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Expression of Bovine *myf5* Induces Ectopic Skeletal Muscle Formation in Transgenic Mice

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***myf5* is one of a family of four myogenic determination genes that control skeletal muscle differentiation. To study the role of *myf5* in vivo, we generated transgenic mice harboring the bovine homolog, *bmyf*, under control of the murine sarcoma virus promoter. Ectopic expression of the full-length *bmyf* transgene was detected in brain and heart tissue samples of F_1 progeny from transgenic founder mice. Ectopic *bmyf* expression activated endogenous skeletal myogenic determination genes in the hearts and brains of transgenic animals. Incomplete skeletal myogenesis in most hearts gave rise to cardiomegaly and focal areas of cardiomyopathy. In brains in which ectopic expression led to a more complete myogenesis, focal areas of multinucleated, striated myotubes containing actin, desmin, and myosin were observed. These unexpected results show that *myf5* can initiate myogenic differentiation in vivo, supporting the hypothesis that *myf5* is responsible for determination of cells to the myogenic lineage in normal embryogenesis.**

Understanding the mechanism of cell type determination in developing multicellular organisms is one of the fundamental problems in biology. Recently, a family of genes which express skeletal muscle-specific transcription factors capable of converting mesodermal cells in vitro to stable myoblasts and myotubes has been identified. These transcription factors represent the first candidates for specific cell type determination factors characterized at the molecular level. The gene family encodes four myogenic determination factors (MDFs), which include MyoD (13, 39), myogenin (15, 44), myf5/bmyf (1, 6, 11), and MRF4/herculin/myf6 (5, 24, 30). Normally, expression of these genes is specific to skeletal muscle and muscle cell precursors. MDFs have been shown to transactivate expression of many muscle-specific genes (e.g., muscle creatine kinase [9, 36], myosin light chain 1/3 [31], and the alpha subunit of the acetylcholine receptor [28]).

Most of the data implicating the MDFs in activation of the mammalian skeletal muscle differentiation program have been generated by in vitro experiments with cultured cells (10, 43). In situ hybridization analysis in mouse embryos has shown that the MDFs are sequentially expressed in somites and limb buds (3, 19, 27, 33). Miner et al. (23) were the first to address the in vivo role of MDFs by targeting the expression of *MyoD* under control of the muscle creatine kinase promoter-enhancer to heart and skeletal muscle in transgenic mice. Ectopic expression of *MyoD* in the heart led to incomplete skeletal myogenesis, cardiomegaly, and death in utero. While some skeletal muscle-specific genes were activated, no organized sarcomeres or multinucleated myotubes were observed in diseased hearts.

myf5 is the first of the MDF genes expressed in the developing murine dermamyotome, suggesting that it could be responsible for initiating activation of the myogenic differentiation program. However, as recently shown by gene knockout experiments (7, 32), *myf5* is not necessary for initiation of myogenesis in mice. In *myf5*-deficient mice, myogenesis proceeded normally after a brief delay in muscle-specific gene expression in myotomes. Therefore, although *myf5* has been shown to transcriptionally activate other muscle-specific genes in vitro, its role in normal myogenesis in the developing animal has not been clearly established. To further elucidate the role of *myf5* in vivo, we have generated transgenic mice harboring the bovine homolog, *bmyf*, which has 88% amino acid homology with mouse *myf5* (*Mmmyf5*) (8). In this paper, we show that ectopic expression of *bmyf* in the heart and brain not only initiated myogenic differentiation but resulted in the appearance of multinucleated skeletal muscle myotubes in the brain. Thus, in the intact animal, *myf5* expression, while perhaps not necessary, is sufficient to initiate activation of a developmental program leading to complete skeletal myogenesis.

