Integrins are cellular adhesion receptors that mediate signaling and play key roles in the development of multicellular organisms. However, their role in the cellular events leading to myotome formation is completely unknown. Here, we describe the expression patterns of the α1, α4, α5, α6, and α7 integrin subunits in the mouse myotome and correlate them with the expression of several differentiation markers. Our results indicate that these integrin subunits may be differentially involved in the various phases of myogenic determination and differentiation. A detailed characterization of the myogenic cell types expressing the α4 and α6 subunits showed a regionalization of the myotome and dermomyotome based on cell-adhesion properties. We conclude that α6β1 may be an early marker of epaxial myogenic progenitor cells. In contrast, α4β1 is up-regulated in the intercalated myotome after myocyte differentiation. Furthermore, α4β1 is expressed in the hypaxial dermomyotome and is maintained by early hypaxial myogenic progenitor cells colonizing the myotome. Developmental Dynamics 231:402–415, 2004.

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INTRODUCTION

The body wall muscles (e.g., abdominal, intercostal) and the deep back muscles originate from the myotomes, embryonic structures derived from the dermomyotome of the mesodermal somites (reviewed in Buckingham, 2001). The myotome can be subdivided into three regions defined either morphologically or by the expression of different markers (Spörle, 2001). These are the epaxial myotome, dorsal to the notochord; the intercalated or adaxial myotome, which is a subdivision of the epaxial domain and probably originates from the early epaxial cells; and the hypaxial myotome, which is last to develop and lies ventral to the notochord (Spörle, 2001).

Several models have been put forth to explain how cells leave the dermomyotome to invade the myotomal space and how the myotome grows. In 1910, L.W. Williams elaborated the first model for epaxial myotomal colonization that suggested that the dorsomedial lip of the dermomyotome was the origin of myogenic progenitor cells (MPCs). Recent studies propose several different pathways of cell translocation from the dermomyotome to the myotome, which suggest possible differences depending on the stage and species studied (Kaehn et al., 1988; Denetclaw et al., 1997; Kahane et al., 1998b; Venters et al., 1999; Eloy-Trinquet and Nicolas, 2002). Although a consensus has not been reached between these different models (reviewed in Hollway and Currie, 2003), they all include the concept that the myotome is formed by several populations of...
MPCs, which arise at different times and express specific factors.

A primary epaxial myotome is formed by MPCs expressing Myf-5 in the mouse or Myf-5 and MyoD in avian embryos. These MPCs subsequently differentiate and elongate to become dermis- and myosin-positive mononucleated myocytes (Kaehn et al., 1988; Ott et al., 1991; Williams and Ordahl, 1994; Denetclaw et al., 1997; Kahane et al., 1998b; Venters et al., 1999; Hirsinger et al., 2000). Several lines of evidence suggest that further growth of the myotome occurs by entry of a second cell population that elongates and differentiates into mononucleated myocytes between, or medial to, the primary myotomal cells, thus forming the secondary myotome (Kahane et al., 1998b; Venters et al., 1999; Eloy-Trinquet and Nicolas, 2002). Although much less is known about the development of the hypaxial myotome, it is commonly accepted that it arises as a mirror image of the epaxial myotome (Dietrich et al., 1998; Cinnamon et al., 1999; Spörlé, 2001). Finally, a third MPC population has been described in the quail. In contrast to the previous two, it inhabits the myotomal space in a proliferating state for some time, expressing FREK (FGFR4) but no myogenic regulatory factors (MRFs) before even-terminated differentiation and survival, depend on integrin-mediated adhesion and/or signaling. Several integrin subunits (α1, α3, α4, α5, α6, α7, α9, αv, β1, β3) are expressed during skeletal muscle development (reviewed in Gullberg et al., 1998). Of these, the roles of the fibronectin receptor α5β1 and the laminin receptor α7β1 are the best studied. α5β1 promotes proliferation and inhibits differentiation of myoblasts in vitro (Blaschuk and Holland, 1994; Boettiger et al., 1995; Sastry et al., 1996), whereas α7β1 is thought to promote the maturation and survival of myotubes (Song et al., 1992; Vachon et al., 1997).

Very few studies have addressed the role of integrins in myotome development. Although the expression of the β1 (Pow and Hendrickx, 1995), α1 (Duband et al., 1992), α4 (Stepp et al., 1994), α6 (Bronner-Fraser et al., 1992; Thorsteinsdóttir et al., 1995; Pow and Hendrickx, 1995), and α7 (Velling et al., 1996) integrin subunits have been described in the myotome, it is not clear whether their expression patterns vary with the differentiation state of the myotomal cells and/or among the different regions of the myotome.

