Localized expression of a myogenic regulatory gene, *qmf1*, in the somite dermatome of avian embryos

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***qmf1* is a quail myogenic regulatory gene that is transcribed in skeletal myoblasts and differentiated muscle and shows sequence homology to *MyoD1* and *Myf5*. We used the *qmf1* transcript as an in situ hybridization marker for determined myogenic cells to study myogenic lineages in developing embryos. We present evidence for the temporal and spatial regulation of *qmf1* mRNA expression and slow cardiac troponin C (TnC), fast skeletal troponin T (TnT), and α -cardiac actin contractile protein mRNA expression in the somite myotome and limb buds. Our results show that *qmf1* is a marker for myogenic lineages during both somite formation and limb development and that *qmf1* mRNAs, but not contractile protein mRNAs, localize in dorsal medial lip (DML) cells of the somite dermatome. We propose that the DML is a site of myogenic lineage determination.**

[Key Words: *qmf1*; myogenesis; avian; somite; dermatome; myotome]

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Skeletal muscles of vertebrates are formed by the differentiation of myoblasts in muscle-forming regions of the embryo. In vitro, these determined myogenic cells proliferate clonally (Konigsberg 1963, 1971; Yaffe and Saxel 1977) and stably inherit the potential to differentiate into myofibers (Shainberg et al. 1969; Buckley and Konigsberg 1974; Kalderon and Gilula 1979) that transcribe a functionally related set of muscle-specific genes (Devlin and Emerson 1978). The mechanisms of skeletal myoblast differentiation and muscle gene transcription have been studied extensively, primarily because of the molecular cloning of muscle-specific genes.

The molecular genetic mechanisms of cell lineage determination and myoblast formation have been more difficult to study. Lineage marking and somite grafting experiments with chick/quail chimeric embryos (Le Douarin and Barq 1969) have established that segmental plate mesoderm and newly segmented somites of stage 13 embryos can give rise to virtually all skeletal muscles of the avian embryo (Chevallier et al. 1977; Christ et al. 1977, 1983; Chevallier 1979; for review, see Konigsberg 1986). In avian embryos, most somites are derived from the anterior portion of the segmental plates (Bellairs 1979; Packard and Meier 1983; Cheney and Lash 1984; Stern and Bellairs 1984), and newly formed somites appear as segmental clusters of cells arranged as a loose epithelium surrounding a core of mesenchyme-like cells (Williams 1910). Somites mature as they progress rostrally, with respect to newly segmented somites. During days 2–4, beginning at about stage 12, somites undergo a

compartmentalization process in which two and, subsequently, three cell layers, including the sclerotome, dermatome, and myotome, are formed. Ventromedial cells of the sclerotome give rise to the connective tissue of the body and limb (Bancroft and Bellairs 1976; Solursh et al. 1979; Chernoff and Lash 1981). The dorsolateral cells of the dermomyotome produce the outer dermatome and inner myotome (Christ et al. 1978). It is the dermomyotomal cell layer that has been suggested as the source of the limb and body skeletal musculature of the embryo (for review, see Christ et al. 1986). Although these embryological studies demonstrate that myogenic progenitor cells are present in the segmental plate mesoderm and, subsequently, in the somite of early-stage embryos, it has not been possible to identify the specific mesodermal cells that give rise to myoblasts in embryos. Furthermore, because there have been no molecular markers to distinguish myoblasts from other mesenchymal cells, it has not been established whether myoblasts originate in the segmental plate mesoderm, after somites have separated from the segmental plates or, at an even later stage, when somites compartmentalize into the myotome, dermatome, and sclerotome.

The multipotential, mouse embryo-derived cell line C3H10T $\frac{1}{2}$ (Reznikoff et al. 1973) has recently provided a unique tissue culture model of an embryonic, multipotential mesodermal cell (Taylor and Jones 1979) for molecular genetic studies of myoblast lineage determination (Konieczny and Emerson 1984) and for identifying regulatory genes that can be used as molecular markers for studies of myoblast determination in embryos. Using the 10T $\frac{1}{2}$ cell as a recipient for DNA transfection, myo-

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genic regulatory genes, including *MyoD1*, *myd*, *myogenin*, and *Myf5* (Davis et al. 1987; Pinney et al. 1988; Braun et al. 1989; Edmonson and Olson 1989; Wright et al. 1989), have been identified. *MyoD1*, myogenin, and *Myf5* share sequence homology and are part of the *myc* family of proteins (Davis et al. 1987). These *myc* proteins are related by a homology in a helix-loop-helix region (Murre et al. 1989a), suggesting that they represent DNA-binding proteins that form homodimers or heterodimers with other protein members of this gene family (Murre et al. 1989b). The helix-loop-helix domain in *MyoD1* is functionally significant because transfection of this domain is necessary to cause myogenic conversion of 10T $\frac{1}{2}$ cells (Tapscott et al. 1988).

RNA transcripts of myogenic regulatory genes such as *MyoD1* provide unique molecular markers to investigate the origins of myogenic lineages because such genes are expressed in both determined skeletal myoblasts and differentiated muscle (Davis et al. 1987). In this study, we characterized the developmental expression of an avian myogenic regulatory gene, referred to as *qmf1* (*quail myogenic factor 1*). *qmf1* was cloned as a cDNA from embryonic myofibers of the Japanese quail (*Coturnix coturnix*), based on its sequence homology to mouse *MyoD1* (Davis et al. 1987; Pinney et al. 1988). Northern RNA hybridization and cDNA sequence analyses show that the *qmf1* gene is expressed in embryonic myoblasts, as well as in differentiated skeletal muscle, but not in nonskeletal muscle tissues. Using an in situ hybridization approach, we have examined *qmf1* and contractile protein mRNA expression in the early quail embryo. Our results show that *qmf1* mRNA expression follows the rostral-caudal gradient of somite compartmentalization in early-stage embryos. *qmf1* mRNA and contractile protein mRNAs localize in the myotome of the more mature, compartmentalized somites. Furthermore, *qmf1* mRNAs are selectively localized over cells of the dorsal medial lip (DML) of the dermatome, suggesting that this region is a site of myogenic lineage determination. *qmf1* is also involved in limb myogenesis. In stage 24 hind-limb buds, *qmf1* mRNA localizes over dorsal and ventral regions that extend along the proximal-distal axis of premuscle mass formation. In contrast to the somite, dorsal and ventral localization of *qmf1* gene expression precedes the initiation of muscle differentiation.