MATERIALS AND METHODS

DNA constructs and transgenic mice. To construct the transgene, a 1,945-bp *Eco*RI restriction fragment bearing a full-length *bmyf* cDNA was inserted into a unique *Eco*RI site in pEMSV scribe (13, 18) as described previously (11). From the resultant plasmid, pEMSV*bmyf* (Fig. 1), a 2,602-bp *Hin*dIII restriction fragment was gel purified for microinjection. This DNA fragment contained the full-length *bmyf* cDNA with 420 bp of 5′ sequence bearing a Moloney murine sarcoma virus (MSV) long terminal repeat (LTR) promoter-enhancer and 237 bp of 3′ sequence bearing the simian virus 40 polyadenylation signal. Transgenic mice were produced by pronuclear microinjection, as described elsewhere (20). Founder animals were initially identified by polymerase chain reaction (PCR) amplification of *bmyf*-specific sequences from tail biopsy samples (forward primer, GCACA

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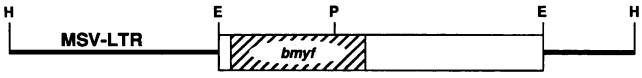


FIG. 1. Diagram of the pEMSV*bmyf* transgene. A 2.7-kb *Hin*dIII fragment containing an MSV LTR promoter-enhancer ligated to a full-length bovine *myf5* cDNA (1.95-kb *Eco*RI insert) as described elsewhere (11) was purified and microinjected into single-cell embryos from homozygous FVB/N mice. The hatched box represents the 765-bp coding region of *bmyf*. E, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

TGATAGATGAGCCTG; reverse primer, GAATGTAACA GCCCTATCTG; the primers spanned nucleotides 581 to 840 of the cDNA; annealing temperature [T_m] = 55°C). Transgenic DNA was further characterized by Southern blot analysis (35). Founder mice were bred to normal FVB/N mice to establish transgenic lines.

RT-PCR analysis of gene expression. For PCR analysis of MDF expression, mouse tissues were collected, rinsed in saline, and immediately submerged in liquid nitrogen. Total RNA was isolated with LiCl-guanidinium isothiocyanate (GuSCN) or phenol-chloroform-GuSCN, treated with DNase (RQ1 DNase; Promega), and reverse transcribed (Superscript Reverse Transcriptase; Gibco BRL). A 5-μl aliquot of the reverse transcriptase (RT) reaction mixture was labeled with [α-^{32}P]dCTP and used to calculate cDNA amounts. Portions (10 to 20 ng) of each resultant template cDNA were amplified by PCR with the appropriate oligonucleotide primers. Each 50-μl reaction mixture contained template, 5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris [pH 8], 15 mM $MgCl_2$, and 0.01% gelatin), 0.5 U of *Taq* polymerase (Amplitaq; Perkin-Elmer Cetus), 200 μM (final concentration) deoxynucleoside triphosphates (Promega), and 10 pmol of each primer. PCR mixtures were amplified for 28 cycles with the following conditions: 95°C for 45 s, T_m for 30 s, and 72°C for 2 min. Oligonucleotide primers for mouse brain amyloid precursor protein cDNA were used to verify the integrity of the cDNAs. The specificity of the amplification products was confirmed by the absence of products in non-RT reactions, as well as by the appearance of predicted-size fragments after restriction enzyme digestion (Fig. 2). Aliquots from PCR mixtures with and without restriction enzyme digestion were electrophoresed on a 6% polyacrylamide–Tris–borate–EDTA gel. The *bmyf* primers are described above. For *MRF4*, the forward (AGAGACTG CCCAAGGTGGAGATTC) and reverse (AAGACTGCTGG AGGCTGAGGCATC) primers spanned nucleotides 491 to 1367 of the gene, which includes a 622-bp intron (T_m = 63°C). For *myogenin*, the forward (GAGCGCGATCTCC GCTCAAGAGG) and reverse (CTGGCTTGTGGCAGCCC AGG) primers spanned nucleotides 470 to 850 of the cDNA (T_m = 69°C). For *MyoD*, the forward (AGGCTCTGCTGC GCGACC) and reverse (TGCAGTCGATCTCTCAAAGC ACC) primers spanned nucleotides 642 to 1129 of the cDNA (T_m = 69°C), and for mouse *myf5* (*Mmmyf5*), the forward (GAGCCAAGCGTAGCAGCCTTCG) and reverse (CTCCA CCTGTTCCCTCAGCAGC) primers spanned nucleotides 136 to 464 of the cDNA (T_m = 63°C).

