Myf-5 is transiently expressed in nonmuscle mesoderm and exhibits dynamic regional changes within the presegmented mesoderm and somites I–IV

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Myf-5 is one of four myogenic regulatory factors that play important roles in skeletal muscle development. This study provides detailed analysis of Myf-5 expression during early chick development using an in situ hybridization technique that has been optimized to detect low level Myf-5 transcripts. This facilitated detection of heretofore unrecognized dynamic changes in Myf-5 expression patterns. Myf-5 expression is first detected at stage 3 in the primitive streak and exhibits transient low-level expression in nonmyogenic mesoderm. Myf-5 is later expressed in the presegmented mesoderm (psm) in a reiterating pattern that is coordinated with somitogenesis and also colocalizes with the Notch ligand C-Delta-1. In somites (S) I–IV, Myf-5 expression exhibits dynamic regional changes, and in somites rostral to S IV, Myf-5 is expressed at higher levels in muscle precursors in the dorsomedial somite. Semiquantitative comparison of Myf-5 mRNA levels in the psm and in myotome-containing somites indicates about a 10-fold difference. The expression pattern of Myf-5 differs from that of MyoD, which we find is expressed only in the dorsomedial somite. These data reveal that Myf-5 is expressed at low levels several stages before muscle differentiation occurs and suggest that only a subset of cells that initially express Myf-5 will upregulate its expression and differentiate as muscle.

Key Words: somite; somitogenesis; myogenesis; muscle; Myf-5; MyoD; Delta-1.

INTRODUCTION

All body skeletal muscle is derived from somites: epaxial (deep back) muscle is derived from the medial somite and hypaxial muscle (limb, abdominal, intercostal) from the lateral somite (Ordahl and Le Douarin, 1992, for review). During somitogenesis in the chicken embryo, a pair of somites buds from the presegmented mesoderm (psm) every 90 min, resulting in a caudal-to-rostral developmental gradient. The most caudal somite is designated somite I (Ordahl, 1993), and in day 2 avian embryos it is an epithelial ball of cells. At the level of somite IV, the dermamyotome (presumptive dermis and muscle) is formed from the dorsal aspect while the ventral aspect undergoes a mesenchymal transition and becomes sclerotome, the precursor to the vertebral column and ribs (Christ and Ordahl, 1995, for review). At approximately somite X, cells from the medial dermamyotome withdraw from the cell cycle and involute beneath the dorsomedial dermamyotome lip to form the epaxial myotome (Denetclaw et al., 1997; Kahane et al., 1998). In thoracic-level somites of day 3 embryos, cells from the lateral dermamyotome involute beneath the ventrolateral dermamyotome lip to form the hypaxial myotome, the precursor to abdominal and intercostal muscle (Christ et al., 1983; Cinnamon et al., 1999; Denetclaw and Ordahl, 2000). In contrast, precursors to limb muscle migrate from the ventrolateral dermamyotome lip of hindlimb level (lumbosacral) somites and forelimb level (brachial) somites and differentiate in the limb bud (Ordahl and Williams, 1998; Ordahl et al., 2000, for review).

The MRFs (Myogenic Regulatory Factors) (Myf-5, MyoD, myogenin, and MRF4) are a family of basic helix-loop-helix transcription factors that regulate muscle development. All four of the MRFs are capable of activating transcription of muscle-specific genes and myogenic differentiation in some...
nonmuscle cell lines (Tapscott et al., 1988; Weintraub et al., 1989). MyoD and Myf-5 specifically regulate early myogenic determination in vivo since mice lacking both of the genes do not develop myoblasts (Rudnicki et al., 1993). Originally it appeared that MyoD and Myf-5 were redundant since single-knockout mice display normal muscle phenotypes at birth (Braun et al., 1992; Rudnicki et al., 1992). However, more detailed examination of the mutant embryos disclosed that mice null for each exhibit developmental delays in different muscle types (Kablar et al., 1997, 1998), implying that the roles for Myf-5 and MyoD in myogenic determination are only partially redundant.

Consistent with the idea of differing developmental roles for the two genes, Myf-5 and MyoD expressions are initiated at different times in development as detected by conventional in situ hybridization. From the onset of somitogenesis in the mouse at day 8, Myf-5 is expressed in the dorsomedial aspect of every somite (Ott et al., 1991; Tajbakhsh et al., 1996). MyoD is also expressed in the dorsomedial somite, but it follows Myf-5 expression and is first detected at day 10.5 (Sassoon et al., 1989). In chick, Myf-5 also precedes MyoD expression (Hacker and Guthrie, 1998).