Results

Characterization of a quail *MyoD1* homolog

A full-length mouse *MyoD1* cDNA, cM213 (Pinney et al. 1988), was used as a probe to isolate a homologous quail sequence. The mouse *MyoD1* cDNA (cM213) was hybridized at low stringency to Northern blots of RNAs isolated from various mouse and quail cultured cells and adult tissues. Figure 1A shows that the cM213 probe hybridizes to a 2-kb mRNA isolated from mouse C3H10T $\frac{1}{2}$ -derived myofiber (23A2 MF) (Konieczny and Emerson 1984) but not to 10T $\frac{1}{2}$ RNA or mouse liver RNA. The cM213 probe also hybridizes to a 1.7-kb

Figure 1. Northern blot analysis of mouse *MyoD1* and quail *MyoD1*-related RNAs. Each lane contains 5 μ g of poly(A)⁺ RNA, except the first two lanes, which contain 10 μ g of total RNA. *MyoD1* and *qmf1* mRNA expression was analyzed in mouse (first three lanes) and quail (remaining lanes) tissues and cultured myoblasts (MB) and myofibers (MF). (A) Identification of quail *MyoD1*-related species. Hybridization was performed at reduced stringency with a random-primed mouse *MyoD1* probe. (B and C) The blot was hybridized to a random-prime-labeled cC509 probe (see Fig. 2A). (B) Filter was exposed to film for 2 hr at -70°C in the presence of an enhancer screen. (C) Exposure was for 12 hr at -70°C with an enhancer screen.

mRNA from cultured quail myoblasts and myofibers. To recover cDNA clones of this 1.7-kb quail mRNA, a λ gt10 cDNA library of quail myofiber RNA was screened under low stringency hybridization conditions, and an 827-bp clone (cC509) was recovered and used to rescreen the library at high stringency. This second screen yielded a longer quail *MyoD1*-related clone of 1238 bp (cC511). The cC509 and cC511 clones have overlapping sequences and encode a *MyoD1*-related protein, called *qmf1* (Fig. 2). As shown in Figure 2A, cC511 extends farthest at the 5' end and encodes a nearly full-length predicted protein. The sequence amino-terminal to cC511 was determined by sequencing a genomic DNA fragment.

MyoD1 and *qmf1* mRNA expression is restricted to myoblasts and to skeletal muscle (Fig. 1) (Davis et al. 1987; Pinney et al. 1988). Figure 1, B and C, shows that cC509 hybridizes to a 1.7-kb mRNA species in myoblasts and myofibers (Fig. 1B) and to mRNA in adult skeletal muscles, but not to RNAs in heart, smooth muscle, or liver (Fig. 1C). The adult pectoralis and adductor muscles express lower levels of *qmf1* mRNA than do embryonic cultured muscle cells. Thus, *qmf1* mRNA and mouse *MyoD1* mRNA have a similar pattern of skeletal myoblast and muscle-specific expression.

Figure 2. (A) Nucleotide sequence and predicted amino acid sequence of *qmfl*. The 827-bp sequence of c509 used for the in situ hybridization analysis is shown. c509 specifically excludes the conserved regions of homology, basic (nucleotides 323–394), and *myc* regions (nucleotides 443–505) to other MyoD1-related sequences. Direct comparison between c511, *CMD1*, and *qmfl* gene sequences indicates that c511 lacks 104 nucleotides that encode the most 5' end of the *qmfl* protein. (B) Protein sequence comparison of mouse MyoD1, *qmfl*, XMyoD, human Myf5, and rat myogenin (*mdfl*). Residues in boldface type represent identical amino acid residues shared by the predicted protein sequences.

Figure 2B shows that the protein *qmfl* shares extensive homology with mouse MyoD1, Myf5, myogenin, and a recently isolated *Xenopus laevis* MyoD1 sequence,

XMyoD (Hopwood et al. 1989), particularly in the basic (residues 102–124) and *myc* helix–loop–helix homology (residues 141–162) regions. These regions of the

MyoD1 protein have been shown to be necessary and sufficient to promote myogenic conversion of 10T½ cells (Tapscott et al. 1988). As demonstrated by the presence of additional segments of conserved residues spanning the amino (42–62 and 77–101) and carboxyl termini (200–215 and 246–260), qmf1 is more closely related to MyoD1, XMyoD, and Myf5 than to myogenin. The extent of homology between qmf1 and MyoD1 (96%) and qmf1 and XMyoD (93%) in these conserved segments is greater than that between qmf1 and Myf5 (57%). Interestingly, however, only qmf1, XMyoD, and Myf5 share a deletion of 14-amino-acid residues immediately 3' of the *myc* homology region (169–187). Therefore, outside the basic and *myc* homology regions, qmf1 shares distinct sequence similarities with XMyoD, Myf5, and MyoD1.