Histology and immunocytochemistry. Twenty F_1 and F_2 hemizygous transgenic animals from lines 25-37, 25-2/6, and 26 were analyzed histologically. Briefly, mice were anesthetized by an intraperitoneal injection of avertin and killed by cardiac perfusion with 2% paraformaldehyde-lysine-periodate. Dissected organs were immersion fixed in 10% neutral buffered formalin or 10% buffered zinc formalin, dehydrated, and embedded in paraffin. Ribbons of 4- to 6-μm-thick sections were cut, floated onto slides, and stained with hematoxylin and eosin. Brain sections were also stained with Mallory's phosphotungstic acid hematoxylin.

In order to determine the presence of specific muscle proteins, brain sections were immunostained with antibodies against muscle actin (monoclonal antibody, 1:400; Enzo Diagnostics) (42), desmin (polyclonal antibody, 1:100; Lipshaw) (42), or muscle myosin (polyclonal antibody, 1:100; Lipshaw) (14). Biotin-conjugated anti-mouse or anti-rabbit immunoglobulin G (1:200; Vector Laboratories) was used as the second antibody. After washing, an avidin-biotin-peroxidase conjugate (Vector Laboratories) was reacted with bound second antibody. Sections were then rinsed in phosphate-buffered saline, developed with diaminobenzidine, counterstained with Gill hematoxylin, dehydrated, and mounted.

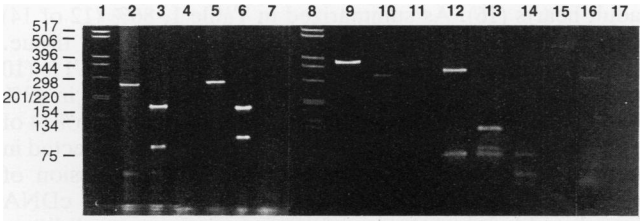


FIG. 2. PCR products following reverse transcription of mRNA from transgenic mice. Total RNA was extracted and reverse transcribed as described in Materials and Methods. Specificity was confirmed by the absence of product in non-RT samples, as well as the generation of predicted-size fragments by restriction endonuclease digestion. Amplified fragments were electrophoresed through a 6% polyacrylamide–Tris-borate-EDTA gel. Lane 1, 1-kb ladder; lane 2, *bmyf* (259 bp); lane 3, *bmyf* plus *Bam*HI (170 and 89 bp); lane 4, non-RT RNA; lane 5, *MRF4* (254 bp); lane 6, *MRF* plus *Pst*I (157 and 97 bp); lane 7, non-RT RNA; lane 8, 1-kb ladder; lane 9, *myogenin* (380 bp); lane 10, *myogenin* plus *Pst*I (299 and 81 bp); lane 11, non-RT RNA; lane 12, *Mmmyf5* (328 bp); lane 13, *Mmmyf5* plus *Hin*dIII (127, 112, and 89 bp); lane 14, non-RT RNA; lane 15, *MyoD* (488 bp); lane 16, *MyoD* plus *Ava*II (296, 132, and 60 bp); lane 17, non-RT RNA.

RESULTS

Four transgenic founder mice were obtained from a total of 10 live-born animals. All four founder animals were determined to be germ line transgenic; however, inheritance of the transgene was stable in only two of the lines (lines 26 and 35). Subsequent breeding of one genotypically unstable founder (line 25) to normal FVB/N mice resulted in the establishment of two stable transgene inheritance patterns (25-2/6 and 25-37) in the F_2 generation. Southern blot analysis revealed that line 35 contained 1 to 5 copies of the transgene, while lines 25-2/6, 25-37, and 26 all contained more than 30 copies of the transgene per genome.