Myf-5 expression has also been detected prior to somitogenesis in both mouse and chick embryos, but only when techniques that are more sensitive than standard in situ hybridization procedures are used. Myf-5 transcripts have been amplified by RT-PCR in gastrula stage (stage 3) chick embryos (Lin-Jones and Hauschka, 1996), in chick psm (Maroto et al., 1997), and in mouse psm (Kopan et al., 1994). Myf-5 transcription in mouse psm has also been detected via lacZ staining of mice with β-galactosidase knocked into the Myf-5 locus (Cossu et al., 1996). Because presomitotic expression of Myf-5 has not been visible by standard in situ hybridization, its detection by more sensitive techniques implies that these early Myf-5 transcripts are in low abundance. The relevance of such low-level MRF expression to muscle precursors of the somite remains unknown.

In an effort to determine the temporal and spatial pattern of Myf-5 expression prior to somitogenesis, we optimized in situ hybridization to detect low abundance Myf-5 transcripts. Our results indicate that in chicken embryos, Myf-5 expression begins in the primitive streak at stage 3. At first, Myf-5 is broadly expressed, including regions of the mesoderm that are not fate-mapped to become muscle. Ultimately, however, Myf-5 expression is upregulated by about 10-fold and becomes restricted to presumptive muscle in the differentiating myotome of older somites. We also demonstrate that Myf-5 expression in the psm occurs in a reiterating pattern that is coordinated with both somitogenesis and the expression pattern of the Notch ligand C-Delta-1 and that Myf-5 expression patterns undergo complex regional changes in somites I–IV. In addition, our findings provide further evidence that the initial steps of myogenesis begin much earlier in development than had previously been thought.

**MATERIALS AND METHODS**

**Eggs and Embryos**

White Leghorn chicken eggs (H and N International) were incubated in a 38°C forced-draft incubator at 100% humidity. Embryos were staged according to Hamburger and Hamilton (1992) and the somite staging system developed by Ordahl (1993) was used for numbering somites.

**RT-PCR**

Paraxial mesodermal tissue was dissected from stage 11–14 embryos as described previously (Stern and Hauschka, 1995) and solubilized immediately in 500 μl of Trizol reagent (Gibco BRL). Tissue samples contained either 10 somites (somites I–IV or somites V+) or 4 pieces of the midsegmental plate, each approximately the length of 4 somites. RNA was isolated from the Trizol mixture according to the manufacturer’s directions and resuspended in 10 μl of water. Five microliters of RNA was reverse transcribed with Sensiscript RT (Qiagen) and 5 μl of RNA was mock reverse transcribed (no RT control). One-fourth of the RT or control reaction was PCR amplified using Taq polymerase (Promega) in 50-μl reactions under the following cycling conditions: denature 5 min at 94°C; amplify 40 cycles of 45 s at 94°C, 1 min at 58°C, 1 min at 72°C; final extension of 10 min at 72°C. Fifteen microliters of each sample was run on a 6% polyacrylamide gel, and the gel was stained for 20 min in SYBR gold nucleic acid stain (Molecular Probes) diluted at 1:5000 in TAE (40 mM Tris acetate, 2 mM EDTA). The gel was scanned with a Storm PhosphorImager (Molecular Dynamics) in the blue fluorescence mode and visualized using ImageQuant software (Molecular Dynamics). The Myf-5 and GAPDH primers used were described previously (Münsterberg and Lassar, 1995).

**In Situ Probes**

The reported Myf-5 expression patterns were confirmed with two in situ hybridization probes. One is a 349-base fragment of Myf-5, 3' of the conserved basic helix-loop-helix region. The fragment was isolated by RT-PCR from RNA from day 11 embryonic chicken breast muscle using the primers (nucleotides 552–571) 5'-AGGGAAACGGTGAAGACTA-3' and (nucleotides 1302–1321) 5'-TCATAGCCGCGTGAAGCTC-3' (GenBank Accession No. X73250). The ends of the fragment and pBluescript KS (Stratagene) were cut with Smal and ligated together. Antisense riboprobes were made by cutting the plasmid with EcoRI and transcribing with T7 RNA polymerase. The second Myf-5 probe is a 336-base fragment spanning the 5' UTR and the 5' coding region. It was RT-PCR amplified from day 2 chicken embryo RNA using the primers (nucleotides 47–66) 5'-ACCTTTGTGTCGGGTTCTGCTAA-3' and (nucleotides 365–383) 5'-CATGTTGCGATTTCTCT-3' and ligated into the pCRII-TOPO TA cloning vector (Invitrogen). The antisense riboprobe was synthesized by linearizing the plasmid with XbaI and transcribing with Sp6 RNA polymerase. Probes were subsequently purified on a 5-10% spin column.