We recently mapped integrin expression patterns in limb MPCs in the mouse (Bajanca and Thorsteinsdóttir, 2002). We showed that limb MPCs up- and down-regulate several integrin subunits during delamination from the dermatomyotome, migration, and early differentiation in the limb muscle masses, suggesting a dynamic regulation of cell-ECM interactions during early skeletal muscle development in vivo. In the present study, we aimed to identify the integrins expressed during the earliest phases of myotome development in the mouse. Our results show that the laminin receptor α6β1 is present on early epaxial MPCs, and we suggest it is a marker for the early Myf-5–positive epaxial lineage. In contrast, the fibronectin receptors α4β1 and α5β1 are only up-regulated in these cells after their differentiation. When hypaxial myotome formation starts, this domain becomes enriched in α4β1, suggesting this integrin may be a marker for undifferentiated cells in the hypaxial lineage. The other laminin receptors, α1β1 and α7β1, were only detected in later stage (from E10.5) myotomes. Our data show important differences in the cell adhesion properties between the epaxial and hypaxial domains of the mouse myotome, as well as between the different stages of myotome development.

RESULTS

Early (Primary) Myotome Formation

We analyzed the expression of α1, α4, α5, and α6 mRNA and α7 protein in embryos at a stage where the primary myotome is starting to form. Over the period from embryonic day (E) 8.5 to E9.0 only α6 was detected in the somites (Fig. 1B), whereas α4 and α5 were expressed in other tissues. α4 was found in the head and branchial arches as described previously (Pinco et al., 2001), and α5 was widespread in all mesenchymal tissues (not shown). Neither α1 nor α7 were detected in the embryo at this stage (not shown). The α1 expression pattern has not been described previously for these stages in the mouse, but the absence of α7 agrees with the detailed study of Velling et al. (1996) who found α7 mRNA expression starting at E10.0/E10.5.

The α6 integrin subunit is present very early in development (Hierck et al., 1993; Thorsteinsdóttir et al., 1995), and until E9.5, it is most strongly expressed in the paraxial mesoderm, heart, headfolds/head, and presumptive limb region (Fig. 1B,D). In the paraxial mesoderm, a faint signal was detected in the rostralmost region of the presomitic mesoderm (PSM), becoming slightly stronger in epithelial somites (not shown, see Fig. 2 for protein localization). Of interest, in E8.5 embryos α6 expression...
Fig. 1. mRNA expression of α1, α4, α5, and α6 integrins in embryonic day (E) 8.5–E10.5 embryos. 

A–B: Late E8.5. Myf-5 (A) and α6 (B) exhibit similar expression patterns in the early myotome (arrows), but α6 is also faintly expressed in epithelial somites. 

C–H: Early E9.0. Myotomal Myf-5 (C) and α6 (D) expression remains very similar, but α6 is also expressed in the overlying dermomyotome (arrows in H) and epithelial somites. 

α-cardiac actin expression reveals elongated myocytes at the forelimb level, starting in somite XIII (arrows in E), a level where α4 mRNA is first observed, in somites XI to XV (arrows in F, blue line in G). 

I–P: Late E9.5. Expression pattern of Myf-5 (I,M), α5 (J,N,P), α4 (K), and MLC1A (L,O). α5 is very strong in the presomitic mesoderm and first epithelial somite (yellow arrow in J, compare with Myf-5 in I). Note narrow, striped myotomal pattern for α5 starting approximately in somite XII (arrows in J,N,P; longitudinal section in N, compare with M; forelimb level in P, compare with O). α4 mRNA is present more caudally (somite IV, black arrow in K) than MLC1A (somite XII, black arrow in L), and anteriorly. It is no longer restricted to elongated cells (compare with I and L). 