F_1 hemizygous offspring (8 to 16 weeks) from three of four stable lines (25-2/6, 25-37, and 26) were shown to express *bmyf* mRNA in brain and heart tissue by Northern (RNA) blot analysis (data not shown). *bmyf* mRNA was not detected by Northern blot analysis in any other tissues examined, including kidney, liver, lung, skeletal muscle, stomach, and small intestine. As shown in Table 1, analysis of reverse-transcribed mRNA amplified by PCR also showed the expression of *bmyf* in most heart and brain samples of transgenic mice. This restricted expression pattern directed by the MSV LTR was similar to that observed in transgenic

TABLE 1. Comparison of the incidence of myogenic regulatory gene expression,[a] myogenesis, and pathology in brains and hearts of *bmyf* transgenic mice[b]

Line	No. of positive[c] animals/no. tested											
	Brain tissue						Heart tissue					
	bmyf	*Mmmyf*	*myogenin*	*MRF4*	*MyoD*	Myotubes	*bmyf*	*Mmmyf*	*myogenin*	*MRF4*	*MyoD*	Pathology
25-2/6	6/6	1/6	4/6	3/6	0/6	5/10	5/6	0/2	2/6[d]	2/6[d]	1/5[d]	7/10
25-37	2/4	3/4	3/4	3/4	1/4	5/5	2/3	0/2[d]	1/2[e]	0/2[e]	1/2[e]	5/5
26	4/4	0/1	3/4	0/4	0/4	1/5	4/4	0/2	0/2	0/2	1/2	3/5

[a] For all negative PCRs, the integrity of the cDNA was confirmed by amplification with primers for the brain amyloid precursor protein.
[b] mRNA expression and histopathology were examined in different groups of animals.
[c] Number expressing the indicated gene or showing myotubes or pathology.
[d] Animals expressing *myogenin*, *MyoD*, and *MRF4* also expressed *bmyf*.
[e] Both animals expressed *bmyf*.

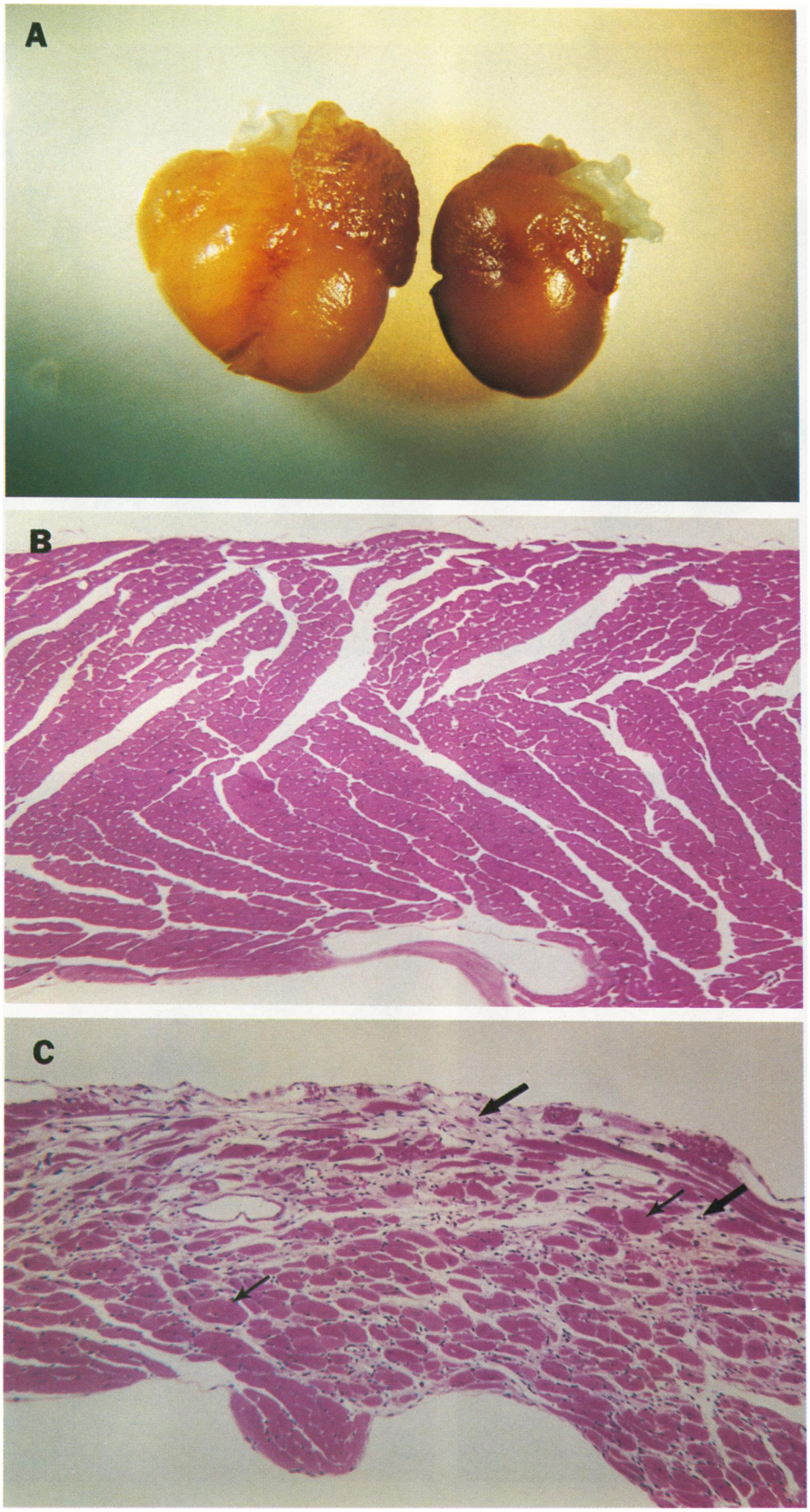
mice expressing v-*ski* under control of an MSV promoter-enhancer (37).