qmf1 is the quail homolog of a recently reported chicken myogenic cDNA, CMD1, also isolated by homology to mouse MyoD1 (Lin et al. 1989). qmf1 and CMD1 share 94% homology throughout their predicted protein sequences. A divergence in amino acid sequences between qmf1 and CMD1 between residues 59 and 68 (Fig. 2A) is due to insertion of three cytosines at positions 221, 223, and 250, respectively, in qmf1. Because qmf1 and CMD1 are homologous sequences, it is likely that the CMD1 gene is also expressed in skeletal myoblasts. As shown for CMD1 (Lin et al. 1989), qmf1 also converts 10T½ cells to muscle, thereby confirming its myogenic regulatory potential. The qmf1 cDNA, under the control of an SV40 promoter, converts 10T½ cells to stable myoblast colonies at a frequency of at least 15% (data not shown). Thus, the results of these RNA hybridization studies, sequence comparisons, and DNA transfection experiments establish that qmf1 encodes a myogenic regulatory protein that has sequence similarities to MyoD1, XMyoD, and Myf5 and is expressed in skeletal myoblasts as well as in differentiated muscles.

Rostral–caudal gradient of qmf1 gene expression in early-stage embryos

qmf1 antisense RNA was used as an *in situ* hybridization probe to investigate the expression of qmf1 mRNA and, thus, the presence of myogenic cell lineages in quail embryos. Our initial experiments focused on *qmf1* gene expression in early-stage embryos in which somites separate from the segmental plates at the caudal end and more mature somites compartmentalize to form the sclerotome, dermatome, and myotome at the rostral end.

Figure 3. (Top) Schematic diagram of a stage 19 quail embryo sectioned in a transverse orientation at the level of the forelimbs. Transverse sections reveal the mediolateral structures of the somite. (Bottom) Schematic diagram of lumbar somites of a stage 19 quail embryo sectioned in a parasagittal orientation. Parasagittal sections reveal the rostral–caudal direction of embryonic development. (D) Dermatome; (M) myotome; (S) sclerotome; (N) neural tube; (L) limb; (n) notochord; (a) descending aorta; (v) posterior cardinal vein; (d*) dorsal medial lip; (v*) ventral lateral lip.

For our studies, staged embryos were sectioned serially in a transverse orientation to reveal the medial–lateral structures of the somite and in a parasagittal orientation to reveal the rostral–caudal gradient of somite maturation (Fig. 3). Our results show that qmf1 mRNA expression is first detectable by *in situ* hybridization in rostral somites of stage 13 and 14 embryos (Fig. 4). At these early stages, the rostral–caudal gradient of somite development is pronounced; the rostral somites begin to

Figure 4. Localization of qmf1 transcripts in parasagittal and transverse sections of stage 13 and 14 embryos. (A) Bright-field and (B) corresponding dark-field micrograph of a stage 13 rostral somite cut in a parasagittal orientation and hybridized to qmf1 probe. (C) Bright-field micrograph of a parasagittal section showing somite 5 ± 1 (arrow) and the adjacent caudal somites of a stage 13 embryo. (D) Dark-field micrograph of section shown in C hybridized with qmf1 probe. (E) Parasagittal section through newly segmented somites of a stage 13 embryo. (F) Same section as in E, seen under dark-field illumination, hybridized to qmf1 probe. (G) Transverse section through somite 4 or 5 of a stage-14 embryo, seen under bright-field illumination, hybridized to the α-cardiac actin probe. Arrows indicate the most dorsal localization of grains in the myotome. (H) Same section as in G, seen under dark-field optics. (I) Transverse section through the mesonephros of a corresponding stage-14 embryo seen under bright-field illumination. Arrows indicate hybridization to qmf1 probe as seen in I when viewed under dark-field illumination. Scale bar, 50 μm.

Figure 4. (See facing page for legend.)

show a separation of the dermomyotome into the dermatomal and myotomal cell layers, and the more caudal somites have just separated from the segmental plates.

In situ hybridization to parasagittal sections of stage 13 embryos with the *qmf1* (cC509) probe permitted an analysis of the spatial localization of *qmf1* mRNAs. Figure 4, A and B, demonstrates that the *qmf1* gene is expressed at detectable levels in the most rostral somites. Figure 4, C and D, shows that *qmf1* mRNA is expressed as caudal as somite 5 ± 1 , but not in the adjacent more caudal somites. We do not detect α -cardiac actin (Hallauer 1984) or *qmf1* hybridization within the central core of mesenchymal cells in the more caudal newly formed somites or in the segmental plate mesoderm. As shown in Figure 4, E and F, *qmf1* transcripts are not detected in caudal newly segmented somites. Interestingly, transverse sections of stage 13 embryos show no detectable hybridization with the *qmf1* probe, suggesting a low density of labeled myogenic cells in the somites. In contrast, transverse sections of stage 14 embryos show low, but detectable, levels of *qmf1* transcript hybridization as caudal as somite 10 ± 2 , at the level of the mesonephric tubules (Fig. 4I,J). At this level, the α -cardiac actin probe also hybridizes weakly to the newly formed myotome (data not shown), whereas at the rostral end of the embryo, α -cardiac actin transcripts are abundant in myotomal cells (Fig. 4G,H). Although *qmf1* and α -cardiac actin gene expression is restricted to the myotome, *qmf1* mRNAs are found over more dorsally localized cells of the compartment (arrows in Fig. 4G,I). These results indicate that hybridization to *qmf1* mRNA is first detected in the myotomes of rostral somites of stage 13 embryos and that the expression pattern of *qmf1* follows the rostral–caudal direction of embryonic development.

