Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4623–4628, May 1996
Biochemistry

Myocyte-specific enhancer factor 2 acts cooperatively with a muscle activator region to regulate *Drosophila* tropomyosin gene muscle expression

(*mef2* gene/transcriptional regulation/myogenesis)

MEEI-HUA LIN*, HANH T. NGUYEN†, CLAIRE DYBALA*, AND ROBERT V. STORTI*‡

*Department of Biochemistry M/C536, University of Illinois College of Medicine, Chicago, IL 60612; and †Department of Cardiology, Children's Hospital, Boston, MA 02115

Communicated by Irving M. Klotz, Northwestern University, Evanston, IL, January 4, 1996 (received for review October 2, 1995)

ABSTRACT **MEF2 (myocyte-specific enhancer factor 2) is a MADS box transcription factor that is thought to be a key regulator of myogenesis in vertebrates. Mutations in the *Drosophila* homologue of the *mef2* gene indicate that it plays a key role in regulating myogenesis in *Drosophila*. We show here that the *Drosophila tropomyosin I (TmI)* gene is a target gene for *mef2* regulation. The *TmI* gene contains a proximal and a distal muscle enhancer within the first intron of the gene. We show that both enhancers contain a MEF2 binding site and that a mutation in the MEF2 binding site of either enhancer significantly reduces reporter gene expression in embryonic, larval, and adult somatic body wall muscles of transgenic flies. We also show that a high level of proximal enhancer-directed reporter gene expression in somatic muscles requires the cooperative activity of MEF2 and a cis-acting muscle activator region located within the enhancer. Thus, *mef2* null mutant embryos show a significant reduction but not an elimination of *TmI* expression in the body wall myoblasts and muscle fibers that are present. Surprisingly, there is little effect in these mutants on *TmI* expression in developing visceral muscles and dorsal vessel (heart), despite the fact that MEF2 is expressed in these muscles in wild-type embryos, indicating that *TmI* expression is regulated differently in these muscles. Taken together, our results show that *mef2* is a positive regulator of *tropomyosin* gene transcription that is necessary but not sufficient for high level expression in somatic muscle of the embryo, larva, and adult.**

Much is now known about axis formation and segmentation of the *Drosophila* embryo; however, relatively little is known about morphogenesis of the internal organs such as muscle. In vertebrates, myogenesis is controlled in part by the MyoD family of basic helix–loop–helix transcription factors and a second factor, the myocyte-specific enhancer factor 2 (MEF2) (reviewed in refs. 1–3). In *Drosophila* the only MyoD muscle homologue identified thus far is the *nau/Dmyd* gene; however, the timing and restricted pattern of expression of the *nau/Dmyd* gene suggests that it probably has a more limited role in muscle differentiation than its vertebrate homologues (4, 5).

A single *mef2* gene has been identified in *Drosophila* (6, 7). The *mef2* gene is expressed in the mesoderm of early embryos shortly after gastrulation, and continues to be expressed in cardioblasts and in visceral and somatic muscle lineages throughout embryogenesis. This pattern of expression suggests that *mef2* may be important in regulating the earlier stages of myogenesis that establish mesoderm and muscle lineages, as well as the later stages of myoblast fusion and differentiation. Mutations in the *mef2* gene, however, suggest that the role of *mef2* in regulating *Drosophila* myogenesis may be limited to later aspects of myogenesis because muscle patterning in *mef2* mutant embryos appears to be normal up to the stage of myoblast fusion and differentiation to form myotubes (8, 9). Thus far no target genes for MEF2 regulation have been identified in *Drosophila*.

The MEF2 factors belong to the MADS box family (named for MCM1, agamous, deficiens, and serum response factor) of transcription factors (reviewed in refs. 3, 10, and 11), and bind DNA through a 56-amino acid MADS box domain that recognizes A+T rich sequences found in the enhancers of many vertebrate muscle genes (3). MEF2 can also form dimers through the MADS box domain and the adjacent 29-amino acid MEF2 domain that is highly homologous among the MEF2 family members. In the experiments presented here, we have examined the role of the *mef2* gene in regulating transcription of the *Drosophila tropomyosin I* (*TmI*) gene. We show that each of two previously identified *TmI* muscle enhancers (12, 13) contains a consensus MEF2 binding site that is a direct target for *mef2*-regulated transcription in somatic body wall muscles during all stages of development. Interestingly, regulation of *TmI* expression in visceral muscle and dorsal vessel (heart) does not appear to require MEF2 despite the fact that it is also expressed in these two muscle types.

MATERIALS AND METHODS

Nuclear Extract Preparation and Gel Mobility Shift Assay. Nuclear extracts were prepared from isolated nuclei as described (14) except that KCl was used instead of $(NH_4)_2SO_4$ to extract the nuclei. Gel mobility shift assays were performed according to Parmacek *et al.* (15) with slight modifications. Gel mobility shift assays of the 1B(c) fragment (see Fig. 2*A*), and those of the double-stranded oligonucleotides (see Fig. 2*B*) were in 5 and 8% polyacrylamide gels, respectively. The rabbit polyclonal anti-MEF2 antibody has been described (8).