Twenty transgenic offspring (8- to 16-week-old F_1 and F_2 progeny from lines 25-2/6, 25-37, and 26) were examined histologically. At necropsy, gross lesions were limited to cardiomegaly in most transgenic mice. Affected hearts were grossly enlarged and dilated, especially the left atria and ventricles (Fig. 3A). Focal areas of myocardial degeneration or hypotrophy, characterized by swollen or missing myofibers and connective tissue fibrosis, were observed (Fig. 3C); however, we found no histological evidence of typical skeletal muscle fiber development. Atrial lesions were occasionally accompanied by filling of the atrial cavity with large, well-organized thrombi. In total, 75% (15 of 20) (Table 1) of the transgenic animals examined exhibited various degrees of cardiomyopathy, typified in Fig. 3C. Such lesions were never observed in nontransgenic littermates (Fig. 3B). We have also noted an unusually high incidence of premature death in mice from lines 25-37 and 25-2/6; these mice never survived beyond 8 months. However, line 26 animals appear to have a normal lifespan, even though cardiomegaly has been observed in these animals. While no direct correlation between transgenic cardiomyopathy and premature death was made, a 100% incidence of cardiomyopathy was observed in moribund animals examined.

Histopathological examination of transgenic brains revealed the presence of striated myotubes in the forebrains of most animals. Myotubes were observed immediately beneath the pia mater in the retrosplenial and frontal cortex, within the dorsal median sulcus, and occasionally along the dorsomedial margin of the ependymal layer of the anterior lateral ventricles. Single or multiple, irregular bundles of myotubes were observed along the Virchow-Robin space and bilaterally within the cingulate cortex, the anterior cortex, the corpus callosum, the hippocampus (Fig. 4A), and the anterior-dorsal region of the caudate putamen. Myotubes were limited to these regions of transgenic brains and were never observed in the brains of nontransgenic littermates. Aside from space occupation and modest disorganization of normal brain architecture, the myotubes caused no apparent injury to adjacent neurologic tissue. When stained with phosphotungstic acid-hematoxylin, the myotubes were found to be clearly multinucleate and contained distinct striations characteristic of striated muscle fibers (Fig. 4B). Immunocytochemical analyses with antibodies directed against muscle-specific actin and myosin, as well as the muscle protein desmin, demonstrated the presence of these proteins in myotubes, confirming the existence of ectopic striated myotubes in the brains of these animals (Fig. 4C and D; myosin immunocytochemistry not shown). These histopathological examinations revealed that 55% (11 of 20) (Table 1) of the animals showed some level of myogenic differentiation in the brain. This ranged from a few isolated cells reacting positively to antibodies against actin, desmin, and myosin to extensive myotube development including organized sarcomeres, as shown in Fig. 4.