MyoD probes consisted of a 647-base PstI to XbaI fragment of the CMD-1 clone (Lin et al., 1989) (nucleotides 609–1256, GenBank Accession No. X16189) inserted into a PstI/XbaI-cut pSPT18 vector (Pharmacia). Antisense probes were synthesized by linearizing with HindIII and transcribing with T7 RNA polymerase. A 200-base probe was also synthesized and yielded the same results. It was
whole-mount in situ hybridization

Whole chick embryos were dissected and their heads pierced with a tungsten needle to reduce entrapment of probe in the forebrain cavity. Frequently, embryos stage 10 and older displayed nonspecific staining in the head and neural tube. Nonspecific staining in the same regions was also seen in control embryos hybridized with sense probe and was absent in embryos bisection through the head and neural tube prior to hybridization. Embryos were fixed overnight at 4°C in 4% paraformaldehyde/0.1 M phosphate-buffered saline (0.1 M PBS)/2 mM EGTA, dehydrated in a methanol series, and stored at −70°C in 100% methanol. Following rehydration, embryos were hybridized with digoxigenin (DIG; Roche)-labeled antisense RNA probes as described previously (Henrique et al., 1995). After hybridization, the DIG-labeled probes were detected using NBT/BCIP (Roche) as substrate for the anti-DIG-antibody coupled to alkaline phosphatase (Roche). Colorimetric reactions were conducted at room temperature with overnight incubations at 4°C and took up to 5 days to complete. When the color reaction buffer turned lavender, the embryos were washed and additional NBT/BCIP was added (up to four times per day). Embryos were then postfixed in 4% paraformaldehyde/PBS for 20 min at room temperature and cleared in 100% methanol. Photographs were taken on a Nikon SMZ-U microscope with a DC-330 CCD color camera (DAGE-MTI, Inc.) using Scion Series 7 image acquisition software (Scion Corp.).

It is believed that the sensitivity of the in situ hybridization was increased by using short riboprobes (about 350 bases) and by frequently adding new substrate to prolong the reaction. Myf-5 staining in mature rostral somites was first detected within 10 min of initiation of the in situ hybridization color reaction, the strong expression domain in somites I–IV and psm was first detected within 1–3 days, and Myf-5 expression within the nonmyogenic regions of the somite (light blue stain in Figs. 8A and 8C) appeared within 2–5 days. MyoD expression in the medial somites was seen within 24 h and no additional staining appeared upon prolonging the color reaction.

sections

Sections of embryos processed by whole-mount in situ hybridization were prepared by dehydration in an ethanol series, washing in xylene, and embedding in Paraplast X-tra (Oxford Labware). Blocks were cut in 10- to 16-μm sections on a Jung Biocut (Leica) rotary microtome. The sections were collected on Superfrost Plus slides (VWR), washed twice for 10 min in xylene, and embedding in Paraplast X-tra (Oxford Labware). Blocks were cut in 10- to 16-μm sections on a Jung Biocut (Leica) rotary microtome. The sections were collected on Superfrost Plus slides (VWR), washed twice for 10 min in xylene, and mounting in Permount (Fisher). Sections were photographed on a Zeiss Axio-phot microscope using Nomarski optics.

Semiquantitative Determination of Relative Myf-5 Transcript Levels

A semiquantitative dot blot method was developed to measure the relative difference between Myf-5 mRNA levels in cells with high and low transcript levels. Myf-5 sense mRNA was transcribed from plasmids containing either 5′ or 3′ fragments of the Myf-5 cDNA, as described above. Strips of nitrocellulose were spotted with twofold serial dilutions of Myf-5 mRNA ranging from 5 to 0.0024 ng, each in a volume of 1 μl. A Stratalinker (Stratagene) was used to UV crosslink the mRNA to the nitrocellulose which was then hybridized to antisense probes. Embryos were hybridized to the same probes, and both embryos and dot blots were processed and developed as described under Whole-mount in situ hybridizations. Development of the color reactions was monitored carefully and digital images were taken of the blots and embryos at five different time points. Subsequently, NIH Image software was used to perform densitometry on the images of the mRNA spots, chick somites, and psm.

The mean optical densities (OD) of the somites and dot blots over time were plotted to determine whether their reaction kinetics were similar. If so, ODs vs amounts of spotted mRNA were graphed, and the linear part of the curve was used to determine amounts of spotted mRNA that had identical ODs at the time points when Myf-5 expression was first detected in rostral somites and in the psm. The resulting calculated fold difference between the two amounts of mRNA was used as an estimate for the relative difference in Myf-5 transcript levels between the rostral somites and the psm.

results

Myf-5 Is Expressed Prior to Somitogenesis

We first investigated the distribution of Myf-5 in stage 11–13 chick embryos by RT-PCR, reproducing the results of Maroto et al. (1997) that Myf-5 mRNA is present in the psm. Myf-5 was also detected in somites I–IV and, as demonstrated previously by in situ hybridization (Borycki et al., 1997), in somites V and older (Fig. 1).