DNA Cloning and Plasmid Preparation. The reporter gene P-element vector construct containing the *Drosophila hsp70* gene promoter and the *Escherichia coli lacZ* gene (pWhsp70lac) and the reporter constructs 1B (proximal enhancer), 1B(c), and muscle activator (MA) DNA fragment [referred to previously as 1B(a)] have been described (12, 13). The 1BΔMEF2(a and b) constructs were made by mutating the 1B plasmid using the Altered Site mutagenesis system (Promega) and the single-stranded oligonucleotides indicated below. The mutated fragments were amplified by PCR and cloned into the *Kpn*I–*Not*I site of the pWhsp70lac transformation vector. The MA+MEF2, MA+ΔMEF2, MEF2, and ΔMEF2 constructs were made using the double-stranded oligonucleotides listed below which contain 5′ *Bam*HI and 3′ *Not*I protruding ends.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: *TmI*, *Drosophila tropomyosin I*; MA, muscle activator; MEF2, myocyte-specific enhancer factor 2; IFM, indirect flight muscles; TDT, tergal depressor of the trochanter (jump) muscles.

‡To whom reprint requests should be addressed at: Department of Biochemistry M/C536, University of Illinois College of Medicine, 1853 West Polk Street, Chicago, IL 60612.

The MA+MEF2 and MA+ΔMEF2 constructs were made by cloning a *Kpn*I–*Bam*HI MA fragment along with the double-stranded oligonucleotide into the *Kpn*I–*Not*I site of the pWhsp70lac vector. The MEF2 and ΔMEF2 constructs were made by cloning the oligonucleotide into the *Bgl*II–*Not*I site of the pWhsp70lac vector. The 3B construct was made by cloning a 3B fragment amplified by PCR into the *Kpn*I–*Not*I site of the pWhsp70lac vector. 3BΔMEF2 was made by PCR amplification of the 3B fragment incorporating the single-stranded mutant oligonucleotide listed as described (16). All clones were sequenced to verify their integrity. 1BΔMEF2(a); single-stranded 5′-CATACGCATTTGAGCTCAACTCTGCCTG-3′ 1BΔMEF2(b); single-stranded 5′- CATACGCATTTCTC-GAGAACTCTGCCTG-3′ MA+MEF2 and MEF2; double-stranded 5′-GATCCGCATTTATTTTTAACTCTGC-3′ MA+ΔMEF2(a) and ΔMEF2(a); double-stranded 5′-GATC-CGCATTTGAGCTCAACTCTGC-3′ MA+ΔMEF2(b); double-stranded 5′-GATCCGCATTTCTCGAGAACT-CTGC-3′ 3BΔMEF2; single-stranded 5′-GCATATAAAGT-TTGAGCTCAACCCCACCG-3′.

P-Element Transformation and Analysis of Transgene and Endogenous *TmI* Expression. P-element transformations were carried out according to Spradling and Rubin (17) and as described (12). Whole mount *in situ* hybridization using digoxigenin-labeled antisense β-galactosidase and *TmI* RNA for analysis of transgene and endogenous *TmI* expression, respectively, was performed as described (18, 19). β-galactosidase enzymatic staining of larvae and adult flies was done as described (13). A rough quantitation of expression levels in larval and adult muscles among different constructs was achieved by the visual monitoring of the timed appearance of the blue reaction products from the 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) substrate/β-galactosidase reaction. Generally, the highest expressing constructs achieved a