Q–W: E10.5. α5 expression remains in an elongated, thin stripe (arrows in Q,S); α4 mRNA (T) is now evident in caudal somites (black arrows), in interlimb level hypaxial myotomes (arrow-heads), in intercalated myotome, and, rostrally, in epaxial dermomyotomal lips (white arrows, see transverse section in R); α6 (U) is down-regulated rostrally (arrows), but α1 (V, transverse section; W, longitudinal section) is strongly expressed in the whole myotome (arrows). Yellow arrows indicate the first clearly epithelial somite.
in the PSM or epithelial somites was never detected, and dermomyotomes are faintly labeled. The dermomyotome expression increases in intensity from E8.5 to E9.5 (Fig. 1B,D). From somite V onward, α6 expression remains in the dermomyotome, is down-regulated in the sclerotome (Fig. 1H), and is strongly present in the nascent myotome (Fig. 1B,D,H). Comparing posterior, immature somites to progressively more rostral and mature somites shows that myotomal α6 expression is first detected in the dorsomedial region of the somite, then also in the mediorostral region and later the expression domain progressively expands ventrolaterally (Fig. 1B,D). Myf-5 is the earliest marker for MPCs in the mouse myotome and is also weakly expressed in the presomitic mesoderm and epithelial somites (Ott et al., 1991; Buckingham et al., 1992; Kiefer and Hauschka, 2001). Its expression pattern in the myotome is very similar to that of α6 mRNA (compare Figs. 1A and B; C and D). The results obtained thus suggest that α6β1 is the first and probably the only integrin present on the earliest myotomal MPCs.

**Differentiation of Epaxial Myogenic Cells Into Elongated Myocytes**

After invasion of the myotomal space, myogenic cells elongate rostrocaudally in the mouse, giving rise to differentiated mononucleated myocytes (Venters et al., 1999). α-cardiac actin (Fig. 1E) is the earliest marker for myocytes, appearing as early as E8.5 in the mouse (Buckingham et al., 1992), but myocytes soon also express MLC1A (Fig. 1L; Buckingham et al., 1992). The expression of α6 mRNA remained strong along the whole extent of the myotome at this stage, always very similar to the Myf-5 pattern (compare Fig. 1D with 1C).

From early E9.5, mRNAs for the α4 (Fig. 1F) and α5 (not shown) integrin subunits were also detected in the central region of rostral myotomes (“central” meaning the middle on a medial to lateral axis). The α4 subunit (Fig. 1F) occurred in a striped pattern, similar to that of α-cardiac actin (Fig. 1E). Moreover, the dorsomedial-most region of the myotome was negative for α4 expression, an observation confirmed in sections of these embryos (Fig. 1G), suggesting an up-regulation in elongated myocytes. This finding was in contrast to the α6 mRNA expression pattern, which was widespread in the myotome at this stage (Fig. 1H). α1 was still not clearly detectable at E9.5 (not shown). Thus, we conclude that differentiating epaxial myocytes up-regulate the α4 and α5 integrin subunits and that α6 expression is maintained throughout the myotome.

**Myotomal Growth**

As the myotomes grow from late E9.5 onward, integrin expression patterns become more dynamic. Expression of α6 mRNA is now clearly present in all myotomes, forming a streak along the mediolateral axis (arrows in Fig. 1J,N,P,Q,S). This unusual mRNA distribution pattern, restricted to a medial to lateral midline in the myotome is similar to the mRNA expression pattern described for slow myosins before myocyte innervation (Sacks et al., 2003). α4 mRNA expression is now evident in much younger somites than before and expression is concentrated in the hypaxial region of these somites (black arrows in Fig. 1K,T). Because expression of the myocyte marker, MLC1A, is only detected more rostrally (Fig. 1L), this finding indicates that α4 is expressed in less differentiated cells than observed at the previous stage. Rostrally, α4 expression is also very strong in the dorsomedial lip of the dermomyotome (Fig. 1R,T).

Expression of α6 mRNA remains strong along the whole extent of the growing myotomes until approximately E10.5, when it starts being down-regulated in rostral myotomes (arrows in Fig. 1U). At approximately E10.5, expression of the α1 integrin subunit is clearly detected in the myotome (Fig. 1V,W), at a time that α7 is also up-regulated (not shown; Velling et al., 1996). This up-regulation of two new laminin receptors and the down-regulation of the early α6-containing laminin-receptor suggest that the myotomes are entering a new myogenic phase.

**α6β1 Protein Is Expressed by Epaxial Myf-5–Positive MPCs and Remains Expressed Through Their Differentiation Into Myocytes**

The similarity between the mRNA expression patterns of α6 and Myf-5 in the early epaxial myotome suggested that this integrin subunit could be a feature of Myf-5–expressing myogenic cells. However, α6 mRNA appeared to be more widely expressed than Myf-5 in epithelial somites and dermomyotomes. By using immunohistochemistry (IHC), we analyzed the distribution of the α6 protein in the paraxial mesoderm, concentrating on early myotomal cells. α6 protein was found to follow the mRNA expression pattern described above for epithelial somites, dermomyotome, and myotomal cells. It was not detected on PSM cells but was concentrated on the basal surface of the cells forming the cleft separating the first epithelial somite from the PSM (Fig. 2A), where its ligand, laminin, was also found (Fig. 2B). α6 is also present on the surface of cells in epithelial somites (Fig. 2C). In 15-somite embryos, α6 is present in the whole dermomyotome but immunoreactivity is clearly stronger in the myotome (Fig. 2D), although at later stages, the dermomyotomal α6 immunoreactivity increases (Fig. 2E).