To investigate further the rostral–caudal gradient of *qmf1* gene activation in the somites, *qmf1* and fast skeletal troponin T (TnT) (Hastings and Emerson 1982; Hastings et al. 1985) probes were hybridized to stage 19 embryos (Fig. 5D–G). At this developmental stage, the rostral somites show advanced myotomal muscle mass formation compared to caudal somites. Comparison of parasagittal sections of stage 19 embryos probed with *qmf1* (Fig. 5D,E) and TnT (Fig. 5F,G) cRNAs reveals a coincidence of grain accumulation in the myotomal region of the somite. Examination of serial parasagittal sections of stage 19 embryos probed alternatively with *qmf1* and TnT probes further establishes the rostral–caudal gradient of somite development. When these sections are probed for TnT mRNA, the myotomal muscle masses of rostral somites label prominently, whereas the myotomes of caudal somites label weakly. In contrast, hybridization with the *qmf1* probe is low, but constant, along this same rostral–caudal axis of the embryo. The results of these in situ analyses at stages 13, 14, and 19 establish that the expression of *qmf1* and contractile protein transcripts are related to the developmental stage of somites along the rostral–caudal axis of the embryo and not to the developmental stage of the embryo.

Differential localization of qmf1 and contractile protein mRNAs in the somites

To determine the spatial localization of *qmf1* and contractile protein mRNAs within the myotomal compartment, we compared stage 24 embryo sections probed alternatively with *qmf1* and TnT cRNAs. For these experiments, sets of serial, transverse sections of stage 24 embryos were hybridized with a cRNA probe of *qmf1* (cC509) and with a cRNA probe of TnT on the adjacent section. In situ hybridization reveals expression of *qmf1* and TnT mRNAs in the myotome region of a caudal somite of a stage 24 embryo (Fig. 5A–C). In all sections examined, *qmf1* probe hybridization in the myotome was consistently lower than that of the TnT probe. This probably reflects a lower abundance of *qmf1* mRNA than TnT mRNA in differentiated myogenic cells, as revealed by the 10- to 20-fold lower recovery of *qmf1* clones compared to TnT clones in screens of several myofiber cDNA libraries. Significantly, however, the spatial distribution of *qmf1* mRNAs, compared to TnT transcripts, in the myotomal compartment is different (cf. Fig. 5B,C). *qmf1* probe hybridization grains are abundant over a histologically distinct group of basophilic cells at the DML of the dermatome (also see Fig. 6), a region that does not hybridize with contractile protein cRNAs. Furthermore, *qmf1* cRNA probes preferentially hybridize to transcripts in the ventrolateral region of the myotome (Fig. 5B), adjacent to, but not within the region of basophilic cells at the ventral lip of the dermatome. The ventrolateral region of the myotome also hybridizes at low levels with the TnT probe. In addition, the *qmf1* probe hybridizes preferentially to cells found along the myotome–sclerotome interface (Fig. 5B; also see Fig. 6B,F,J). In contrast, TnT mRNA is expressed over the entire mediolateral width of the myotomal compartment (cf. Fig. 5B,C).

To identify the earliest stage of development at which localization of *qmf1* mRNA occurs over the basophilic cells of the DML of the dermatome, we analyzed serial transverse sections of embryos ranging from stage 14 to stage 26 probed with *qmf1* cRNA probe. The earliest stage at which *qmf1* mRNAs clearly accumulate over epithelioid cells of the dorsomedial dermatome occurs at stage 19. Figure 5, H and I, shows that *qmf1* transcripts accumulate over the DML region of a lumbar somite. Therefore, these results indicate that *qmf1*, but not contractile protein, cRNA probes hybridize preferentially to dorsomedial cells of the dermatome and ventrolateral and ventromedial cells of the myotome.

DML cells express qmf1 mRNA but not muscle protein mRNAs

We examined in detail *qmf1* and contractile protein mRNA expression in the DML region of somites. Because this region does not express contractile protein mRNAs, we reasoned that this may be a region where myogenic lineage determination occurs in the embryo.

Figure 5. Localization of *qmfl* and *TnT* mRNAs in stage 19 and stage 24 quail embryos by in situ hybridization histochemistry. (A) Transverse section through the mid-portion of a stage 24 embryo hybridized with *qmfl* probe. Arrows indicate the dorsomedial and ventrolateral regions of the dermatome. (B) Same section as in A viewed under dark-field illumination. (C) Dark-field micrograph of section adjacent to A hybridized with *TnT* probe. (D) Bright-field photograph of a parasagittal section through lumbar somites of a stage 19 embryo hybridized with *qmfl* probe. (E) Same section as in D seen under dark-field illumination. (F) Parasagittal section through lumbar somites of a corresponding stage 19 embryo hybridized with *TnT* probe. (G) Same section as in F seen under dark-field illumination. Bright-field (H) and corresponding dark-field (I) micrographs of a transverse section of a stage 19 embryo hybridized to the *qmfl* probe. Scale bar, 100 μ m (A–C) and 50 μ m (D–I).

Figure 6. (See facing page for legend.)

Stage 24 embryos were sectioned serially in a transverse orientation, and adjacent sections were hybridized with qmf1 probe and with differentiated muscle contractile protein antisense probes. The four probes, qmf1, TnT, slow cardiac troponin C (TnC) (Bucher et al. 1988), and α -cardiac actin, hybridize to cells of the differentiated myotome (Fig. 6A–L). The TnT, TnC, and α -cardiac actin probes hybridize to mRNAs throughout the dorso-ventral length of the myotome. In contrast, the qmf1 probe preferentially hybridizes to the most dorsal and medial regions of the myotome and weakly to the central core of the myotome (Fig. 6A,B,E,F,I,J). In >80% of the sections examined, qmf1 hybridization leaves a dense accumulation of grains (arrow) over the DML cells, whereas TnC and TnT probes hybridize only over the more ventrally located myotomal cells (Fig. 6C,D,G,H). Although moderate levels of qmf1 transcripts are seen over the myotome, this compartment is intensely labeled after hybridization with the α -cardiac actin probe (Fig. 6K,L). Localization of qmf1 in the dorsal medial region of the dermatomyotome is also observed in somites of stage 14 embryos (Fig. 4I,J). The DML is therefore a zone of qmf1-expressing cells that form an anatomically discrete band of cells across the dorsal region of the somite. Because DML cells preferentially accumulate qmf1 mRNA, but not muscle protein transcripts, we conclude that the DML delineates a region where qmf1-expressing myoblasts are found.