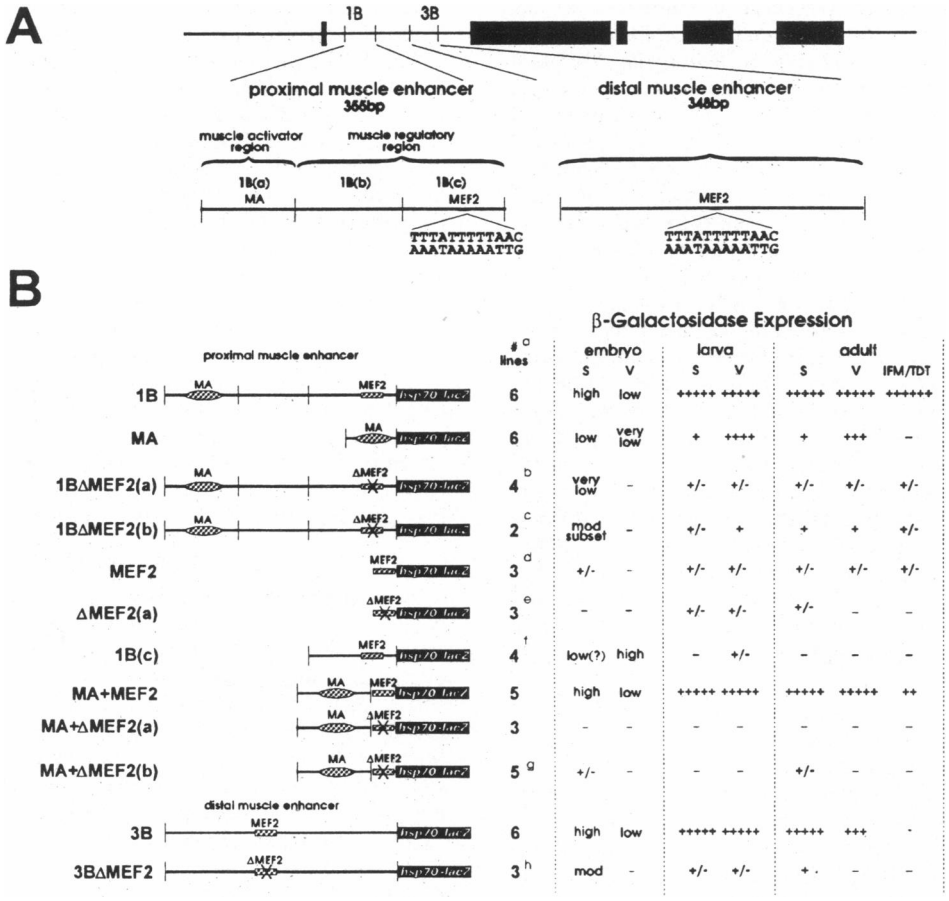

FIG. 1. Organization of muscle enhancers within the first intron of the *TmI* gene and expression of MEF2 transgenes. (*A*) Schematic diagram of the proximal and distal enhancers. The proximal enhancer 1B(a), 1B(b), and 1B(c) fragments are indicated, and their functions were described (13). The 12-bp conserved sequences in the proximal and distal enhancers that contain the consensus MEF2 binding site sequence are indicated. (*B*) Expression levels of β-galactosidase mRNA in embryonic and β-galactosidase enzyme in larval and adult muscles that are observed with specific enhancer and MEF2 transgene constructs. S and V refer to somatic and visceral muscles, respectively. The + and − measurement of expression levels is described in *Materials and Methods*. Unless otherwise indicated, all of the lines for a construct showed the indicated expression pattern. [a], Number of independently transformed lines obtained and analyzed. [b], Three lines showed no expression; one line showed very weak expression in embryonic body wall muscles and larval and adult somatic and visceral muscles as well as moderate expression in adult IFM/TDT muscles. [c], Both lines showed moderate embryonic expression in a subset of ventral body wall muscles and one line also showed ectopic expression in the epidermis; also one line showed weak visceral muscle expression in larvae and adults, whereas the other showed moderately high expression in all larval and adult muscles. [d], Two lines showed no expression; one line showed weak expression in somatic muscles of embryos, in somatic muscles, and hindgut visceral muscles of larvae and adults, including IFM and strong expression in TDT muscles. [e], One line showed mid- and hindgut endodermal and epidermal expression and one line showed epidermal expression in embryos; two lines showed no expression and one line showed very weak expression in a subset of larval dorsal body wall muscles and midgut visceral muscles. All three lines showed very low expression in adult abdominal muscles. [f], One line showed no expression and three lines showed expression in embryonic visceral muscles and in larval hindgut visceral muscles. [g], One line showed light body wall, one line showed epidermal, and two lines showed peripheral nervous system expression in embryos; no lines showed larval expression but all lines did express in the adult abdominal body wall muscles. [h], All three lines showed moderate expression in embryonic body wall muscles; two of three lines showed no larval body wall or visceral muscle staining and one line showed very weak expression in larval body wall and visceral muscles; all three lines showed weak expression in the adult abdominal body wall muscles.

maximal blue intensity for most muscles in ≈2–3 hr of staining and are referred to as "+++++." Indirect flight muscles (IFM) often reached maximum staining sooner. Transgenic flies containing lower expressing constructs were allowed to incubate in stain until they reached an equivalent intensity of blue up to a limit of 20 hr of staining and are referred with a "+." A "±" designation refers to constructs in which most of the lines showed no or variable expression. A "-" means no lines expressed.

***Drosophila* Stocks.** Fly stocks were maintained at 22°C or 25°C on standard media. The deficiency stocks *Df(2R)X1* and *Df(2R)P520* and the null allele $mef2^{22-21}$ have been described (8, 20).

RESULTS

Organization of the *TmI* Intron Enhancer Region and MEF2 Binding. Previous deletion analysis and expression studies of reporter genes in transgenic flies have identified two muscle enhancer regions within the first intron of the *TmI* gene (Fig. 1*A* and refs. 12 and 13). Indeed, both the proximal and distal enhancers in the intron can direct high level expression of a heterologous *hsp70* promoter/β-galactosidase reporter gene in the somatic body wall and visceral muscles of the embryo, larva, and adult. However, unlike the proximal enhancer, the distal enhancer cannot direct expression in the adult IFM/tergal depressor of the trochanter (jump) (TDT) muscles.

On the coding strand the proximal enhancer fragment contains a 12-bp sequence gTTAAAAATAAa with a perfect match of the 10-bp vertebrate MEF2 consensus binding sequence (3, 6, 7). To determine whether the proximal enhancer could be a target for MEF2 binding, the 120-bp 1B(c) fragment containing the putative MEF2 binding site was used in a gel mobility shift assay to test for MEF2 binding activity in embryo nuclear extracts. Fig. 2*A* shows that several bands are retarded when nuclear extract is mixed with labeled 1B(c) DNA (lane 2). The binding activity in the extracts is due at least in part to MEF2, since MEF2 antibody specifically supershifts one major and two minor bands (lanes 3 and 4). The supershifted bands are not seen with preimmune serum (lanes 5 and 6), are competed by a double-stranded oligonucleotide containing the 10-bp MEF2 consensus binding sequence (lanes 7 and 8), and not competed by an oligonucleotide containing a 6-bp mutation within the consensus sequence (lanes 9 and 10). Further analysis of the 1B(c) fragment showed that the MEF2 binding activity was in a fragment containing the consensus MEF2 binding sequence and a mutation in this sequence in the 1B(c) fragment eliminated the supershifted products (not shown). As with the proximal enhancer the distal enhancer also contains a 12-bp sequence with a consensus MEF2 binding site sequence. Similar results were obtained when the distal enhancer 3B fragment was assayed for MEF2 binding (not shown). Accordingly, a double-stranded oligonucleotide containing the consensus MEF2 binding and flanking sequences was assayed for binding activity. Fig. 2*B* shows that, upon incubation of nuclear extract with the labeled consensus sequence oligonucleotide, three major bands are retarded (lane 2), which are supershifted with MEF2 antibody (lanes 7 and 8). The shifted bands are competed by a wild-type sequence oligonucleotide (lanes 3 and 4) and not competed by a mutated oligonucleotide (lanes 5 and 6). The highest molecular weight supershifted product may not be specific, because a band at this position is also detected with preimmune serum (lanes 9 and 10). The fact that multiple bands are shifted in both experiments suggests that MEF2 may be bound to DNA as a monomer, homodimer, or a complex with additional proteins in the extract.