Double IHC for α6 and Myf-5 showed that all Myf-5–positive cells in the dorsomedial lip of the dermomyotome and in the myotome express α6 (Fig. 2D). This result confirms that α6 is present very early in myotomal myogenesis.

Because the α6 mRNA pattern suggested that this integrin subunit is not restricted to young MPCs, we determined whether it remains present as they differentiate into elongated myocytes. Double IHC for α6 and the MRF myogenin, which comes up later than Myf-5 (Smith et al., 1993), or myosin, which is specific for differentiating myocytes and myofibers (Venters et al., 1999), confirms that α6 is not lost immediately after differentiation of myotomal cells (Fig. 2F–J). Cell dissociation experiments performed on interlimb
level somites from E9.5 embryos confirmed that elongated, differentiated myocytes express α6 (Fig. 2G–J).

In agreement with the in situ hybridization data, α6 immunoreactivity starts to decrease at rostral levels from E10.5 onward. Most of the myogenin-expressing cells either no longer express α6 or the level of immunoreactivity is faint (compare Fig. 2K with 2L).

α6 dimerizes with either the β1 or the β4 integrin subunit (Sonnenberg et al., 1990). To identify the particular α6-containing integrin present in the early myotome, we exposed sections of E9.5 embryos to antibodies against β1 and β4 (Fig. 2M,N). β1 is strongly present in myotomal cells, while β4 was absent, confirming that E9.5 myotomal cells express the laminin receptor α6β1 on their surface. Immunoreactivity of laminin is seen lining the myotome medially at this stage, and some punctate staining is present within the forming myotome (Fig. 2O). Thus, laminin is present in close proximity to the α6-positive cells.

Taken together, our results show that α6β1 is expressed very early in myogenesis. It is strongly expressed on early epaxial MPCs, remaining in myocytes while they differentiate, but is then gradually down-regulated.

MyoD-Positive Cells Express Different Laminin Receptors

MyoD starts being expressed in rostral myotomes at E10.5 in the mouse (Buckingham et al., 1992; Sassoon, 1993). Because α6 expression decreases at the same time, we hypothesized that α6 could be down-regulated in myogenic cells as they start expressing MyoD. Double IHC for α6 and MyoD at E11.5 shows that the majority of MyoD-positive cells do not express this integrin subunit (Fig. 3A,B). This absence of α6 was recorded on young MyoD-positive cells located either epaxially (Fig. 3A) or hypaxially (arrow in Fig. 3B) as well as along the dorsoventral extent of the mature myotome (Fig. 3B). In some cases, both proteins were present in the same region (Fig. 3C). This region corresponds to a small area along the anterior-posterior axis where MyoD has already been up-regulated in the somites but α6 has not yet been down-regulated. However, due to the density of cells, it was not possible to distinguish whether the two proteins were coexpressed in the same cells. It is possible, thus, that a subpopulation of MyoD-positive cells in the central myotome maintains α6 expression.

Interestingly, the largest MyoD-positive, α6-negative area is the ventrolateral, or hypaxial, region of the myotomes (see arrow in Fig. 3B). This observation led us to investigate whether the α6 protein was reduced or absent in the hypaxial side of myotomes. An analysis of α6 expression at interlimb (trunk) levels in E11.5 embryos reveals strong expression in the intercalated myotome but low or almost undetectable expression levels in the hypaxial region (Fig. 3D–F). Together, our results suggest that α6 is not expressed by the MyoD-positive epaxial MPCs and that its expression in the hypaxial myotome is either transient or absent.