RT-PCR determination of *bmyf* or endogenous MDF transcripts was not done for animals examined histologically. We have shown, however, that in a separate group of transgenic animals of comparable age, 85% (11 of 13) expressed *bmyf* in the heart compared with the 75% incidence of cardiomyopathy in transgenic animals examined histologically (Table 1). Endogenous *myogenin* was activated in 30% (3 of 10), endogenous *MRF4* was activated in 20% (2 of 10), endogenous *MyoD* was activated in 33% (3 of 9), and *Mmmyf5* was activated in none of the transgenic hearts examined (Table 1). Preliminary results indicate that the skeletal muscle myosin heavy-chain gene is also activated in adult hearts (16). As summarized in Table 1, 86% (12 of 14) of transgenic mice expressed *bmyf* mRNA in brain tissue. Endogenous *myogenin* transcripts were detected in 71% (10 of 14), endogenous *MRF4* transcripts were detected in 43% (6 of 14), and *Mmmyf5* transcripts were detected in 36% (4 of 11) of the transgenic brains examined. *MyoD* was detected in only 1 of the 14 brain samples examined. Expression of endogenous MDF mRNAs was never observed in cDNA samples of hearts or brains of nontransgenic (control) littermates run simultaneously. The integrity of all RT-PCR cDNAs that did not contain MDF transcripts was confirmed by amplification of endogenous amyloid precursor protein transcripts. All samples assayed were positive for amyloid precursor protein.

FIG. 3. Pathology of hearts from *bmyf* transgenic mice (line 25-2/6). (A) Gross pathology of a heart from a 7-week-old transgenic mouse (left) compared with a heart from an age-matched nontransgenic littermate. (B) Paraffin-embedded section from the ventricular wall of a nontransgenic littermate heart stained with hematoxylin and eosin. Magnification, ×147. (C) Section from the ventricular wall of a *bmyf* transgenic mouse heart similarly stained. This section shows typical areas of myocardial degeneration, characterized by swollen (small arrows) or missing (large arrows) myofibers and by connective tissue fibrosis, associated with the presence of the transgene. Magnification, ×147.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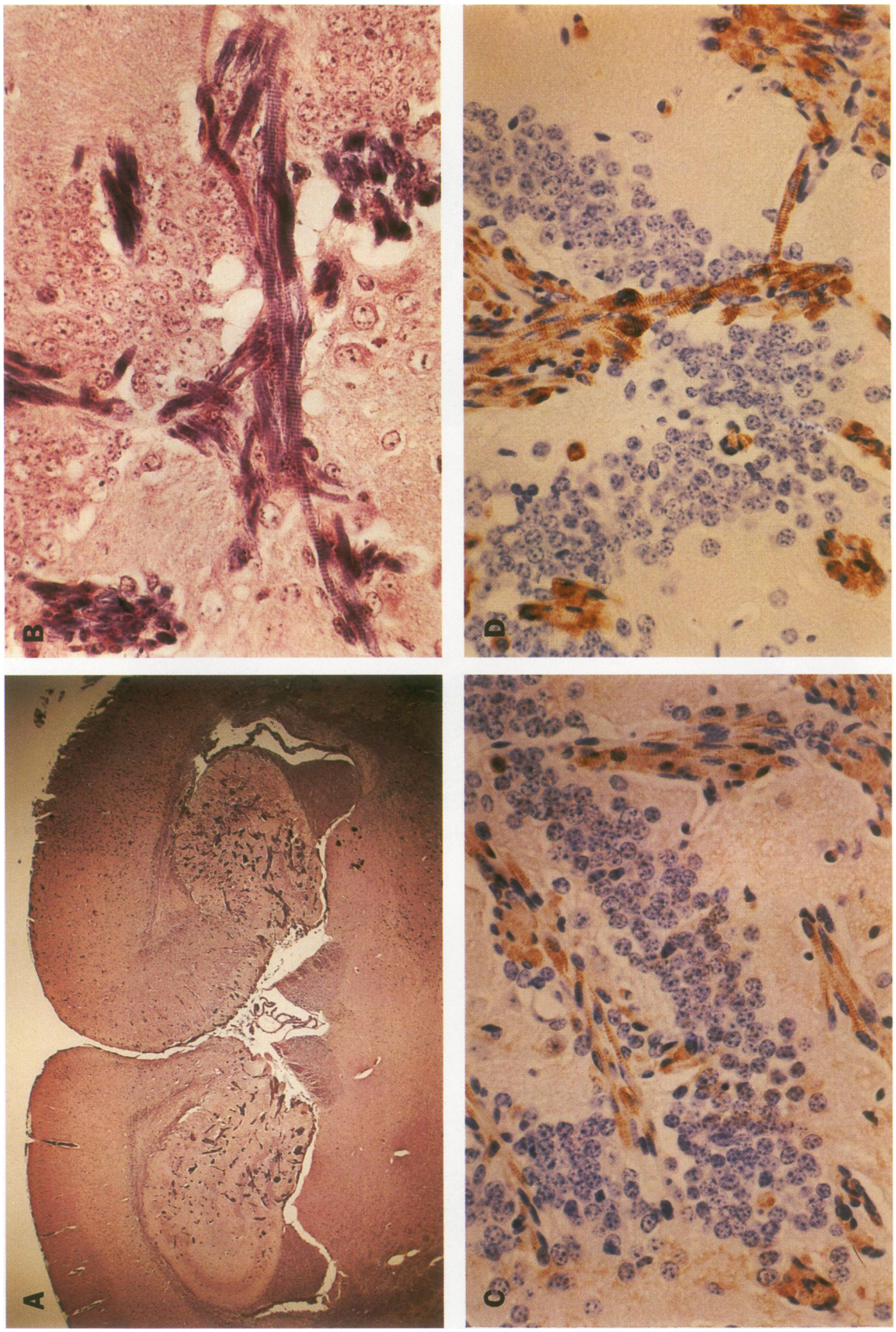