MEF2 Is Necessary but Not Sufficient for High Level Muscle Expression in Transgenic Flies. To determine the role of *mef2* in regulating enhancer activity, we made reporter gene constructs in which the MEF2 binding site was altered in sequence or position, and tested their effects on enhancer

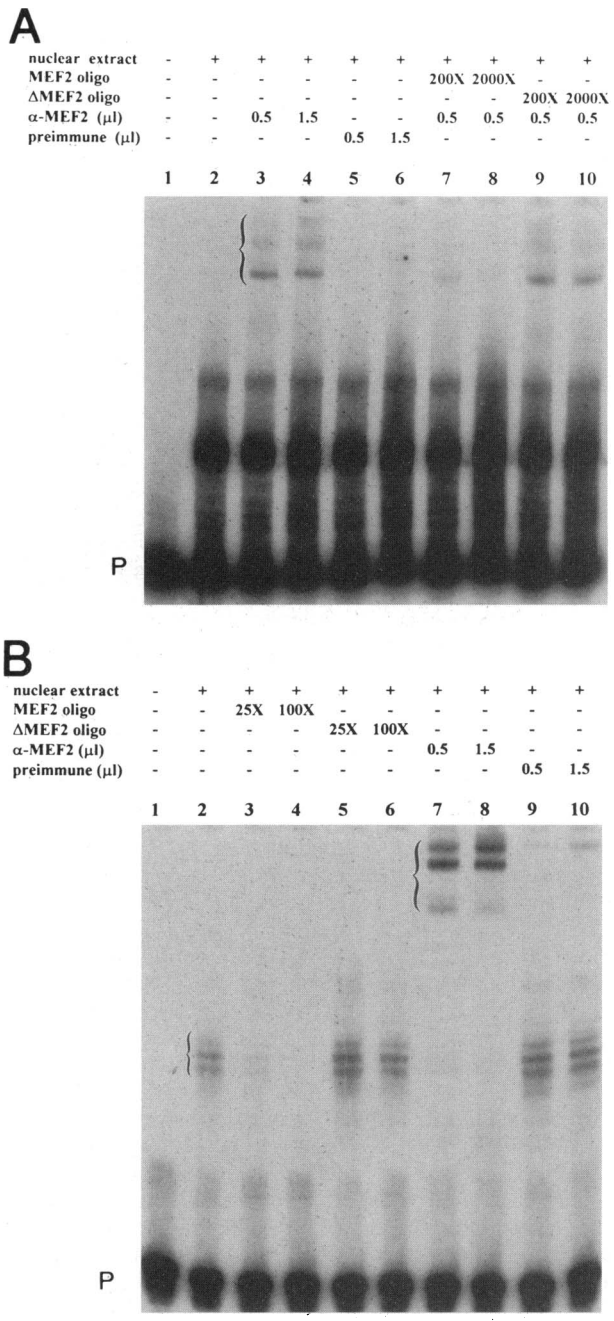

FIG. 2. Gel shift analysis of MEF2 binding to the 1B(c) fragment DNA (*A*) and a MEF2 double-stranded oligonucleotide binding sequence (*B*). Nuclear extracts prepared from 0- to 24-hr embryos were used and the MEF2 and ΔMEF2(a) competitor oligonucleotides are as described. The MEF2 antiserum (α-MEF2) and preimmune serum have been described (7). Brackets in *A*, lane 3, and *B*, lane 7, show the MEF2 supershifted products. Bracket in *B*, lane 2 shows the gel retardation products. P refers to the position of the probe.

function in transgenic flies (Fig. 1*B*). As shown previously (13), the proximal enhancer fragment (1B construct in Fig. 1*B*) can direct low level visceral muscle expression and high level expression in somatic body wall muscles in embryos (Fig. 3*A*), and high level expression in the body wall and visceral muscles of larvae (Fig. 4 *A* and *B*), and adults (not shown). By contrast, the first 90-bp of this fragment [MA fragment in Fig. 1*B* and previously referred to as 1B(a)] can direct only basal level (about 10%) expression in body wall muscles (Figs. 3*B* and 4*C*). Expression in visceral muscle by the proximal enhancer is too low to reliably quantitate in embryos. However, staining of