Expression and localization of qmf1-expressing cells precedes differentiation in the limb bud

To examine the temporal expression and spatial localization of qmf1 and contractile protein transcripts in growing limb buds, embryos ranging from stages 19 to 26 were hybridized in situ with probes specific for qmf1 and TnT mRNAs. The earliest detection of qmf1 transcripts in the hind limb occurred at stage 24 (Fig. 7A,B). qmf1 hybridization is restricted to dorsal and ventral regions of the limb. In several sections, we observed a continuous proximal–distal gradient of labeled cells extending from the ventrolateral region of the somite to the dorsal region of the limb bud (data not shown). Stage 24 embryonic hind limbs show no hybridization specific to the TnT probe, suggesting that myogenic differentiation has not yet taken place (Fig. 7C). By stage 26, however, qmf1 and TnT mRNAs colocalize in the dorsal and ventral premuscle masses (Fig. 7D–F), establishing the onset of differentiation in the hind limb between stages 24 and 26. Therefore, our results demonstrate that by stage 24, qmf1 mRNA expressing myogenic cells are spatially segregated in the dorsal and ventral regions of the limb mesenchyme prior to differentiation, as assayed by TnT mRNA expression.

Discussion

In this study we investigated the developmental expression of a quail myogenic mRNA and contractile protein mRNAs in the somites and limbs of developing quail embryos. qmf1 mRNA is expressed in myoblasts and myofibers, but not in smooth or cardiac muscle or liver. The encoded qmf1 protein is a helix–loop–helix protein, homologous to CMD1 (Lin et al. 1989) and showing extensive similarity to three other myogenic proteins: mouse MyoD1 (Davis et al. 1987; Tapscott et al. 1988), human Myf5 (Braun et al. 1989), and *X. laevis* MyoD (Hopwood et al. 1989). Because qmf1 mRNA is expressed in embryonic myoblasts, as well as in differentiated myofibers, qmf1 mRNA has been used as a molecular marker to investigate the developmental appearance and localization of undifferentiated, but determined myogenic cells in developing embryos.

qmf1 mRNA is first detected in the rostral somites of stage 13 embryos, showing that the activation of qmf1 mRNA is relatively coincident with the process of somite compartmentalization and myotome formation. These findings are consistent with immunological studies showing that myosin expression is first detected in the rostral somites of stage 13 embryos (Holtzer et al. 1957). *qmf1* gene expression is undetectable in the segmental plate mesoderm or in the newly segmented somites, even though quail/chick grafts of these tissues have been shown to give rise to differentiated muscles at later stages of development. Therefore, these results may indicate that the myogenic cells in tissue grafts represent earlier stage mesodermal cells that do not express qmf1. We also show that activation of qmf1 mRNA expression follows the rostral–caudal gradient of somite maturation. This result indicates that activation of *qmf1* gene expression is regulated locally in the somites in relation to the events and processes that control somite compartmentalization and not by stage-specific processes in the embryo.

qmf1 mRNAs localize initially over more dorsomedial cells of the myotome when compared with contractile protein mRNAs and accumulate preferentially, by at least stage 19, in histologically distinct cells of the DML of the dermatome. In addition, qmf1 transcripts colocalize with contractile protein mRNAs in the myotome. The specific localization of qmf1 mRNA in DML cells of the dermatome suggests that determination of qmf1-expressing cells is a spatially controlled process. The finding that cells in the DML of the dermatome are myogenic is consistent with results of cytological and immunological studies that have implicated the DML in myotome formation (Williams 1910; Boyd 1960; Hamilton 1965; Miki and Mizoguti 1982; Ede and El-Gadi 1986). Somite grafts in chick/quail chimeras also show

Figure 6. DML cells express qmf1 transcripts. Bright-field (A, C, E, G, I, and K) and corresponding dark-field (B, D, F, H, J, and L) micrographs of transverse sections of a stage 24 embryo hybridized with the [³⁵S]UTP-labeled probes (right). For direct comparison, A and C, E and G, and I and K show adjacent sections. Sectioning and spreading artifact caused compression of I tissue section. Arrows indicate regions of the most dorsal expression of the RNAs probed. DML cells express qmf1 mRNA (A, E, and I) whereas differentiation-specific mRNAs localize in the myotome (C, G, and K). (N) Neural tube; (SG) spinal ganglion; (dml) dorsal medial lip. Scale bar, 50 μ m.

Figure 7. Localization of *qmf1* and TnT transcripts in stage 24 and stage 26 hind limb buds. (A) Bright-field photograph of a longitudinal section of a stage 24 limb bud hybridized with *qmf1* probe. (B) Corresponding section viewed under dark-field illumination. (C) Section adjacent to A hybridized with TnT probe. Bright-field (D) and corresponding dark-field (E) photograph of a stage 26 limb section hybridized with *qmf1* probe. (F) Section adjacent to D hybridized with TnT probe. Hybridization to muscle-specific transcripts is restricted to ventral (*left*) and dorsal (*right*) axes of the limb. Scale bar, 100 μ m.

that both the dermatome and myotome contribute cells to the formation of abdominal muscle (Chevallier 1979; Christ et al. 1983, for review, see Christ et al. 1986), and the dermatome has been implicated as the source of myogenic cells that locate in the wing bud (Christ et al. 1974, 1977; Chevallier et al. 1976, 1977). Our observations on *qmf1* gene expression therefore provide direct evidence for the myogenic potency of the dermatome. With the development of *qmf1*-specific antibodies, im-

munocytochemical studies should better define the specific cells that express *qmf1* in the dermatome.

