate products; lanes 2–4, immunoprecipitation of E12R (7) plus MASH1; lane 5, MASH1 alone; lane 6, E12R alone. Wild-type (wt) or mutant (mt) MCK oligonucleotide was added to lane 3 or 4, respectively.

examine interactions between MASH1 and E12 *in vivo*, due to an inability to immunoprecipitate MASH1 from whole cell lysates with our monoclonal antibody (T.S., unpublished results).

MASH1 and MASH2 Activate Transcription from the MCK Enhancer. Previous studies of bHLH proteins have demonstrated that DNA binding is necessary but not sufficient for transcriptional activation (6, 11). We therefore asked whether MASH1 and MASH2 were able to influence transcription of a CAT reporter gene under the control of the MCK enhancer. This promoter, comprising 3300 base pairs (bp) of MCK 5′ flanking DNA, is normally inactive in 10T½ cells but is strongly activated by cotransfection of MyoD expression constructs (6, 10). Both MASH1 and MASH2 activated transcription from the 3300MCKCAT reporter gene in transiently transfected 10T½ cells (Fig. 3*A*, lanes 4–6). By contrast, E12 did not transactivate this gene (Fig. 3*A*, lane 2). The activity of MASH1 was not significantly affected by deletion of a 558-bp fragment containing the 5′ untranslated region and the first six amino acids (Fig. 3*A*, compare MASH1 and MASH1-E). The extent of transactivation by MASH1 and MASH2 (80- and 160-fold relative to the RSVL control, respectively) appears 20- to 40-fold lower than that obtained with MyoD in parallel (Fig. 3*A*, lane 3 and data not shown). However, in this experiment, expression of the *MASH* genes and *MyoD* was driven by different promoters. In subsequent experiments, when both *MyoD* and *MASH1* were under the control of the RSV enhancer, transactivation of 3300MCKCAT by MASH1 was 60% of that obtained with MyoD (Fig. 4*A*, lanes 1 and 4; Table 1). Transactivation by *MyoD* and the *MASH* genes did not require exogenous E12, suggesting that 10T½ cells contain endogenous E12-like activity, consistent with previous data (12).

To determine whether E-box elements in the MCK enhancer were responsible for transactivation of MCKCAT by MASH1 and MASH2, we used a reporter construct with two adjacent E-box-containing oligonucleotides placed upstream of a minimal 80-bp MCK promoter [(2E)80MCKCAT]. This E box–reporter construct was transactivated by MASH1 and MASH2 to an extent similar to 3300MCKCAT (Fig. 3*B*, lanes 4–6; Fig. 4*B*, lanes 1 and 4; Table 1). In contrast, transfection of the rat E12 expression vector did not activate the minimal E-box dimer–reporter construct (Fig. 3*B*, lane 2). Transactivation of these minimal constructs is dependent upon the E-box sequence, since a mutation in this site that eliminates MASH1 or MASH2 DNA binding *in vitro* (Fig. 1, lanes 9, 10, 14, and 15) greatly reduces (by 20-fold) transactivation by these proteins *in vivo* (Fig. 3*C*, lanes 4–6). Taken together, these results indicate that MASH1 and MASH2 not only bind