We next optimized in situ hybridization conditions to visualize the localization of presomitic Myf-5 expression in the developing chicken embryo. Myf-5 expression is not detected in stage 1 or 2 embryos, but is first visible along the nascent primitive streak in stage 3 embryos (Fig. 2A). As the primitive streak regresses in stages 4–10, Myf-5 expression remains posterior to Hensen’s node (Figs. 2B and 2F–2H). Cross sections show that Myf-5-positive cells re-
Myf-5 expression mainly in the mesoderm of stage 3 (Fig. 2C) and stage 5 embryos (Fig. 2D), but they are also detected in the epiblast at both stages (stage 5 Myf-5 epiblast expression not shown). There is little to no expression in the hypoblast (compare Figs. 2D and 2E). At stage 5, Myf-5 expression also extends laterally and posteriorly from Hensen's node (Fig. 2B), and by stage 7 (Fig. 2F) this lateral expression resolves into discrete stripes in the psm and first somite. Thereafter, Myf-5 is expressed within the rostral 2/3 of the psm but is strikingly absent in an approximately somite-length segment of the most rostral psm (arrows, Figs. 2F–2H). Myf-5 is also expressed in every somite of all embryos examined (up to stage 25) (Figs. 2F–2H). There is no specific staining in embryos probed with a sense control (Figs. 2E, 2I, and 2J). Apparent staining in the head of st. 10 embryo (J) is nonspecific (see Materials and Methods).

**FIG. 2.** Myf-5 expression prior to and during somitogenesis demonstrated by in situ hybridization. Whole mounts (A, B, F, G, H, I, J) and cross sections (C, D, E) taken after in situ hybridization. Stage (st.) 3 (A): Myf-5 is expressed in the primitive streak (ps). (C) Cross section through the primitive streak (dotted line in A) shows expression mainly in the mesoderm (m) layer (bracket), but it is also expressed in the epiblast layer (e) of the primitive streak and is not expressed in the hypoblast (h) (arrow). st. 5 (B): Myf-5 is expressed posterior to Hensen’s node (asterisk). (D) Cross section through the primitive streak posterior to Hensen’s node (dotted line in B) reveals expression mainly in the mesoderm (bracket). st. 7 (F): Myf-5 is expressed posterior to Hensen’s node and in the psm and first somite. There is no expression at the rostral end of the psm (arrow). st. 8 (G): Myf-5 is expressed posterior to Hensen’s node, in the psm except at the rostral end, and in all somites. st. 10 (H): Myf-5 is expressed in the psm except at the rostral end and in all somites. Control embryos probed with sense Myf-5 had no specific staining at st. 3 (I), st. 5 (E, cross section), or st. 10 (J). Apparent staining in the head of st. 10 embryo (J) is nonspecific (see Materials and Methods).
tory steps pertinent to myogenesis are initiated at least as early as stage 3; several stages prior to somite formation and many stages before terminal muscle differentiation in the somite.

Relative Levels of Myf-5 Expression in the Psm and Somites I–IV Are Approximately 10-fold Less Than in the Rostral Somites

Using in situ hybridization, the psm and S I–IV took considerably longer than the rostral somites to stain positively for Myf-5 expression. This suggests that there is weak Myf-5 expression in the psm and S I–IV compared to expression in rostral somites. To estimate the relative fold difference of Myf-5 transcripts in these two tissues, serial dilutions of Myf-5 sense mRNA were spotted onto strips of nitrocellulose and processed as dot blots in parallel with chicken embryo Myf-5 in situ hybridizations. The optical densities (ODs) of the mRNA spots on the dot blots were determined at the same times that the first Myf-5 signal was detected in rostral somites (30 min, Figs. 3) and psm and S I–IV (42 h., Fig. 3). For example, a roughly 16-fold difference in Myf-5 mRNA concentration between the rostral somites and the psm and S I–IV was calculated based on detecting similar ODs for the 2.5-ng spot developed for 30 min and the 0.16-ng spot developed for 42 h. The assay was performed two times with each of two antisense probes made against different regions of the Myf-5 transcript. Taking into consideration the data from each experiment, we estimate that the relative difference of Myf-5 mRNA levels in the rostral somites vs psm and S I–IV is approximately 10-fold.