Two possibilities could explain the localized expression of *qmf1* mRNAs in the DML region of the dermatome. Determination of myogenic cells may be a single-step process in the early somite prior to or coincident with somite compartmentalization to give rise to a distinct population of myogenic cells that then proliferate along the DML and migrate into the muscle-forming re-

gions of the embryo. Alternatively, determination of myogenic cells may be occurring constantly at the DML, continuously giving rise to myogenic cells. These possibilities cannot be distinguished by our present data. However, because *qmf1* is a marker for determined myogenic cells, we can conclude that the process of myogenic determination is initiated early in development. In fact, if *qmf1* gene activation is coincident with the determination of cells to myogenesis, then determination occurs by stage 13 and progresses along the rostral–caudal axis of somite development.

qmf1 mRNA is also abundant, relative to contractile protein mRNAs, in the ventrolateral region of the myotome and along the interface between the myotome and sclerotome. These regions of *qmf1* mRNA accumulation likely are sites where undifferentiated myogenic cells predominate over differentiated muscle cells, and these may be regions where *qmf1* myogenic cells proliferate, pathways of myogenic cell migration to the abdominal and limb regions of muscle formation in the embryo, or both. Somite grafting experiments have established previously that limb muscles derive from the somite (for review, see Christ et al. 1986), probably from the ventrolateral region of the dermatome (VLL). Our studies show that the *qmf1* probe does not hybridize to VLL cells of the dermatome, implicating the DML as the sole source of *qmf1*-expressing cells that become localized in the myotome as well as in the abdominal and limb regions. Another possibility to be considered, however, is that the VLL cells of the dermatome represent a different or earlier lineage of myogenic cells that do not express *qmf1* mRNAs but that come to occupy the limb mesenchyme to give rise to muscle cells distinct from *qmf1*-expressing cells or to pre-*qmf1*-expressing myogenic cells. In support of this latter view, studies of clonal cell cultures have suggested the existence of different types of myoblasts which, when differentiated, express specific subsets of muscle myosin heavy-chain isoforms (Miller and Stockdale 1986). Although our data suggest the former idea, these possibilities cannot be resolved until the relationship between somitic precursor cells and limb or abdominal muscle cells is clearly established.

Consistent with our work, *in situ* hybridization analyses to examine the spatial expression of *myogenin* and *MyoD1* genes during mouse embryogenesis (Sassoon et al. 1989; Wright et al. 1989) show that both mRNAs localize in the somite myotome. Unlike *MyoD1* and *qmf1* mRNAs, which are expressed in skeletal myoblasts and differentiated muscle, *myogenin* mRNA becomes abundant when mammalian and avian myoblasts initiate the early stages of differentiation (Wright et al. 1989; M.E. Pownall and C.P. Emerson, unpubl.). Interestingly, these analyses show that *MyoD1* mRNA is not detected as early as *myogenin* or α -cardiac actin in the somite of mouse embryos but appears in the limb at the same time as *myogenin*. These results suggest that *MyoD1* and *myogenin* may function differently at different developmental stages and that different populations of muscle cells may be present in the embryo (Sassoon et al. 1989).

In contrast to *MyoD1* in mouse embryos, *qmf1* is a marker for the early events of myogenesis in both the somite and the limb, suggesting that *qmf1* in the avian embryo may not be functionally equivalent to *MyoD1*. The distinct regions of homology that *qmf1* shares with both *Myf5* and *MyoD1* may indicate that *qmf1* is a composite avian myogenic regulatory protein functionally related to both *MyoD1* and *Myf5* proteins. Furthermore, *myogenin* and *MyoD1* gene expression in mouse embryos has not been examined in transverse sections, which reveal the mediolateral structures of the somite. Therefore, it is not yet known whether *MyoD1*, *myogenin*, or *Myf5* gene expression occurs in cells of the DML. *In situ* hybridization with these other myogenic regulatory genes may provide additional information on the early events of myogenic determination in mammalian and avian embryos.

Recently, Hopwood et al. (1989) showed that *XMyoD* gene expression in *Xenopus*, like mouse *MyoD1* and quail *qmf1*, is restricted to skeletal muscle lineages. They also demonstrated that the *XMyoD* gene becomes actively transcribed ~2 hr before cardiac actin mRNAs accumulate. Our *in situ* results show relatively coincident expression of *qmf1* and α -cardiac actin mRNAs in the somite myotome. However, we cannot exclude the possibility that *qmf1* mRNA expression in the somites, like that of *XMyoD*, slightly precedes α -cardiac actin mRNA expression. In light of these findings, therefore, it will be of interest to examine the temporal sequence of activation of *qmf1* and contractile protein genes more precisely in very early-stage quail embryos using sensitive polymerase chain reaction (PCR) and RNA hybridization techniques.