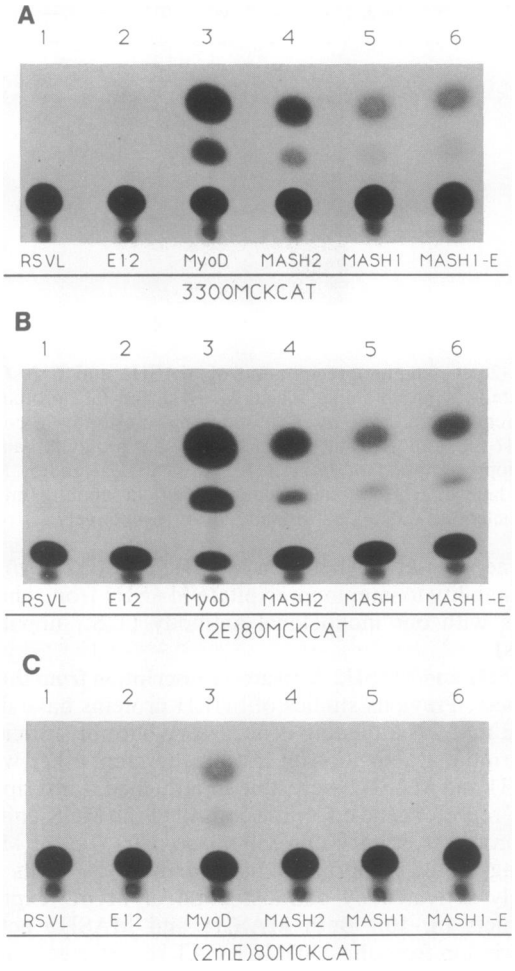

FIG. 3. Transactivation of MCKCAT reporter genes by the *MASH* genes and *MyoD*. CAT activity was assayed in extracts from 10T½ cells transiently cotransfected with 5 μg of the reporter gene 3300MCKCAT (*A*), (2E)80MCKCAT (*B*), or (2mE)80MCKCAT (*C*) and 10 μg of an expression construct containing either luciferase (RSVL) (lanes 1), rat E12 (lanes 2), MyoD (SVC11s) (lanes 3), MASH2 (lanes 4), MASH1 (lanes 5), or a truncated MASH1 lacking the 5′ nontranslated region and the first six amino acids (MASH1-E) (lanes 6). 3300MCKCAT contains 3300 bp of MCK 5′ sequence. (2E)80MCKCAT contains two E-box oligonucleotides from the MCK enhancer fused to 80 bp of MCK basal promoter. (2mE)-80MCKCAT is similar to (2E)80MCKCAT except that mutant E-box oligonucleotides are present. Note that all expression constructs used the Rous sarcoma virus (RSV) long terminal repeat except MyoD, which was expressed from the simian virus 40 early promoter. Equal amounts of protein were added to each reaction mixture.

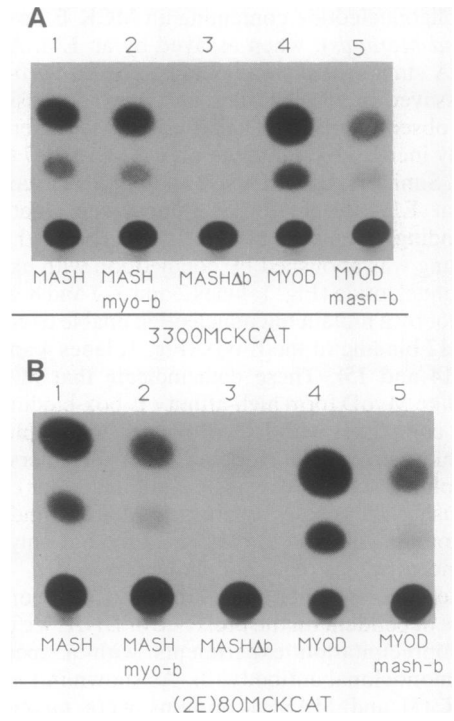

FIG. 4. Activity of basic region exchange mutants of MASH1 and MyoD. CAT assays were performed on extracts of 10T½ cells transiently cotransfected with 5 μg of the reporter gene 3300MCK-CAT (*A*) or (2E)80MCKCAT (*B*) and 10 μg of an RSV expression vector containing either MASH1 (lanes 1), MASH1 with the MyoD basic region (MASHmyo-b; lanes 2), MASH1 with the basic region deleted (MASHΔb; lanes 3), MyoD (lanes 4), or MyoD with the MASH1 basic region (MYODmash-b; lanes 5). Equal amounts of protein were added to each lane.

the MCK E box but also act to enhance transcription of a gene that contains these sites. As in the case of MyoD, transactivation of MCK by the *MASH* genes was abolished by cotransfection of Id (data not shown), a mammalian homologue of *Drosophila* extramacrochaete (12). Thus, the genetic and biochemical interactions between achaete-scute, daughterless, and extramacrochaete in *Drosophila* (23) may be reflected in functional interactions between their mammalian homologues MASH, E12, and Id, respectively. Whether such interactions are important *in vivo* remains to be determined.

MASH1 Activates Endogenous MCK Expression, but Not the Entire Myogenic Program, in 10T½ Cells. The foregoing results raised the question of whether the *MASH* genes, like *MyoD* (21), could induce expression of the endogenous myogenic differentiation program in fibroblasts. Transient transfection of *MASH1* into 10T½ cells failed to induce muscle differentiation as indicated by the lack of cell fusion or expression of myosin heavy chain, a muscle-specific marker (15) (Fig. 5*C*). By contrast, abundant expression of myosin was detected after *MyoD* transfection (Fig. 5*F*). Nevertheless, transfection of *MASH1* activated expression of the endogenous MCK gene in at least 10% of the transfected cells, as determined by staining with a specific anti-MCK antibody (16) (Fig. 5 *A* and *B*; Table 1). Thus, MASH1 activates transcription of both exogenous and endogenous MCK. However, this activity is not accompanied by full myogenic conversion of the 10T½ cells.