FIG. 3. Serial dilutions of Myf-5 sense mRNA were spotted onto strips of nitrocellulose and processed as dot blots in parallel with stage 12–14 chicken embryo Myf-5 in situ hybridizations. The left column indicates time elapsed during the colorimetric detection reaction for both dot blots and embryos (3 of 6 time points examined in one representative experiment are shown). The middle column indicates which tissues are positive for Myf-5 expression at the indicated assay time point. The right column shows the dot blots at each assay time point. At 7.5 min there is no staining in the embryo, and the dot blot shows a concentration-dependent decrease in intensity over the 5–1.3 ng range of mRNA spots. At 30 min only the rostral somites (somites rostral to VI, VII, or VIII) are positive for Myf-5 expression and there is a concentration-dependent decrease in intensity over the 5–0.63 ng range of mRNA spots. At 42 h Myf-5 expression is first detected in the psm and somites I–IV (only the strong domain of Myf-5 expression in S I–IV is detected at this time). The signal intensity over the 5–1.3 ng range is saturated, and there is a concentration-dependent decrease in intensity over the 0.63–0.04 ng range of mRNA spots. The 2.5- and 1.3-ng mRNA spots at 30 min have an intensity similar to that of the 0.16- and 0.08-ng mRNA spots at 42 h. respectively. The difference in amounts of mRNA with like ODs is 16-fold, but was as little as 4-fold in other experiments, suggesting that the relative difference between Myf-5 mRNA levels in the rostral somites vs psm and S I–IV is approximately 10-fold.

Myf-5 Expression Changes as the Paraxial Mesoderm Develops into a Stage IV Somite

Myf-5 expression in the psm occurs in a periodic pattern that is similar to C-Delta-1 and is coordinated with somitogenesis. Upon observing Myf-5 expression in several embryos, we noticed that its pattern in the psm was not uniform, even among embryos with identical numbers of somites. An approximately somite-sized segment in the rostral psm, which is presumably the region that will bud off as the next somite, is always negative for Myf-5 expression (arrows in Figs. 2F–2H, 4A–4C, and 5A). However, beginning just caudal to the negative expression domain, Myf-5 is either expressed in a diminishing gradient toward the caudal psm (Figs. 4A, bracket, 2F–2H, and 5A) or expressed at relatively higher levels within a less than one somite-sized domain of the psm (Fig. 4B, asterisk). Embryos
with the latter expression pattern often have an additional
Myf-5-expressing region that begins over one somite-length
caudal to the Myf-5-positive domain (Fig. 4B, bracket).
Thus, the patterns of Myf-5 expression in Figs. 4A and 4B
resemble one another, except that the pattern in Fig. 4B is
displaced caudally by about one somite-length (Fig. 8A).

Another gene that has a reiterating expression pattern
during somitogenesis is the Notch ligand C-Delta-1.
C-Delta-1 is excluded from the rostral end of the psm and,
coincident with somitogenesis, gradually narrows its do-
main of expression. As a new somite buds off, C-Delta-1 is
weakly expressed in a stripe at its caudal end, a pattern that
is visible only upon prolonged development of the in situ
hybridization (data not shown) (Palmeirim et al., 1998). To

FIG. 4. Myf-5 and C-Delta-1 expression in the psm. In these experiments, Myf-5 in situ hybridizations were developed for extended periods of time to emphasize weak regions of expression in the somites. (A) Myf-5 is expressed in S I but is not expressed in an approximately somite-length segment of the rostral psm (arrow). Caudal to the Myf-5-negative domain, Myf-5 is expressed broadly in a decreasing gradient toward the caudal psm (bracket). (B) Myf-5 psm expression is slightly different in another embryo of the same stage (stage 13). As in A, Myf-5 expression is absent from the rostral psm (arrow). However, immediately caudal to this domain Myf-5 is expressed at relatively higher levels within a nearly somite-sized domain of the psm (asterisk). Over one somite-length caudal to this, Myf-5 is expressed in a decreasing gradient (bracket). Note that psm expression in B is similar to that in A only it is staggered caudally by about one somite-length. (C) Myf-5 expression in the psm is similar to the strong expression domain of C-Delta-1, a gene whose expression pattern is coordinated with somite formation. Arrow points to the Myf-5-negative segment of the most rostral psm. Arrowheads point to the cleavage furrow between S I and the psm. Embryos were bisected longitudinally through the neural tube and the halves hybridized with either C-Delta-1 or Myf-5 probes. Due to a short development time of C-Delta-1 in situ hybridizations, its strong expression domain is emphasized while the weaker domain in caudal S I is not visible. Rostral is up.

FIG. 5. Myf-5 and MyoD expression in S I–IV. (A) Strong Myf-5 expression changes from a caudal domain in S I to a medial domain in S IV. Myf-5 is also expressed just caudal to the most rostral psm (arrow), which is just starting to bud off as a new somite (asterisk marks developing cleavage furrow). Only strong Myf-5 expression domains in somites and psm are visible due to the shorter development time of this in situ. (B) MyoD is expressed in the medial somite of somites II+ in a stage 12 embryo. Rostral is up. Roman numerals denote somite stage.
test whether the changes observed in Myf-5 psm expression are similar to those described for C-Delta-1, embryos were bisected through the neural tube and each half was assayed via in situ hybridization for either Myf-5 or C-Delta-1 expression. Expression of C-Delta-1 and Myf-5 localized to equivalent regions of the psm (Fig. 4C) in all seven embryos examined. These results suggest that, like C-Delta-1, Myf-5 is expressed in a periodic fashion that is coordinated with somitogenesis.