MyoD and myogenin are reported to activate expression of the α7 integrin subunit in C2C12 skeletal myoblasts (Ziobor and Kramer, 1996). α7 is not present in the mouse myotome when MyoD starts being expressed (see above), but its up-regulation coincides in time with the appearance of MyoD (Velling et al., 1996). Thus, we used double IHC for MyoD and the α7 subunit to determine whether these are expressed by the same cells in vivo. In trunk-level somites of E10.5 embryos, some myotomal cells only express MyoD (Fig. 3G). However, in more rostral somites, α7 was detected epaxially in elongated mononucleated myocytes with MyoD-positive nuclei (Fig. 3H). In the mature myotomes of

![Fig. 2](image-url)

**Fig. 2.** α6β1 is present in epithelial somites and then becomes strongly expressed in all cells of the early epaxial myotome. A,B: Longitudinal section of embryonic day (E) 11.5 embryo tail bud showing α6 protein in the cleft separating the last formed somite and the presumptive mesoderm (arrows in A) and laminin (B) assembled around tail bud and somites. C: Sagittal confocal section shows α6 protein expression in an epithelial somite of an E11.5 embryo. D: Transverse section of a 15 somite embryo shows that all the Myf-5-positive (red) cells in the myotome are α6-positive (green); note that, at this stage, the myotome shows a stronger immunoreactivity for α6 than the dermomyotome. E: Caudal section of an E11.5 embryo labeled for Pax-3 (red) and α6 (green) shows that α6 expression is strong in the dermomyotome (arrows) at this stage. F: Transverse confocal section of an E9.5 embryo shows that α6 protein (green) is present on myogenin-positive (red) cells in the myotome. G–J: Dissociation of trunk somites of an E9.5 embryo confirms the presence of elongated, mononucleated cells that express both α6 (green) and myogenin (red). Nuclei are visualized with 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) staining (blue). K,L: Sagittal sections of an E11.5 embryo showing trunk (K) and rostral (L) myotomes labeled with myogenin (red) and α6 (green). Note the weaker, almost absent, immunoreactivity of α6 in rostral myotomes. M–O: Transverse sections of an E9.5 embryo show the presence of the β1 integrin subunit on myotomal cells (arrows in M), whereas the β4 subunit is not expressed in the myotome (arrows in N). At this stage, laminin lines the myotome medially (arrows in O) and some punctate staining is present within the forming myotome. bv, blood vessels; dm, dorsomedial; vl, ventrolateral. Scale bars = 100 μm in A–F, J (applies to G–J), K–O.

![Fig. 3](image-url)

**Fig. 3.** Expression of laminin receptors by MyoD-positive cells and in the hypaxial myotomes. A–C: Sagittal sections of an embryonic day (E) 11.5 embryo show that most MyoD-expressing cells (red) are negative for α6 (green), both in young epaxial cells near the dorsomedial lip (A) and in rostral mature myotomes (B). Note that the hypaxial domain is clearly α6-negative (arrow in B). Some MyoD-positive nuclei, however, are detected in trunk myotomes in areas containing α6-positive elongated myocytes (C). D–F: Sagittal section of an E11.5 embryo at interlimb (trunk) level showing faint immunoreactivity for α6 (D, green in F) in the ventrolateral (hypaxial) portion of the myotome, whereas the whole myotome stains for myogenin (E, red in F). G–I: Immunohistochemistry for α7 (green) and MyoD (red) shows that α7 is specific for MyoD-positive myocytes and myotubes. G: Myoid is clearly expressed in trunk-level myotomes of E11.0 embryos, before α7 starts being expressed on those cells. H: α7 first appears in elongated, MyoD-positive cells epaxially. Sagittal section of mature E11.5 myotomes shows extensive α7 immunoreactivity on elongated myocytes, which always stain for MyoD. I: Binucleated MyoD- and α7-positive cells (arrows) are also detected at this stage. dm, dorsomedial; drg, dorsal root ganglia; vl, ventrolateral. Scale bars = 100 μm in A–C, F (applies to D–F), G–I.
Fig. 2.

Fig. 3.
E11.5 embryos, α7 was widely expressed in myocytes with MyoD-positive nuclei (Fig. 3I). We never detected MPCs expressing α7, suggesting that it is up-regulated in differentiated, elongated cells. In E11.5 embryos, some binucleated cells express both α7 and MyoD (arrows in Fig. 3I). Our results suggest that α7β1, together with α1β1 (see above), are the main laminin receptors present in late myotome development, when α5β1 is being down-regulated.