Detailed mutagenic studies have suggested that the MyoD basic region is not only necessary for DNA binding but also contains a "recognition code" necessary for myogenic activity (6). As expected, deletion of the MASH1 basic region abolished its ability to transactivate the exogenous and endogenous MCK enhancer (Fig. 4 *A* and *B*, lanes 3; Table 1). Substitution of the MASH1 basic region into MyoD reduced the ability of MyoD to activate exogenous (Fig. 4 *A* and *B*, lanes 5) and endogenous (Fig. 5*H*) MCK transcription, but clearly preserved some activity (Table 1). However, this replacement mutation abolished myogenic activity (Fig. 5*I*), indicating that MyoD containing a MASH1 basic region behaves similarly to intact MASH1. To determine whether the MyoD basic region was sufficient to convert MASH1 to a myogenic protein, we substituted the MyoD basic region for that of MASH1 (Table 1). Although such chimeric constructs activated transcription of both exogenous (Fig. 4 *A* and *B*, lanes 2) and endogenous (Fig. 5*K*) MCK genes, they failed to induce complete myogenesis as assayed by myosin expression (Fig. 5*L*) or cell fusion, again behaving qualitatively like intact MASH1. These data support the idea that the MyoD basic region is necessary for myogenesis, but they reveal that

Table 1. Sequence and activity of MASH1 and MyoD basic region exchange mutants

Construct	Amino acid sequence*	CAT activity†		% β-gal⁺ cells‡	
		3300MCKCAT	(2E)80MCKCAT	MCK⁺	Myosin⁺
	basic region				
MASH1	...FSGFGY**SLPQQQPAAVARRNERERNR**VKLVNL...	60	24	11	0
MASH1Δb	...FSGFGY—————————VKLVNL...	0.5	0.2	0	0
MASH1myo-b	...FSGFGY**KRKTTNADRRKAATMRERRR**VKLVNL...	34	5.0	25	0
MyoD	...**WACKACKRKTTNADRRKAATMRERRR**LSKVNE...	100	100	>100	>100
MYODmash-b	...**WACKACSLPQQQPAAVARRNERERNR**LSKVNE...	11	1.8	3	0
RSVL (control)		0.7	0.2	0	0

*The amino acids exchanged in the expression constructs used for the experiments illustrated in Figs. 4 and 5 are shown in boldfaced type. The line over the sequence shows the basic region conserved between members of the bHLH family.

†CAT activity is shown relative to that of MyoD, which was set to 100. The data are the average of two independent experiments, one of which is shown in Fig. 4.

‡The percentage of transfected (β-gal⁺) cells expressing MCK or myosin immunoreactivity is shown. The data are the average of two independent experiments, one of which is shown in Fig. 5.

this domain is insufficient to confer full myogenic activity upon a heterologous tissue-specific bHLH protein.

DISCUSSION

***MASH1* and *MASH2* Encode Transcriptional Regulatory Proteins.** We have analyzed the DNA-binding and transcriptional regulatory properties of MASH1 and MASH2, two mammalian homologues of achaete-scute, by using the MCK E box as a model target site. *In vitro*, both MASH1 and MASH2 bind the MCK E box with high affinity when complexed with E12. *In vivo*, both *MASH* genes cause a transcriptional activation of reporter constructs containing the MCK E box. This activation is dependent upon the E-box sequence, since mutations in that site that abolish binding of the MASH proteins *in vitro* abolish transactivation *in vivo*. It is possible that the action of the transfected *MASH* genes on MCK is indirect and mediated by induction of endogenous myogenic regulators. We consider this possibility unlikely, however, as myogenic bHLH proteins activate not only MCK but also myosin expression and myoblast fusion in