A strong domain of Myf-5 expression shifts in somites I–IV from a caudal to medial domain. In addition to Myf-5’s reiterating expression pattern in the developing psm, there is also a dramatic shift in its expression pattern as S I matures to S IV. In S I, there is a region of strong Myf-5 expression along the caudal end. In S II and III, however, the strong expression domain shifts rostrally and medially (Fig. 5A) such that by S IV, the shift of the strong Myf-5 expression domain to the medial somite is complete. As previously described in avian embryos (Borycki et al., 1997), Myf-5 is also strongly expressed in the medial somite of S V and older (Fig. 2H). Furthermore, after extended development of in situ hybridizations, low-level Myf-5 expression is also detected throughout the somites (Figs. 4A and 4B).

The expression pattern of MyoD in the psm and S I–III differs greatly from that of Myf-5. Similar to previous findings (Borycki et al., 1997; Hacker and Guthrie, 1998), we detect the earliest MyoD expression in S IV of stage 10 embryos (data not shown). Thereafter, MyoD expression is initiated in S II or III of stage 11 and 12 embryos (Figs. 5B, 6A, and 8B) and in somite I or II of stage 13 embryos and older (data not shown). Expression in the caudalmost somite is often weaker than in older somites (data not shown). Unlike for Myf-5, we find that MyoD is always expressed in the medial somite and is never expressed in the psm or along the caudal border of the somite (compare Figs. 5A and 5B, Figs. 8A and 8B). Thus, despite similar expression patterns in S IV and older, MyoD and Myf-5 expressions in the psm and S I–III are quite different.

Myf-5 is expressed broadly in the psm and becomes progressively restricted to the myotome as the somite matures. Cross sections of embryos were examined to determine the dorsoventral distribution of Myf-5 expression in the paraxial mesoderm (psm and somites). Myf-5 expression becomes gradually restricted as the paraxial mesoderm matures from the caudal psm to myotome-containing somites. The caudal psm does not express Myf-5 (Fig. 7A), but the rostral psm caudal to the Myf-5-negative region (Fig. 7B) and epithelial somites (Fig. 7C) are stained throughout. As the ventral somite begins to de-epithelialize, it displays little to no Myf-5 expression, although the developing dermamyotome in the dorsal somite remains Myf-5 positive (Figs. 7D–7H). The cells closest to the neural tube in
the dermamyotome (Figs. 7E–7G) and the myotome (Fig. 7H) stain the most intensely for Myf-5. Notably, cells in the hypaxial (lateral) dermamyotome also express Myf-5 (Figs. 7G and 7H) with a stronger expression domain at the ventrolateral lip of thoracic level somites (Fig. 7H) (Fig. 8C for model). These results show that Myf-5 is transiently expressed in the ventral somite, a region that is not destined to contribute to the myogenic lineage but is instead fate mapped to become sclerotome. As the somite matures, Myf-5 is expressed exclusively in cells that will give rise to muscle.

In contrast, MyoD is expressed only in the dorsomedial epithelial somite, in what appears to be a subset of Myf-5-expressing cells (compare Fig. 6C with 7D and 7E). Later MyoD is expressed in the medial dermamyotome lip, just under the dermamyotome (data not shown), and in more mature somites it is expressed throughout the myotome (Figs. 6D and 8D). This demonstrates that MyoD expression is restricted to presumptive muscle and that it appears to be expressed in a small proportion of Myf-5-expressing cells.

**DISCUSSION**

The aim of this work was to investigate the localization of presomitic Myf-5 expression in the developing avian embryo and to determine its relevance to Myf-5 expression in muscle precursors of the somite. We observe a continuously changing pattern of Myf-5 expression that begins in the primitive streak and culminates in the myotome. This study reveals several heretofore unknown temporal and regional features of Myf-5 expression.

One surprising finding is that Myf-5 is expressed in the primitive streak of gastrula stage chick embryos (stage 3), demonstrating that the initial steps of myogenesis occur long before myotome formation and muscle differentiation.
in the somite. Prior to myotome formation, Myf-5 expression is not localized exclusively to presumptive muscle. At stage 4, Myf-5 mRNA is found in both presumptive paraxial and lateral plate mesoderm, since its expression resembles fate maps that depict where these tissues originate (Selleck and Stern, 1991; Schoenwolf et al., 1992; Tam et al., 2000). After stage 6, when the psm and somites first emerge, Myf-5 continues to be expressed in cells that are not destined to become muscle, including the ventral psm and epithelial somite, which are instead fated to give rise to sclerotome (Christ et al., 1992). It is only when the dermamyotome begins to develop that Myf-5 expression is downregulated in the ventral somite, and it is ultimately expressed at high levels and exclusively in the presumptive muscle of the myotome (Fig. 8C). In contrast to earlier reports (Tajbakhsh et al., 1992; Borycki et al., 1997; Daubas et al., 2000), we never detect Myf-5 expression in the neural tube or brain (data not shown), a finding that could be attributed to differences in experimental technique.