FIG. 4. Striated skeletal myotubes in the forebrain of *bmyf* transgenic mice (line 25-37). (A) Coronal section from the hippocampal region of an 8-week-old transgenic mouse brain, stained with phosphotungstic acid-hematoxylin. Magnification, ×27. (B) Higher magnification (×540) of the hippocampal region showing areas of striated myotube formation, similarly stained. (C) Immunocytochemical analysis of hippocampal myotubes with anti-muscle actin antibodies. Magnification, ×540. (D) Immunocytochemical analysis of hippocampal myotubes with antidesmin antibody. Magnification, ×540.

DISCUSSION

Expression of the MSV LTR-driven *bmyf* transgene was restricted to the hearts and brains of adult animals in three genotypically unique founder lines. Other transgenic studies using the MSV LTR promoter-enhancer have obtained expression in kidney and skeletal muscle as well (25, 29). Sutrave et al. (37) noted a restricted pattern of v-*ski* expression limited to brain tissue, heart tissue, and skeletal muscle, while Theuring et al. (41) observed expression of T antigen in brain, salivary gland, and pancreas tissues only. The factors controlling tissue specificity of MSV-driven transgene expression are unknown but may be explained, in part, by a juxtaposition of unrelated genetic elements in these gene constructs that results in unique expression patterns (38).

The incidence of *bmyf* expression in the hearts of adult transgenic mice in this study appears to be tightly linked to the appearance of cardiomyopathy (85 versus 75%). This indirect correlation suggests that *bmyf* alone may be sufficient to cause the observed histopathology. Our data also show that *bmyf* transgene expression can transactivate the endogenous skeletal muscle-specific genes *myogenin*, *MRF4*, and *MyoD*, but not *Mmmyf5*, in adult mouse hearts, although expression of the endogenous MDFs was not well correlated with the incidence of cardiomyopathy (Table 1). These observations are consistent with the report demonstrating skeletal muscle-specific gene activation by ectopic expression of *myoD* in transgenic mouse hearts (23). We also found no histological evidence of skeletal muscle differentiation in *bmyf* transgenic hearts, supporting the suggestion by Miner et al. (23) that cardiac tissue-specific differentiation may interfere with activation of the complete skeletal muscle program. In vitro studies have shown that skeletal muscle gene expression in response to forced *MyoD* expression is transient in some cell types (43) and the endogenous developmental program is not superseded. Moreover, hepatocytes (HepG2) and kidney cells (CV-1) have been reported to be refractory to the activity of *MyoD* (22, 34). It is possible that the cardiomyopathy we observed in *bmyf* transgenic mice resulted from the development of mixed skeletal-cardiac myofibers that are unable to meet the demands of cardiac function. This eventually leads to fiber degeneration, cardiac dilation, congestive heart failure, and premature death.