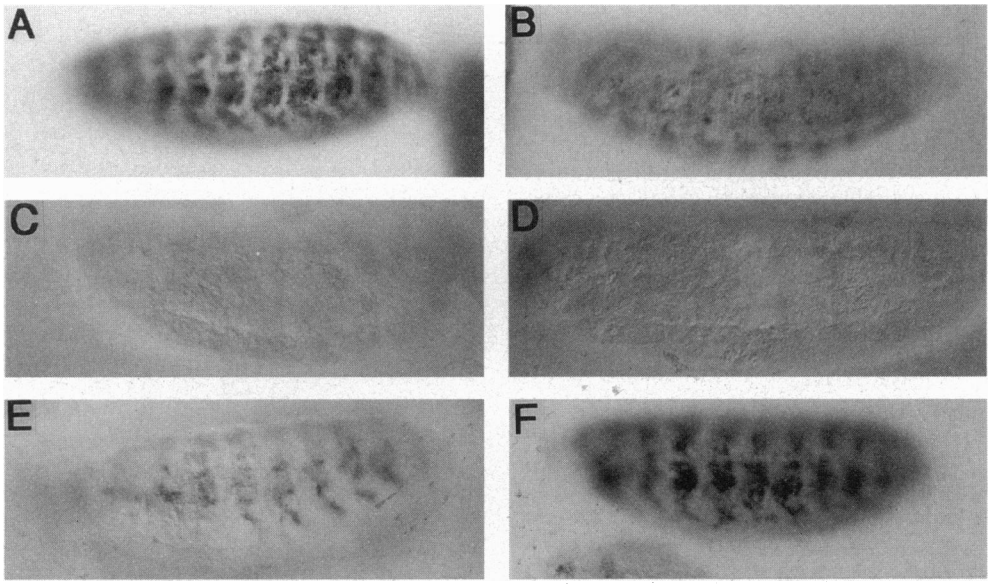

FIG. 3. Whole mount *in situ* hybridization analysis of embryonic body wall expression of MEF2 transgene constructs. The probe for detecting transgene expression was an antisense β-galactosidase riboprobe. (*A*) 1B transgene; (*B*) MA transgene; (*C*) 1BΔMEF2 transgene; (*D*) MEF2 oligonucleotide transgene; (*E*) 3BΔMEF2 transgene; (*F*) MA+MEF2 transgene. All embryos are approximately stage 16 and are positioned with anterior to the left and dorsal up.

larval visceral muscle fibers, which are more distinct, shows that MA fragment-directed expression is only slightly reduced as compared to that obtained with the entire proximal enhancer (Fig. 4 *B* and *D*). The MA region construct does not drive any detectable expression in adult IFM/TDT muscles (13).

We first tested the effect of mutating the MEF2 binding site on enhancer function by making two different 6-bp mutations in the MEF2 site in the context of the entire proximal enhancer [1BΔMEF2(a) and (b) in Fig. 1*B*]. Both mutations eliminated MEF2 binding activity in nuclear extracts (not shown). Transgenic flies carrying the 1BΔMEF2(a) construct show either no expression or basal level expression in the somatic body wall and visceral muscles of embryos (Fig. 3*C*), larvae (Fig. 4 *E* and *F*), and adults (not shown). Both of the 1BΔMEF2(b) lines showed moderate embryonic expression in a subset (mostly ventral) of body wall muscles, and one of the 1BΔMEF2(b) lines also expressed at a low basal level in larval and adult visceral muscle only and the other at a moderately high level in body wall and visceral muscles in larvae and adults. These observations probably reflect differences in the mutated sequences between the two 1BΔMEF2 constructs and/or by the integration of the transgene in the higher expressing line in a chromosomal region influenced by a nearby enhancer. Reporter gene expression in adult somatic and visceral muscle was quantitatively the same as in larvae for all of the constructs tested and therefore only the larval results are shown. In summary, mutation of the MEF2 binding site severely reduced or eliminated muscle expression directed by the proximal enhancer in embryonic, larval, and adult body wall and visceral muscles.

We next tested whether a single MEF2 site alone is sufficient to drive muscle expression. The reporter gene was placed under the control of a single copy of a 20-bp oligonucleotide containing the 10-bp proximal enhancer MEF2 site (MEF2 construct in Fig. 1*B*) and its expression determined in transgenic flies. In the three lines containing this construct, two showed no detectable expression in embryonic (Fig. 3*D*), larval (Fig. 4 *G* and *H*), or adult muscles (not shown). One line showed very low body wall expression in embryos, larvae, and adults, including IFM and strong expression in TDT muscles. We also analyzed the transcriptional activity of the 20-bp sequence oligonucleotide containing the same mutation of the MEF2 site as in 1BΔMEF2(a) construct. Two of three lines of this ΔMEF2(a) construct (Fig. 1*B*) showed no expression; however, one line showed very low expression in a subset of dorsal body wall muscles and midgut visceral muscle of the larva, and all three lines showed very low expression in adult abdominal muscles, suggesting that the mutated MEF2 site or sequences on either side of it contain some transcriptional activity.

We examined further the activity of the single MEF2 site in the context of the larger 120-bp 1B(c) fragment to test the possibility that the MEF2 site needs to be buffered from the P-element sequences and/or the promoter [1B(c) in Fig. 1*B*]. Three of the four lines containing this construct showed what may have been low level expression in embryonic body wall muscle; however, each line also showed considerable ectopic nonmuscle staining, thus making it difficult to determine if there was some low level staining in muscle. We did not detect any body wall or midgut visceral muscle staining in larvae or adults but did detect expression in larval hindgut muscles in these three lines. The fourth line showed no expression at any stage. We conclude that a MEF2 site oligonucleotide alone cannot drive expression in body wall or visceral muscles. However, in the context of the larger 1B(c) fragment, the MEF2 site or other sequences in this fragment may be capable of directing low level expression in embryonic body wall muscles but not in larval or adult muscles. Thus, MEF2 must work in conjunction with additional sequence elements in the proximal enhancer to direct high level muscle expression.

The distal enhancer, which also contains a MEF2 binding site, can direct expression in somatic and visceral muscles to comparable levels as the proximal enhancer (3B in Fig. 1*B*). The effect of mutating the MEF2 binding sequence on distal enhancer-directed expression in larval and adult muscles was the same as with the proximal enhancer. All three of the lines showed either no or basal level expression in larval and adult muscles (Fig. 1*B*, line 3BΔMEF2). Surprisingly, and unlike the results obtained with the proximal enhancer, all three lines showed a moderate level of expression in embryonic body wall muscles (Fig. 3*E*), suggesting that MEF2 may not be the only factor required for distal enhancer-directed high level expression in the embryo.