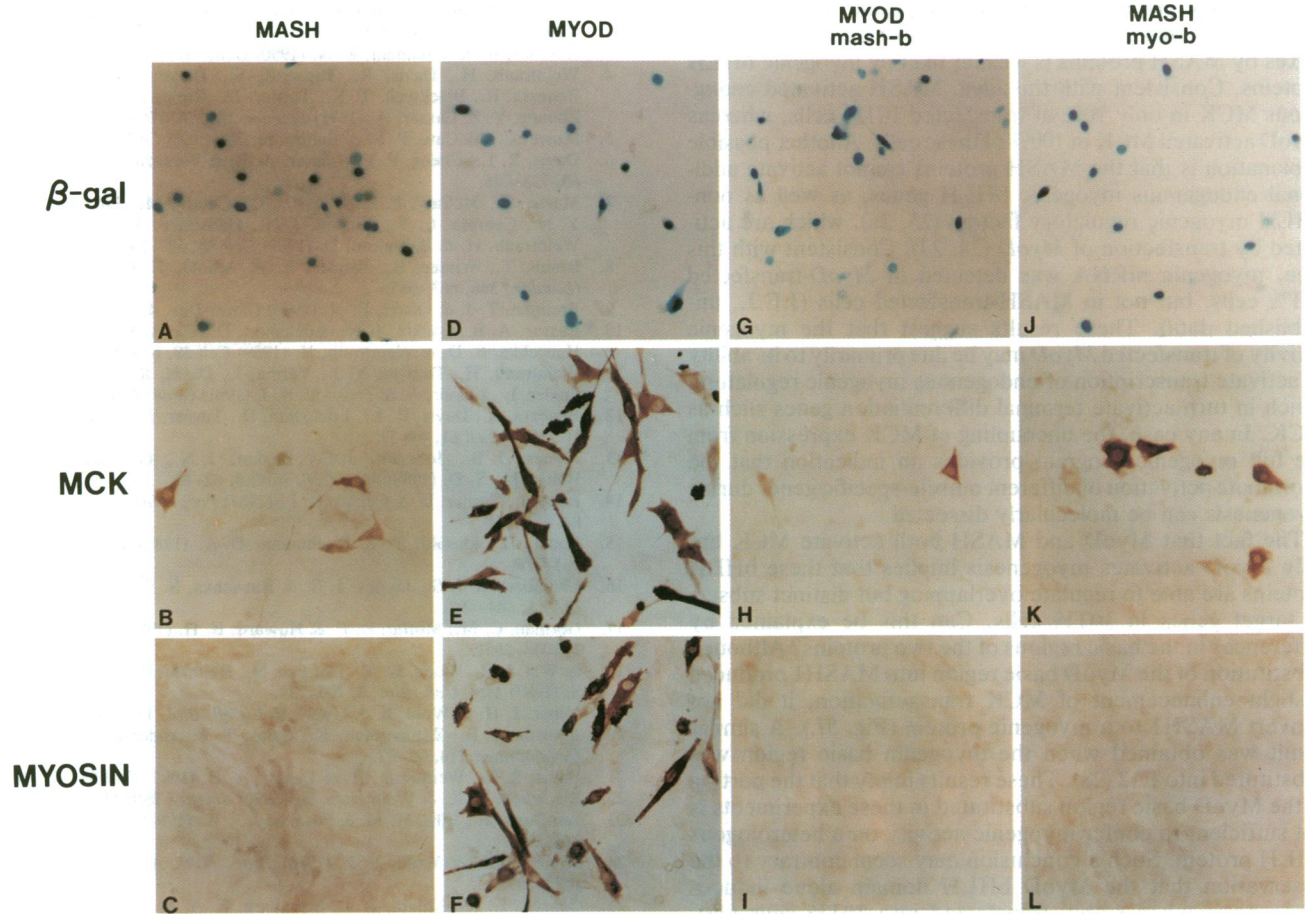

FIG. 5. Induction of endogenous MCK expression but not myogenesis in 10T½ cells expressing MASH1. 10T½ cells were transiently cotransfected with 5 μg of pMaori3 (cytomegalovirus-promoted nuclear-localized *lacZ*) and 20 μg of an RSV expression vector containing either MASH1-E (*A–C*), MyoD (*D–F*), MyoD with the MASH1 basic region (MYODmash-b) (*G–I*), or MASH1 with the MyoD basic region (MASH1myo-b) (*J–L*) (see Table 1 for the sequences of the exchange mutants). Parallel wells were assayed for expression of β-galactosidase (β-gal) (*A*, *D*, *G*, and *J*), MCK (*B*, *E*, *H*, and *K*), or myosin (*C*, *F*, *I*, and *L*) (see *Materials and Methods*). The percentage of MCK-positive and myosin-positive cells present in each condition is given in Table 1.