α4 Labels the Hapaxial MPCs and Central Elongated Myocytes

The in situ hybridization analysis above showed that the α4 integrin subunit has a dynamic expression pattern in the myotome, appearing first in elongated myocytes as the first myosin transcripts are detected, but by late E9.5, α4 appears more caudally than the earliest myocyte markers, suggesting an earlier expression in MPCs. By using IHC, we further characterized the myogenic cells that express α4. In E10.0 embryos, the most posterior dermomyotomes showed intense reactivity for α4 (Fig. 4A). The first myogenic cells expressing α4 were present in a central position in young myotomes, intercalated with nonexpressing cells (arrows in Fig. 4B). In more rostral somites of E10.0 embryos, α4 is also present in the dermomyotomal lips, first appearing in the ventrolateral and then in the dorsomedial lip (Fig. 4C,D). As the myotomes grow and differentiate, α4 is present more widely but it is always negative in the dorsomedial myotome (Fig. 4B,D,F). In a confocal analysis of caudal myotomes of an E11.5 embryo, it is clear that α4 is enriched in elongated myocytes (Fig. 4E). In addition, the ventrolateral lip of the dermomyotome shows immunoreactivity for α4, and surprisingly, MPCs in the hypaxial myotome are also α4-positive (Fig. 4E). These observations suggest that the α4 integrin subunit has two distinct patterns of expression in the myotome. First, it is up-regulated in elongated myocytes, and second, it is expressed by hypaxial MPCs.

To characterize further these two domains of α4 expression, we performed double IHC for α4 and the MRFs Myf-5, MyoD, and myogenin. Double IHC for Myf-5 and α4 in caudal myotomes at E11.5 shows that these two proteins are expressed together in most, but not all, cells in the central myotome, and only Myf-5 is present epaxially (Fig. 4F). At this same stage, myogenin and α4 are coexpressed in the central myotome (Fig. 4G), suggesting that all differentiated cells are α4-positive. With the markers used, we could not confidently detect undifferentiated cells in the myotome expressing α4 at this stage. However, on the hypaxial side, α4 immunoreactivity extends further ventrally than the myogenin-positive nuclei (data not shown). At E11.5, α4 is absent in a subset of MyoD-positive myogenic cells located dorsomedially, but it is present in the cells whose MyoD-positive nuclei are aligned centrally in the myotome (Fig. 4H). However, due to the density of myocytes in this area of the myotome, it is not clear whether α4 is present in all or just in some of these MyoD-positive cells.

The α4 expression domain in the differentiated areas of the myotome was analyzed in detail. In E11.5 embryos, an extensive number of cells in the myotome are already differentiated, fully elongated, and express myosin (Fig. 5A). Myosin-positive cells are also α4-positive; however, some cells that are myosin-negative express α4. These latter cells are detectable in the less mature, caudal myotomes in a medial position relative to the myosin-positive myocytes and near the myotomal basal lamina, (arrows in Fig. 5A). This pattern shows that α4 is up-regulated before full elongation of these cells.

Some elongated α4-expressing cells show an enrichment of α4 immunoreactivity on their tips (Fig. 5B,C). This strong α4 immunoreactivity is distal to the myosin labeling and reveals cell processes that penetrate between the cells of the dermomyotome and come close to the tips of elongated myocytes from adjacent somites. This finding suggests that α4β1 could play a role in the elongation and/or attachment of fully elongated myotomal cells. The α4 subunit must be dimerizing with β1 in the myotome, because its other possible partner, β7, is specific for hematopoietic cells (Postigo et al., 1993).