MEF2 Can Act Cooperatively with the MA Region to Restore Proximal Enhancer Activity. The results from the

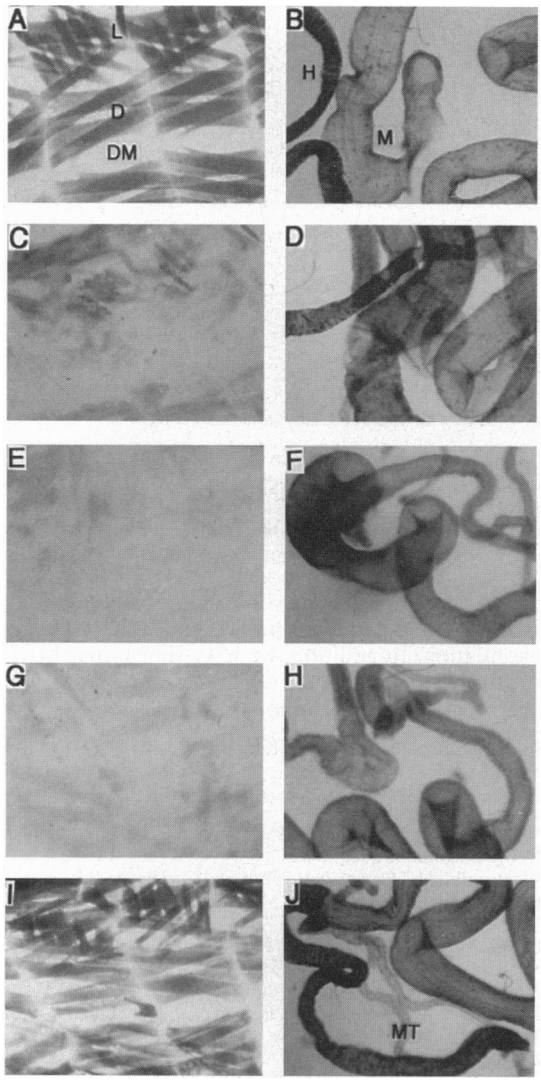

FIG. 4. Larval body wall and visceral muscle expression of MEF2 transgene constructs. (*A*, *C*, *E*, *G*, and *I*) Three-hour β-galactosidase-stained larval body wall muscles. (*B*, *D*, *F*, *H*, and *J*) Three-hour β-galactosidase-stained larval visceral muscles. (*A* and *B*) 1B transgene; (*C* and *D*) MA transgene; (*E* and *F*) 1BΔMEF2 transgene; (*G* and *H*) MEF2 oligonucleotide transgene; (*I* and *J*) MA+MEF2 transgene. L, lateral muscles; D, dorsal muscles; DM, dorsal midline; H, circular muscles of the hindgut; M, longitudinal muscles of the midgut; MT, unstained malpighian tubules.

present and previous studies suggest that MEF2 may act cooperatively with the MA region to facilitate high level expression. To test this notion, a 20-bp oligonucleotide with the MEF2 site from the 1B(c) fragment was cloned downstream of the MA region fragment of the proximal enhancer (MA+MEF2 in Fig. 1*B*) and transgenic flies containing this construct were analyzed for muscle expression. All five of the MA+MEF2 transgenic lines showed high level β-galactosidase staining in the body wall muscles of embryos (Fig. 3*F*), body wall and visceral muscles of larvae (Fig. 4 *I* and *J*) and adults (not shown) at a level indistinguishable from the entire proximal enhancer. All lines also showed low expression in the IFM/TDT muscles, which is in agreement with other ongoing experiments that indicate that high level IFM/TDT expression requires an additional cis-acting element located at the boundary of the 1B(b) and 1B(c) fragments (A. Wohlwill and R.V.S., unpublished work). Thus, MEF2 can act cooperatively with the MA region factors to restore full enhancer activity. Interestingly, two different constructs [MA+ΔMEF2(a) and (b) in Fig. 1*B*] containing the MA fragment ligated to an oligonucleotide with a mutated MEF2 site showed no expression (see *Discussion*).

***TmI* Expression in *mef2* Mutant Embryos.** To assess the role of *mef2* regulation of *TmI in vivo*, we examined *TmI* expression in *mef2* loss of function mutations. Embryos transheterozygous for the deficiencies *Df(2R)X1* and *Df(2R)P520* are null for MEF2 protein (8). *TmI* expression in the body wall muscles of these transheterozygous deficiency embryos is greatly reduced when compared to wild-type embryos (compare Fig. 5 *A* and *B*). The observed low level of *TmI* expression is either in mononucleated myoblasts or in the small number of multinucleated muscle fibers that do form. This low level of body wall muscle *TmI* RNA can be accounted for by the reduced number of myoblasts and myotubes in the mutants and is most likely the expression directed by the MA region. No *TmI* expression was detected in the pharyngeal muscle in *mef2* mutants. Interestingly, *TmI* expression in the visceral muscles (Fig. 5*C*) and dorsal vessel (Fig. 5*D*) approximates the expression levels observed in wild-type embryos, although the gut of *mef2* mutant embryos does not form normally (8, 9). Thus high level *TmI* expression in these abnormally formed visceral muscles does not require *mef2*. Similar results (not shown) were obtained when *TmI* expression was analyzed in the $mef2^{22-21}$ null allele (8).