In the brain, expression of the transgene (*bmyf*) appears to be closely correlated with the incidence of *myogenin* expression but less well correlated with expression of the other endogenous MDFs or with myogenic differentiation (Table 1). This is particularly evident in line 26. Within the limits of the number of animals observed, expression of the transgene nearly always activated *myogenin* (Table 1), but this combination alone was apparently not always sufficient to induce myogenic differentiation in the brain (note the data for line 26). Conversely, the expression of *MRF4* appears to be more closely correlated with the incidence of brain myotubes (Table 1), suggesting that *MRF4* activation may be required for full myogenic differentiation. Our data also suggest that the transgene is capable of transactivating endogenous *Mmmyf5* in some mouse brains. This observation apparently conflicts with a previous in vitro study which demonstrated that *myf5* is not *trans* regulated by myogenic basic helix-loop-helix (bHLH) proteins (4). The discrepancy may be explained by differences in the cellular history and/or environment (in vivo versus in vitro). Endogenous *MyoD* expression was detected in only 1 of the 14 transgenic brain samples studied, supporting the recently proposed hypothesis that *MyoD* is dispensable for skeletal myogenesis (32). In total, our observations support the notion that in vivo myogenesis is a hierarchial process which may be initiated by *bmyf* transgene expression but which requires transactivation of other, endogenous MDF genes to permit activation of the full program of myogenic differentiation.

It is apparent from our data that a defined population of cells in the mouse brain is permissive for expression of the *bmyf* transgene and transactivation of endogenous skeletal muscle-specific genes leading to full myogenic differentiation. The origin of the discrete cell population in the anterior brain which was determined to the myogenic lineage in response to *bmyf* is presently unknown. In vitro transfection of *MyoD*, under control of the MSV LTR, has resulted in activation of previously silent muscle-specific genes in a variety of cell types, including neuroblastoma cells and primary brain cells (10, 39). In certain cell types, e.g., fibroblasts, adipocytes, chondrocytes, and smooth muscle cells, *MyoD* transfection can activate the entire program for muscle differentiation (9), as occurred in the anterior brain of *bmyf* transgenic mice. The influence of growth factors on myogenic HLH genes has been recently reviewed (26). Response to growth factors (through intracellular signalling pathways leading to phosphorylation) and other environmental cues (e.g., neurotransmitters), the presence or absence of HLH-inhibitory proteins such as Id (2) or E1A (4), and the influence of factors such as fos, ras (21), or Rb (12) that can regulate the cell cycle have all been implicated in modulation of HLH gene transactivation and activation or suppression of myogenic differentiation. The *bmyf* transgenic model described in this report offers a potential new tool for further elucidation of factors that regulate myogenic differentiation. Determination of the developmental origin of the cells which become striated myotubes in the brain and characterization of the factors mediating ectopic myogenesis may lead to fundamental new insights into the regulation of skeletal muscle differentiation.

To our knowledge, this is the first example in transgenic mammals of ectopic expression of a myogenic determination factor resulting in activation of the entire sequence of events leading to apparent complete skeletal myogenesis. These observations show that *myf5* and *bmyf* can initiate the myogenic-differentiation program in intact animals, ultimately leading to the appearance of multinucleated, striated myotubes. *myf5* had been previously proposed as the gene responsible for determination of mesodermal cells to the myogenic lineage on the basis of the observations that *myf5* transcripts can be localized to the developing dermamyotome (27) and that it is the earliest MDF gene expressed in the developing mouse embryo (17). In this report, we have demonstrated that ectopic expression of the bovine homolog of *myf5*, under control of a retroviral LTR, is capable of activating the entire myogenic program to produce differentiated skeletal muscle myotubes in the anterior brain in transgenic mice. These observations support the hypothesis that *myf5* is sufficient to initiate skeletal myogenesis in vivo.

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