Although progressive changes in MyoD expression throughout development have not been well characterized, our description of Myf-5 expression is similar to recent reports of MyoD expression in stage 4 embryos and in regions of the embryo that are not fated to become muscle (Gerhart et al., 2000). Furthermore, our finding that rostral somites have approximately 10-fold more Myf-5 mRNA than the psm and I–IV is similar to the 15- to 30-fold difference found between MyoD mRNA levels in Xenopus embryos at developmental stages before and after somitogenesis (Steinbach et al., 1998). Taken together, these data demonstrate that Myf-5 and MyoD are both transiently expressed in nonmyogenic mesodermal lineages and undergo a gradual restriction to, and upregulation of, expression in presumptive muscle.

In the process of becoming restricted to the muscle lineage, the expression of Myf-5 undergoes complex changes in the psm. We demonstrate that Myf-5 psm expression colocalizes with C-Delta-1, a ligand for the Notch receptor that regulates somite segmentation (Irvine, 1999; Pourquier, 1999, for review). This result implies that like C-Delta-1, Myf-5 psm expression changes in concert with somitogenesis. It should be noted that the periodic expression pattern of Myf-5 is different than the waves of expression from the anterior-to-posterior psm displayed by Lunatic fringe (Forsberg et al., 1998) and c-hairy1/HES (Palmeirim et al., 1997; Holley et al., 2000; Jouve et al., 2000). Based on our observations of Myf-5 psm expression and on the known expression pattern for C-Delta-1, we propose a model whereby Myf-5 in the psm is expressed in a reiterating fashion that is coordinated with somitogenesis and that this results in its localization to the caudal end of each newly formed somite I (Fig. 8A).

In the epithelial somite, Myf-5 expression undergoes yet another change in position as the strongly expressing domain gradually rotates from the caudal edge of somite I to the medial edge of somite IV (Fig. 8A). In contrast, MyoD is continually expressed in a medial stripe adjacent to the axial tissues, the neural tube and notochord (Fig. 8B). Known signaling molecules that promote Myf-5 and MyoD expression emanate from the neural tube and notochord, i.e., Wnts and Shh (Borycki and Emerson, 2000, for review), IGF-II and insulin (Pirskanen et al., 2000), and FGF (Stern et al., 1997). The temporal and regional expression patterns of these signaling molecules together with batteries of signal transduction molecules and transcription factors, are thought to coordinate somite myogenesis (Murphy et al., 1994; Pourquie et al., 1996; Hiringer et al., 1997; Marcell et al., 1997; CaoDevila et al., 1998; Heanue et al., 1999). The more dynamic expression pattern of Myf-5 compared to MyoD suggests that Myf-5 gene expression may be responsive to a more complex array of environmental signals. This would be consistent with the much greater number of control regions that have been delineated within the Myf-5 gene (Summerbell et al., 2000).

An additional regulator of Myf-5 may be the Notch pathway. This is based on our finding that Myf-5 expression in the psm colocalizes with C-Delta-1, suggesting that Myf-5 expression in these regions may be responsive to the Notch pathway. Although Delta-1 regulates somite formation (Hrabe de Angelis et al., 1997), Myf-5 knockout mice do not have defects in somitogenesis (Braun et al., 1992; Kablar et al., 1997) and thus it is unclear why Myf-5 expression is coordinated with somite formation. Nevertheless, the Notch pathway can regulate myogenesis in vitro (Kopan et al., 1994; Shawber et al., 1996; Nofziger et al., 1999), suggesting that in the chick embryo Myf-5 may be responding to “myogenic” Delta-1 signals in the psm. In vivo, expression of the MRF myogenin is unaffected in mice lacking Notch1 (Conlon et al., 1995) or Delta-1 (Hrabe de Angelis et al., 1997), although it remains to be determined whether the Notch pathway affects Myf-5 expression.