DISCUSSION

***mef2* Regulation of *TmI* Transcription in Somatic Body Wall Muscles.** MEF2 is necessary but not sufficient for regulation of many skeletal muscle genes in vertebrates (1–3). Similarly, the results presented here demonstrate that MEF2 is necessary but not sufficient for the regulation of the *Drosophila TmI* gene in muscle. High levels of *TmI* gene transcription, which are mediated by the proximal and distal enhancers in body wall muscles of embryos, larvae, and adults, require a functional MEF2 site. Furthermore, our analysis of the proximal enhancer demonstrates that MEF2 can act cooperatively with a second cis-acting enhancer region, the MA region, to generate full muscle enhancer activity. Our results suggest a model whereby transcriptional regulation of the proximal enhancer in somatic body wall muscles is controlled by the cooperative activity of MEF2 and factors that bind to and regulate MA region function.

However, this model for *TmI* muscle enhancer regulation, which is similar to models in which MEF2 interacts cooperatively with basic helix–loop–helix and other myogenic factors to regulate vertebrate muscle genes (21–23), is likely to be too simplistic. For instance, we have shown in previous studies that the basal level activity of the MA region is inhibited when ligated to the 1B(b) fragment, indicating that a repressor is located in the 1B(b) fragment that can inhibit MA region function (13). We do not know if the MEF2 site within the 1B(c) fragment in the context of the entire proximal enhancer is responsible for overcoming this inhibition; however, if so, it would suggest that MEF2 may act through a negative regulatory element(s) within the 1B(b) fragment. Our results would support such a hypothesis and would explain why most of the 1BΔMEF2 transgenic lines showed no or very weak transcriptional activity in both visceral and body wall muscle rather than a level of expression comparable to the MA fragment alone. This cannot, however, explain why all of the MA+ΔMEF2 lines were transcriptionally silent since the putative 1B(b) repressor is not contained within this construct. One possible explanation is that the mutated MEF2 oligonucleotides used in this experiment, which contain additional 1B(c) DNA flanking the mutated MEF2 site, may bind a repressor that inhibits MA function. A similar mechanism has been proposed for MEF2 regulation of the vertebrate creatine kinase muscle enhancer (24). In this example, MHox competes for and inhibits MEF2 binding to its target sequence.

The analysis of MEF2 regulation of distal enhancer activity indicates that MEF2 may not be required for full enhancer

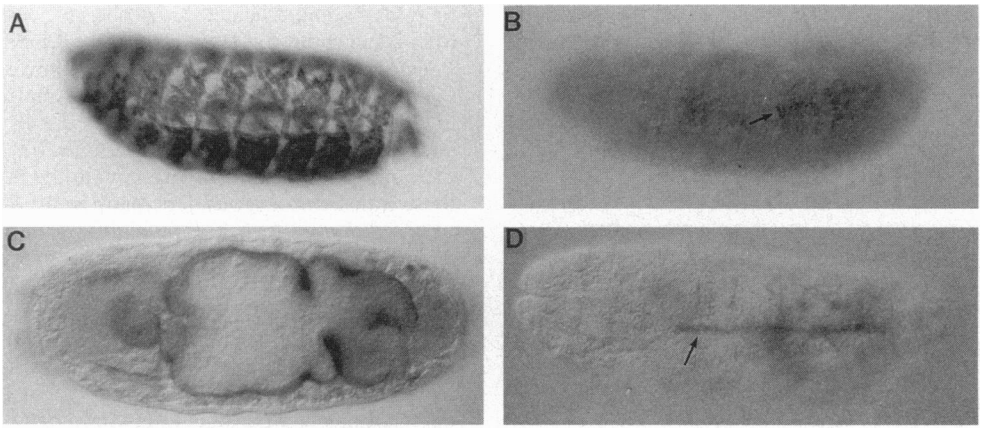

FIG. 5. Whole mount *in situ* hybridization analysis of *TmI* expression in *mef2* mutant embryos. Antisense *TmI* was used as a riboprobe. (*A*) Endogenous *TmI* expression in stage 16 wild-type embryo. (*B*) Stage 16 *Df(2R)X1/Df(2R)P520* transheterozygous embryo, showing reduced *TmI* expression in body wall muscles; arrow shows *TmI* expression in a residual fused muscle fiber. (*C*) Stage 16 *Df(2R)X1/Df(2R)P520* transheterozygous embryo, showing visceral muscle staining. (*D*) Stage 16 *Df(2R)X1/Df(2R)P520* transheterozygous embryo, showing dorsal vessel (arrow) staining. All embryos are orientated with anterior to the left. In *A* and *B* embryos are lateral views, in *C* and *D* they are ventral and dorsal views, respectively.

activity in embryonic body wall muscles although it is required for high level enhancer function in larval and adult body wall muscles. The observed difference between proximal and distal enhancer-mediated expression in embryonic muscle suggests that the distal enhancer is regulated differently. Therefore, it will be important to determine whether the distal enhancer contains a separate activator function, which depends upon different cis-regulatory elements than those in the proximal enhancer, or if it contains an MA region with greater activity that is not dependent on MEF2.

Regulation of *TmI* Expression in Visceral and Cardiac Muscle. *TmI* expression in visceral muscles and the dorsal vessel is largely unaffected in *mef2* mutant embryos. This agrees with the observation that the MA region, which does not contain a MEF2 binding site, can direct expression in visceral muscles at almost the same level as the entire proximal enhancer. We have noted that sequences in the 1B(b+c) region can also contribute to embryonic visceral muscle expression (unpublished). Thus, despite the fact that *mef2* is expressed in visceral muscles and the dorsal vessel, *mef2* does not appear to regulate *TmI* expression in these two muscles as it does in somatic muscles. Interestingly, it has been documented that in *mef2* mutants, myosin heavy chain expression is not detected in the dorsal vessel and is greatly reduced in visceral muscles (8, 9), thus suggesting that myosin heavy chain is regulated differently than *TmI* in these two muscles. Alternatively, myosin heavy chain protein, which was measured in these studies, was unstable because thick filaments may not form in the dorsal vessel and visceral muscles of *mef2* mutant embryos (25).