We analyzed the distribution pattern of the αβ1 ligands fibronectin and VCAM-1 in the myotome. Fibronectin is faintly expressed between the myotome and the sclerotome and is strongly expressed between adjacent somites (Fig. 5D), providing a potential extracellular support for the attachment of the α4-positive elongated cells. VCAM-1 immunoreactivity is present in the mesenchyme surrounding the somites and in the sclerotome (Fig. 5E). Therefore, α4-positive myotomal cells present near the sclerotomal border could be attaching to VCAM-1-positive cells in the sclerotome. Some cells already expressing myosin are located in a close proximity with the VCAM-1-positive sclerotomal cells (Fig. 5F). Taken together, these results point to different roles for α4β1-mediated adhesion in elongated myotomes of adjacent segments in embryonic day (E) 11.5 embryos make contact with each other through α4-containing structures: longitudinal sections (A–E) and sagittal section (F). A: Myosin-positive (red) myocytes of the intercalated myotome extend from rostral to caudal dermomyotome lips and express α4 (green). Note that some α4-positive cells do not express myosin (arrows), indicating that they are younger and less differentiated. B,C: Higher amplification showing myosin (red in B), α4 (green in B,C), and VCAM-1 (green in B,C). D: In some myotomal cells, VCAM-1 expression is colocalized with α4 (green) in those areas (arrows). E: VCAM-1 is present in the menenchymal cells surrounding the dermomyotome (arrowheads) and in the anterior portion of the sclerotome that is closest to the myotome (arrows) but is absent between segments (asterisks). F: Sagittal section through the most ventromedial part of the myotome, shows that the myosin-positive (red) cells of each myotome are in close approximation to VCAM-positive (green) cells in anterior sclerotome. dm, dorsomedial; vl, ventrolateral. Scale bars = 100 μm in A–F.
Fig. 4. α4 is dynamically expressed in the myotome and dermomyotome. A–D: Transverse sections of embryonic day (E) 10.0 embryo showing different axial levels. B–D: Double immunohistochemistry (IHC) for α4 (green) and fibronectin (red). A: Caudally, dermomyotome stains strongly for α4, but myotome is negative. B: At the hindlimb level, some cells in the central myotome express α4 (arrows), but these are intercalated with nonexpressing cells. C: At forelimb level, α4 is strongly expressed in the intercalated myotome (arrows) and hypaxially (arrowheads). D: Further rostrally, the epaxial lip (large arrow), intercalated myotome (arrows) and hypaxial region (arrowhead) are α4-positive. Note that fibronectin lines the myotome medially (B–D) and α4 is absent dorsomedially (asterisks, B–D). E: Confocal optical section of E10.5 embryo shows strong α4 expression in intercalated myotome (between white arrows) and on cells in hypaxial myotome (arrowheads) and dermomyotomal lip (yellow arrow), but α4 is absent between these two domains (asterisks). F–H: Double IHC for α4 and differentiation markers. F,G: Transverse adjacent sections of an E11.5 embryo through a caudal somite shows that Myf5-positive cells near the epaxial lip are α4-negative (arrow in F) and α4 is expressed by myogenin-positive cells (G). H: Sagittal section of E11.5 embryo shows absence of α4 in epaxial MyoD-positive cells (asterisks), but its presence on cells whose MyoD-positive nuclei are aligned along the myotomal midline (arrows). dm, dorsomedial; vl, ventrolateral. Scale bars – 100 μm in A–H.

Fig. 5.
myocytes and in the early hypaxial lineage.

**DISCUSSION**

We have shown that the α1, α4, α5, α6, and α7 integrin subunits have very specific expression patterns during the formation of the mouse myotome, suggesting myogenic cells alter their integrin expression repertoire during their differentiation into myotomal myocytes. We further show that clear regional differences exist in the cell adhesion properties of myotomal cells along the medial to lateral axis in that the αβ1 integrin is expressed by all epaxial cells, while the α4β1 integrin is expressed hypaxially as well as in the intercalated myotome. These regional differences in αβ1 and α4β1 expression are schematically represented in Figure 6.

**Laminin Receptor α6β1 in Epaxial Myotome Formation**

α6β1 was the only integrin subunit detected on early epaxial MPCs, and it is present on all cells that express the MRF Myf-5. The α6 subunit is also detected in the dermomyotome, but staining is much stronger in the Myf-5-positive myotomal cells, both at the mRNA and protein level, suggesting that α6β1 could play a role in events occurring as the MPCs colonize the myotome (see Fig. 6).

In vitro studies have shown that α6β1 is able to inhibit proliferation and stimulate differentiation of myoblasts (Sastry et al., 1996, 1999) and laminin promotes myoblast differentiation in vitro, inducing them to become postmitotic, fusion-capable, and myosin- and desmin-positive (von der Mark and Ocalan, 1989). In fact, it has been demonstrated recently that the ECM is necessary for skeletal muscle differentiation, independently of MRF expression, suggesting that, upon binding to the ECM, integrins generate signals that drive skeletal muscle differentiation (Osses and Brandan, 2002).

A laminin-containing basement membrane lines the myotome, both in avian and mouse embryos (Leivo et al., 1980; Krotoski et al., 1986; this study) and, early myotomal MPCs become postmitotic as they enter the myotomal space, where they soon differentiate into desmin- and myosin-positive myocytes (Kahane et al., 1998b; Venters et al., 1999). Thus, the increased α6 expression observed in early MPCs suggests that the α6β1 integrin could play a role in epaxial MPC differentiation.