10T½ cells (4, 21, 24)—responses not observed upon *MASH* transfection. Indeed, we observe that endogenous MyoD and myogenin mRNAs are not induced by transfection of *MASH* genes (J.E.J., unpublished data). Therefore, the MCK enhancer behaves as a transcriptional activation target of the *MASH* genes, although based upon its expression pattern (2), MCK is unlikely to be an authentic target of MASH1 *in vivo*.

MCK Expression Can Be Uncoupled from the Full Myogenic Program. If most or all bHLH proteins can bind the MCK E box *in vitro*, why do they not all activate myogenesis *in vivo*? Previous studies have explained this paradox by the fact that proteins such as E12 or T4 bind the MCK E box but do not activate transcription. Such an explanation cannot account for the lack of myogenic activity by the *MASH* genes, however, because they activate transcription of both exogenous and endogenous MCK. Given that the MASH and scute (T4) proteins differ in the basic region by only 2 out of 11 amino acids (1), it is curious that these regions had different effects when substituted for the basic region of MyoD. While we cannot rule out differences in transfection or assay conditions as the source of this apparent inconsistency, it may be explained by the fact that not only the basic region but also the adjacent N-terminal 9 amino acids have been replaced in both substitution mutations (Table 1 and ref. 6). MASH1 and scute differ at 8 out of 9 positions in this adjacent region. This lack of conservation may explain the difference in the ability of these two genes to activate transcription of the MCK enhancer.

If the *MASH* genes are able to activate MCK expression, why do they not activate the full myogenic program? One possibility is that the *in vivo* activation of muscle-specific E boxes by MASH proteins is weaker than by myogenic bHLH proteins. Consistent with this idea, MASH activated endogenous MCK in only 10% of transfected 10T½ cells, whereas MyoD activated MCK in 100% of these cells. Another possible explanation is that the MASH proteins cannot activate additional endogenous myogenic bHLH genes, as well as non-bHLH myogenic regulatory factors (25, 26), which are activated by transfection of *MyoD* (24, 27). Consistent with this idea, myogenin mRNA was detected in *MyoD*-transfected 10T½ cells, but not in MASH-transfected cells (J.E.J., unpublished data). These results suggest that the myogenic activity of transfected *MyoD* may be due primarily to its ability to activate transcription of endogenous myogenic regulators, which in turn activate terminal differentiation genes such as MCK. In any case, the uncoupling of MCK expression from the full myogenic program provides an indication that the coordinate activation of different muscle-specific genes during myogenesis can be molecularly dissected.

The fact that MyoD and MASH both activate MCK but only MyoD activates myogenesis implies that these bHLH proteins are able to regulate overlapping but distinct subsets of target genes in 10T½ cells. Can this be explained by differences in the basic regions of the two proteins? Although substitution of the MyoD basic region into MASH1 produced a slight enhancement of MCK transactivation, it did not convert MASH1 to a myogenic protein (Fig. 5*L*). A similar result was obtained when the myogenin basic region was substituted into E12 (28). These results imply that the portion of the MyoD basic region substituted in these experiments is not sufficient to confer myogenic activity on a heterologous bHLH protein. Such a conclusion may seem contrary to the observation that the MyoD bHLH domain alone induces myogenesis when stably transfected into 10T½ cells (29). However, recent data suggest that the inclusion of amino acids adjacent (C-terminal) to the MyoD basic region confers myogenic activity on an E12–MyoD basic chimeric protein (H. Weintraub, personal communication). Thus, residues of MyoD flanking the basic region are also needed for its myogenic activity. Myogenic activity may also depend on other functional domains of MyoD, as suggested by detailed studies of a MyoD–E12 basic chimeric protein (11).

The studies presented in this paper confirm that the *MASH* genes encode transcriptional regulatory proteins, as predicted by their deduced amino acid sequences. This activity was revealed by using a model enhancer from a gene that is unlikely to be an authentic target of *MASH* regulation *in vivo*. However, it provides the opportunity to examine the functional interactions between *MASH* and other genes, as well as to dissect functional domains within the MASH proteins. This information should be helpful in designing experiments aimed at interfering with MASH function *in vivo*, as well as in achieving a clearer understanding of how the functional specificity of bHLH proteins is determined by their structure.

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