qmf1 mRNA expression in the leg bud is detected first along the dorsal and ventral axes of premuscle mass formation at stage 24 prior to muscle differentiation, as assayed by TnT mRNA expression. *qmf1* mRNA expression is highest in the proximal region of the limb and decreases in a proximal–distal direction. Hybridization is undetectable in the most distal portion of the limb. These findings support results of cell culture studies reporting an absence of myogenic precursor cells in the so-called progress zone beneath the apical ectodermal ridge (Newman et al. 1981; Rutz et al. 1982; Hauschka and Rutz 1983). Consistent with our analyses of TnT expression, it has been shown that skeletal and α -cardiac actin mRNAs are undetectable in stage 24 chick limbs but are present by stage 26 (Ordahl 1986). Immunofluorescence and immunochemical analyses of myosin heavy-chain isoform expression in chick embryos indicate further that the forelimb muscle masses express contractile myosin first at stage 25 (Sweeney et al. 1989). Interestingly, a clonal analysis of cells derived from serially sectioned chick limbs has demonstrated the presence of myoblasts in stage 21 leg buds (Bonner and Hauschka 1974; White et al. 1975). *qmf1* mRNAs have not been detected in limbs at this stage by *in situ* hybridization, but it seems likely that myogenic cells in stage 21 limb buds are too few or too dispersed to be detectable by this *in situ* hybridization method. Hybridization

signals at stage 24 are only about fourfold above background; therefore, only two cell divisions of myogenic cell population would be sufficient to bring *qmf1* to a detectable level. Another possibility is that myogenic cells in the limb, which originate in the somite (Christ et al. 1974, 1977; Chevallier et al. 1976, 1977), do not express *qmf1* mRNAs until stage 24. Immunohistological studies of *qmf1* protein expression in the limb bud prior to stage 24 may distinguish these possibilities.

Our findings demonstrate that myogenic cells in stage 24 limbs become organized along the appropriate dorsal and ventral axes of muscle formation, preceding terminal muscle differentiation. Myogenic determination and terminal differentiation, therefore, are temporally separated processes in the limb. Also, the processes that control the spatial organization of primary muscle masses in the limb reflect the organization of the myogenic precursor cells, independent of the terminal differentiation process. Furthermore, the temporal separation of the processes of localization of *qmf1* myogenic cells at stage 24 and the initiation of terminal differentiation by stage 26 imply that muscle differentiation in the limb is a precisely regulated process. Tissue culture experiments show that myoblast differentiation is controlled by concentrations of extracellular growth factors (Kongsberg 1971) such as fibroblast growth factor (Lathrop et al. 1985; Clegg et al. 1987). Such growth factors have been detected in limb buds and may be the regulators of this stage-specific differentiation in the limb bud. In this regard, it is interesting to note that our results show that differentiation of cells in the myotome in stage 13 rostral somites does not appear temporally dissociated from the activation of *qmf1* gene expression, suggesting that if differentiation in the somite and limb is subject to similar extracellular controls, the developmental events leading to the differentiated state are accelerated in the somite.

Materials and methods

Isolation and sequencing of *qmf1* cDNA

A representative embryonic myofiber cDNA library of cultured muscle from Japanese quail embryos (*C. coturnix*) (Bucher et al. 1989) was screened at low stringency with a full-length mouse MyoD1 cDNA probe (Davis et al. 1987; Pinney et al. 1988). Approximately 1×10^6 plaques were screened on duplicate nitrocellulose BA85 filters (Schleicher & Schuell) with ^{32}P -random-primed (Feinberg and Vogelstein 1984) mouse MyoD1. Filters were prehybridized, then hybridized at 55°C and washed at the same temperature, as described (Peden et al. 1982). MyoD1-positive clones were subcloned into the *EcoRI* site of pEMBL 18 (Dente et al. 1983) or Bluescript M13⁺ (Stratagene), and a set of exonuclease III deletions was generated with the Erase-A-Base method, as recommended by the supplier (Promega). The nucleotide sequences of the deletions generated for cC509 and cC511 were determined according to Sanger et al. (1977).

A *qmf1* genomic clone was isolated by the screening of a genomic library of partial *EcoRI* restriction fragments of quail embryo DNA cloned into the λ Charon 4A vector (Baldwin et al. 1985), using the cC509 clone and a 160-bp *EcoRI*-*SphI* fragment of cC511. The *EcoRI* fragments were subcloned into Bluescript KS (Stratagene) for restriction mapping analysis. The nu-

cleotide sequence of a genomic subclone containing the *SphI* site that is present in cC511 was determined using the procedures of Sanger et al. (1977).

Northern blots

RNA was isolated by guanidine thiosulfate extraction (Chirgwin et al. 1979), electrophoresed on 1% (wt/vol) agarose MOPS-formaldehyde gels, and transferred to Zetabind matrix (AMF Cuno, Meriden, Connecticut). The blot was hybridized first with a random-primed, 1.4-kb *EcoRI*-*HindIII* fragment of MyoD1 cDNA for 48 hr at 55°C (Pinney et al. 1988), washed as described by Church and Gilbert (1984), and exposed to Kodak XAR film for the times specified in the text. The hybrids were denatured twice for 30 min each time at 70°C in $0.1 \times$ Denhardt's (0.002% each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone), 10 mM Tris-HCl (pH 7.9), and 0.05% SDS to prepare blots for rehybridization with cC509 at 65°C.