Regulation of *TmI* Expression in Adult Muscles. *mef2* has been shown to be expressed in embryos and larvae (6, 7). Our results indicate that *mef2* is required for high level expression in the somatic muscles of adults, thereby suggesting that *mef2* is expressed in these adult muscles and has a similar function in regulating *TmI* expression in developing adult muscles as it does in embryos and larvae. Furthermore, the low level expression by the MA+MEF2 construct in the IFM/TDT muscles indicates that *mef2* is probably expressed in these muscles or their precursors as well. However, it appears that cooperative interactions between MEF2 and the MA region is not sufficient for high level expression in these muscles and that additional sequences spanning the 1B(b) and 1B(c) fragments are also required for high level expression in the IFM/TDT muscles (A. Wohlwill and R.V.S., unpublished work).

In summary, the results presented here show that the *Drosophila mef2* gene is required for transcriptional regulation of the *TmI* gene during embryonic, larval, and adult myogenesis, thus identifying *TmI* as the first reported target gene for *mef2* regulation. In addition, we have shown that MEF2 acts cooperatively with a second cis-acting region MA region to regulate high level transcriptional activity.

This work was supported by Research Grant GM27611 from the National Institutes of Health to R.V.S. and an American Heart Association Grant-in-Aid (Massachusetts Affiliate) to H.T.N.

1. Gossett, L. A., Kelvin, D. J., Sternberg, E. A. & Olson, E. N. (1989) *Mol. Cell. Biol.* **9,** 5022–5033.
2. Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Edmondson, D. G. & Olson, E. N. (1993) *J. Biol. Chem.* **268,** 755–758.
3. Olson, E. N. & Klein, W. H. (1994) *Genes Dev.* **8,** 1–8.
4. Michelson, A. M., Abmayr, S. M., Bate, M., Arias, A. M. & Maniatis, T. (1990) *Genes Dev.* **4,** 2086–2097.
5. Paterson, B. M., Walldorf, U., Eldridge, J., Dübendorfer, A., Frasch, M. & Gehring, W. J. (1991) *Proc. Natl. Acad. Sci. U S A* **88,** 3782–3786.
6. Lilly, B., Galewsky, S., Firulli, A. B., Schulz, R. A. & Olson, E. N. (1994) *Proc. Natl. Acad. Sci. U S A* **91,** 5662–5666.
7. Nguyen, H. T., Bodmer, R., Abmayr, S. M., McDermott, J. C. & Spoerel, N. A. (1994) *Proc. Natl. Acad. Sci. U S A* **91,** 7520–7524.
8. Bour, B. A., O'Brien, M. A., Lockwood, W. L., Goldstein, E. S., Bodmer, R., Taghert, P. H., Abmayr, S. M. & Nguyen, H. T. (1995) *Genes Dev.* **9,** 730–741.
9. Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B. M., Schulz, R. A. & Olson, E. N. (1995) *Science* **267,** 688–693.
10. Coen, E. S. & Meyerowitz, E. M. (1991) *Nature (London)* **353,** 31–37.
11. Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H. & Sommer, H. (1990) *Science* **250,** 931–936.
12. Schultz, J. R., Tansey, T., Gremke, L. & Storti, R. V. (1991) *Mol. Cell. Biol.* **11,** 1901–1911.
13. Gremke, L., Lord, P. C. W., Sabacan, L., Lin, S.-C., Wohlwill, A. & Storti, R. V. (1993) *Dev. Biol.* **159,** 513–527.
14. Lichter, J. B. & Storti, R. V. (1991) *Biochim. Biophys. Acta* **1088,** 419–424.
15. Parmacek, M. S., Ip, H. S., Jung, F., Shen, T., Martin, J. F., Vora, A. J., Olson, E. N. & Leiden, J. M. (1994) *Mol. Cell. Biol.* **14,** 1870–1885.
16. Michael, S. F. (1994) *BioTechniques* **16,** 411–412.
17. Spradling, A. C. & Rubin, G. M. (1982) *Science* **218,** 341–345.
18. Tautz, D. & Pfeifle, C. (1989) *Chromosoma* **98,** 81–85.
19. Jiang, J., Kosman, D. & Levine, M. (1991) *Genes Dev.* **5,** 1881–1891.
20. O'Brien, M. A., Roberts, M. S. & Taghert, P. H. (1994) *Genetics* **137,** 121–137.
21. Li, H. & Capetanaki, Y. (1994) *EMBO J.* **13,** 3580–3589.
22. Kaushal, S., Schneider, J. W., Nadal-Ginard, B. & Madhavi, V. (1994) *Science* **266,** 1236–1240.
23. Naidu, P. S., Ludolph, D. C., To, R. Q., Hinterberger, T. J. & Konieczny, S. F. (1995) *Mol. Cell. Biol.* **15,** 2707–2718.
24. Cserjesi, P., Lilly, B., Hinkley, C., Perry, M. & Olson, E. N. (1994) *J. Biol. Chem.* **269,** 16740–16745.
25. Epstein, H. F. & Bernstein, S. I. (1992) *Dev. Biol.* **154,** 231–244.