It is surprising that a gene known to be involved in muscle development is expressed long before myotome formation in the somite and within broad nonmyogenic regions of the early embryo. While the levels of Myf-5 transcripts detected in the psm are about 10-fold lower than those in the myotomal regions of rostral somites, even weaker Myf-5 signals are detected in the nonmyogenic regions of somites I–IV (light blue stain in Fig. 8A). Due to uncertainties in whether Myf-5 protein is translated or is active in these tissues, the functional significance of these differences is not yet possible to evaluate. These reservations notwithstanding, might Myf-5 expression in a cell signify that it is “primed” to respond to subsequent myogenic cues even though the cell may not eventually be exposed to them? Indeed, evidence suggests that MRF expression denotes myogenic competence (i.e., the ability to become muscle) under the right conditions. For example cells in the anterior primitive streak of stage 3-5 embryos, which we show are Myf-5 positive, give rise to muscle when transplanted into chicken wing buds (Krenn et al., 1988). Other tissues in which we demonstrate Myf-5 expression, and that have recently been shown to express MyoD (Gerhart et al., 2000), namely the epiblast from stage 4 chick
FIG. 8. Schematic comparison of temporal and regional expression of Myf-5 and MyoD in the paraxial mesoderm in whole mount (A, B) and cross section (C, D) of a stage 12 chicken embryo. Only one half of the bilaterally symmetrical paraxial mesoderm is represented. (A) Changes in Myf-5 expression in the psm and somites I–IV over the course of one somite formation (90 min). (Left) At time 0, Myf-5 is expressed throughout somites I–IV. A strong domain of expression in the caudal portion of somite I rotates medially and rostrally in progressively more mature somites, such that by somite IV it lies along the medial somite. The rostral 2/3 of the psm expresses Myf-5 in

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embryos and the psm, can also differentiate as muscle when cultured in vitro (George-Weinstein et al., 1994; Stern and Hauschka, 1995; George-Weinstein et al., 1996). Furthermore, the Myf-5/MyoD-expressing ventral somite I (this paper and Gerhart et al., 2000), which is fated to become sclerotome, can become muscle if it is transplanted in place of the dorsal somite (Aoyama and Asamoto, 1988; Christ et al., 1992; Dockter and Ordahl 2000). These data are consistent with the idea that low level expression of Myf-5/MyoD confers myogenic competence but does not commit cells to the myogenic lineage.

Multiple studies also show that MRF expression does not always signify a cell’s commitment to myogenesis, suggesting that despite being primed, such cells have not received sufficient cues to induce myogenic differentiation. For example, Myf-5- and MyoD-expressing somites that are surgically separated from neural tube/notochord signals during development lose the expression of both genes and fail to differentiate as muscle in vivo (Borycki et al., 1998; Dietrich et al., 1998; Teillet et al., 1998; Marcelle et al., 1999). Dissected somites also lose MyoD/Myf-5 expression in vitro, unless they are exposed to signaling molecules that are thought to be myogenic inducers in vivo, such as Wnts, Shh, and insulin-like growth factors (Maroto et al., 1997; Reshef et al., 1998; Pirskanen et al., 2000). Taken together these data suggest that early Myf-5/MyoD expression is not sufficient to trigger muscle differentiation in the developing embryo, but that instead Myf-5/MyoD-expressing cells are competent to process inductive myogenic signals. This type of regulation has been demonstrated in other tissues as well. Mash1, a bHLH transcription factor that is required for autonomic neurogenesis, also plays a role in neuronal competence. Nonneuronal precursors expressing low levels of Mash1 are competent to become neurons, and furthermore a decrease in Mash1 expression parallels a decrease in neuronal competence (Lo et al., 1997).

In contrast to our observations in chicken embryos, we and others have not been able to detect Myf-5 expression prior to somitogenesis in mouse embryos by conventional in situ hybridization (data not shown) (Summerbell et al., 2000). This is despite the fact that Myf-5 expression has been detected in the psm by RT-PCR (Kopan et al., 1994) and via lacZ staining of mice with β-galactosidase knocked into the Myf-5 locus (Cossu et al., 1996). This suggests that some of the apparent differences in Myf-5 expression patterns reported for mouse and chick (Borycki et al., 1997; Hacker and Guthrie, 1998) may be a consequence of the techniques used for its detection. Using these more sensitive techniques, it remains to be determined whether Myf-5 is expressed prior to somitogenesis in mouse embryos.

Similarly, the apparent contrast between our observation of MyoD expression initiating in somite IV at stage 10 and the recent report of low level MyoD transcripts in the psm and stage 1 embryos is clearly due to the use of an even more sensitive detection method (Gerhart et al., 2000). It is not yet known whether Myf-5 transcripts can be detected earlier than stage 3 with even more sensitive techniques. The results of Gerhart et al. also suggest that the MyoD expression we detect at stage 10 represents an upregulation of expression. Furthermore, since the MyoD-expressing cells we observe in the dorsomedial somite seem to represent a subset of Myf-5-expressing cells (compare Figs. 8C and 8D), this implies that the MyoD-positive cells have progressed further along the myogenic program.

Our studies indicate that Myf-5 gene expression precedes overt myogenesis by many stages, occurs in some mesodermal tissues that are not destined to become skeletal muscle, and undergoes rapid temporal and regional changes associated with somite formation and maturation. We also show that the temporal and regional pattern of Myf-5 expression differs significantly from that of MyoD. How Myf-5 expression patterns are regulated and the function of these dynamic changes in myogenesis remain to be determined.
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