In situ hybridization

Procedures for in situ hybridization have been described by Cox et al. (1984) with some modifications. Briefly, quail embryos were dissected in PBS [0.13 M NaCl, 7 mM Na₂PO₄, 3 mM NaH₂PO₄·2H₂O, (pH 7.4)] and fixed for 12–14 hr at 4°C in 4% paraformaldehyde in PBS. Embryos were staged according to the number of pairs of somites. To facilitate comparison with chick developmental stages, embryos were staged according to Hamburger and Hamilton (1951); therefore, stage 13 corresponds to 19 somite pairs, stage 14 to 22 somite pairs, and stage 19 to ~37 pairs of somites. Beyond stage 14, external structures, including limb buds, were also used to determine the developmental stage of the embryo. Fixed embryos were washed twice in PBS (10 min each), dehydrated through increasing concentrations of ethanol, cleared twice in xylenes (20 min each), and embedded into paraplast. Serial sections of 6–8 μm were cut, placed onto covered polylysine-coated slides, drained, and dried for 36–48 hr at 45°C. Subbed slides were prepared by soaking in a 10 mM Tris (pH 8.0) solution containing 50 μg polylysine/ml for 10 min. Sections were prepared for hybridization as described in Cox et al. (1984). Hybridization mixtures (60 μl) were applied to slides, covered with a coverslip, and sealed with rubber cement. The slides were incubated in a humidified chamber at 60°C for at least 12 hr. After hybridization, the slides were washed in $4 \times$ SSC (0.6 M sodium chloride, 0.06 M sodium citrate at pH 7.0) three times for 5 min, during which time the coverslips were carefully removed. This was followed by 30-min RNase A digestion [20 $\mu\text{g}/\text{ml}$ in 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0)] at 37°C and a 30-min rinse, also at 37°C in the buffer alone. Slides were washed under high stringency conditions in $2 \times$ SSC, 50% formamide for 30 min at 55°C, followed by three washes in $0.1 \times$ SSC (30 min each) at the same temperature. After a brief wash in $0.1 \times$ SSC at room temperature, the slides were dehydrated in alcohol containing 0.3 M ammonium acetate, and allowed to air-dry for 20 min. For autoradiography, the slides were dipped in Kodak NTB2 liquid nuclear track emulsion diluted 1:1 (vol/vol) with 0.3 M ammonium acetate, dried for 2 hr at room temperature before being placed in light-proof boxes with dessicant, and exposed for 7 days at 4°C. Following exposure, the slides were developed for 2.5 min at room temperature in a 1:2 (vol/vol) dilution of Kodak Dektol developer, stopped in Kodak stop bath for 15 sec, and fixed in Kodak Rapid Fix for 2.5 min. Sections were stained with hematoxylin and eosin, dehydrated, cleared in xylenes, and mounted under coverslips. Serial sections of one stage 10 embryo [by longitudinal sectioning (LS)], two stage 12 embryos

[one by LS and one by transverse sectioning (XS)], two stage 13 embryos (one by LS and one by XS), two stage 14 embryos (both by XS), one stage 15 (by XS), one stage 17 (by XS), four stage 19 embryos (two by LS and two by XS), one stage 21 embryo (by XS), two stage 23 embryos (both by XS), six stage 24 embryos (one by LS and five by XS), and three stage 26 embryos (all three by XS) were prepared for these experiments.

Probe preparation

cDNA clones isolated from Japanese quail myofiber cDNA libraries have been described previously (Hastings and Emerson 1982; Bucher et al. 1989). Sequence analyses of cC156 (cC118), cC147 (cC122), and cC111 have shown that these clones encode protein isoforms for α -cardiac actin, TnT, and TnC, respectively (Wilkinson 1980; Hastings and Emerson 1982; Hastings et al. 1985). S1 nuclease protection analysis of RNAs from cultured embryonic skeletal muscle, embryonic, and adult muscles show that TnT (cC122) expression is restricted to skeletal muscle, whereas TnC (cC111) is also expressed in cardiac muscle (Bucher et al. 1988). High stringency Northern analysis shows that cC156 cardiac α -actin probe hybridizes with RNA from day-10 embryonic breast muscle, cultured myofibers, and adult heart muscle, but not with RNA from cultured myoblasts or the adult skeletal muscle tissues (Hallauer 1984). The lack of hybridization with RNA from myoblasts and adult skeletal muscle demonstrates that under high stringency conditions, α -cardiac actin cC156 does not cross-hybridize with the cytoplasmic and skeletal muscle actin mRNAs. The 335-bp TnT (cC147) and 590-bp α -cardiac actin (cC156) cDNA inserts cloned into the *Pst*I site of Bluescribe M13⁺ (Vector Cloning Systems) were linearized by digestion with *Eco*RI and *Bam*HI restriction enzymes, respectively, to prepare cRNA probes using the T3 polymerase. The control sense RNA probes were synthesized in a T7 polymerase reaction after digestion of the plasmid constructs with *Hind*III. The 192-bp (cC159) *Pst*I fragment of the TnC cDNA (cC111) cloned into the *Pst*I site of Bluescript KS⁺ (Stratagene) was linearized by digestion with *Bam*HI to synthesize the antisense probe using T3 polymerase or *Hind*III to prepare the mRNA sense control probe in a T7 polymerase reaction. The 832-bp qmf1 cDNA (cC509) was subcloned into the *Eco*RI site of Bluescript M13⁺ (Stratagene). The plasmid was linearized with *Hind*III to prepare the cRNA probe using T7 polymerase. The control mRNA probe was synthesized using T3 polymerase after *Pst*I restriction enzyme digestion. For all constructs, the orientation of the inserts was determined by sequencing and by restriction enzyme mapping. High specific activity RNA probes were synthesized using ³⁵S-labeled rUTP (sp. act. >1000 Ci/mmol, NEN) according to the supplier's recommendations. Template was digested with RNase-free DNase (Promega). The RNA was extracted and precipitated with 2 M ammonium acetate and ethanol to remove unincorporated nucleotides. The labeled probe was hydrolyzed to a mean length of 100 bases by incubation in 0.1 M NaHCO₃/Na₂CO₃ (pH 10) at 60°C for an appropriate length of time (Cox et al. 1984) and then ethanol-precipitated. For hybridization, the RNA probes were dissolved in medium consisting of 50% formamide, 300 mM NaCl, 5 mM EDTA (pH 8.0), 20 mM Tris-HCl (pH 8.0), 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10% dextran sulfate, 10 mM DTT, and 500 μ g/ml yeast tRNA. Final activity of probe in the hybridization mixture was 1.3×10^4 cpm/ μ l. The specificity of hybridization was established using sense strand probes, which did not hybridize above background levels of hybridization observed with antisense probes.

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Note added in proof

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries.

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