It has been proposed recently that α6β1 may mediate the assembly of laminin-1 into a matrix (Henry et al., 2001). This study showed that, although laminin-1 bound to mouse embryonic stem (ES) cells by means of dystroglycan, an organized matrix

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**Fig. 6.** Summary of α6β1- and α4β1-expressing cell dynamics during dermomyotome and myotome development (embryonic day [E] 8.5–E10.5). Left panels shows dermomyotomal expression patterns in whole-mounts (without the underlying myotomal patterns), whereas the right panels only demonstrates representative myotomal patterns. The presence of α6β1 is represented in green and of α4β1 in blue; nuclei expressing one or more myogenic regulatory factors are red. A: At E8.5, α6β1 is faintly expressed in epithelial somites and dermomyotomes (left). It becomes strongly expressed in early epaxial myogenic progenitor cells (MPCs) and expression is maintained during myocyte differentiation (right). B: At E9.5, down-regulation of α6β1 starts hypaxially in limb level dermomyotomes and α4β1 is first detected in caudal dermomyotomes (left). Some epaxial myocytes up-regulate α4β1 at late E9.0, and soon after, all myocytes in the adaxial (intercalated) myotome express α4β1 (right). C: At E10.5, both α6β1 and α4β1 are present in caudal dermomyotomes. At interlimb levels, α4β1 is expressed in the hypaxial dermomyotomal lip and rostrally in the epaxial dermomyotomal lip. Dermomyotomal α6β1 expression has diminished rostrally (left), α4β1 is strongly expressed in epaxial myocytes, while α6β1 expression is becoming reduced. Hypaxial myotome formation has started, and α4β1 is expressed on hypaxial MPCs, whereas α6β1 is weak or absent (light green). For schematic simplicity, α6β1- and α4β1-positive myocytes are shown as separate cells. DM, dermomyotome; ECM, extracellular matrix; NT, neural tube.
Specific Adhesion Properties of the Intercalated Myotome

The $\alpha 4$ subunit, in contrast with $\alpha 6$, is not present during early epaxial myotome formation, as evidenced by its timing of appearance and by the consistent absence of $\alpha 4$-positive cells in the dorsomedial myotome. However, $\alpha 4$ is up-regulated in the fully extended cells in the central myotome (see Fig. 6). An extensive work by Spörrle (2001) on the characterization of different regions of the somite, defines an intercalated (adaxial) region in the myotome. It is hypothesized that this region is composed of early epaxial myotomal cells, which arise dorsomedially and due to further growth of the myotome become intercalated between the more recently formed dorsal and the hypaxial myotome regions (Spörrle, 2001). Early expression of Fzd9 and Cx40, markers for differentiated myocytes, is initially restricted to the intercalated myotome and only later found in the dorsal-most epaxial and hypaxial myotome regions (Spörrle, 2001). Thus, $\alpha 4$ seems to be specifically up-regulated in the intercalated (early epaxial, then adaxial) cells as they differentiate (see Fig. 6).

The up-regulation of $\alpha 4$ integrin in differentiating myocytes suggests a role in the process of elongation and/or in the attachment of fully elongated cells. In fact, the strong $\alpha 4$ immunoreactivity at the tips of myocytes and the formation of plaques between somites is consistent with a role in cell attachment. The $\alpha 7\beta 1$ receptor mediates cell–ECM interactions by binding to fibronectin (Wayner et al., 1989) and cell–cell interactions by interacting with VCAM-1 on adjacent cells (Rosen et al., 1992). Our results show that $\alpha 4$-positive myocytes may bind to the fibronectin matrix surrounding the myotome, which is enriched at intersomitic borders. However, it is also possible that these myocytes interact with VCAM-1-positive cells in the mesenchyme around the somite.

Although the exact function of $\alpha 4$ in the intercalated myocytes remains unknown, our results demonstrate a developmental change in the adhesive properties of early epaxial MPCs. They first express a laminin receptor, $\alpha 6\beta 1$, and then, as they differentiate into myocytes, i.e., in a phase of terminal differentiation. This observation shows clear differences between hypaxial and epaxial trunk myogenesis in relation to cell adhesion properties (see Fig. 6).

Hypaxial Myotome Is Not a Mirror Image of the Epaxial Myotome in Relation to Integrin Expression Patterns

We show that the integrin subunit $\alpha 4$ can be used as a marker for hypaxial MPCs while $\alpha 6$ is only transiently and faintly expressed here. It was surprising to find that $\alpha 4$ was present in hypaxial MPCs when in the epaxial myotome it is up-regulated only in elongated myocytes, i.e., in a phase of terminal differentiation. This observation shows clear differences between hypaxial and epaxial trunk myogenesis in relation to cell adhesion properties (see Fig. 6).

The medial and lateral regions of the dermomyotome and myotome receive distinctly different signals for myogenic induction. Shh and Wnt-1 produced from axial structures act as medializing factors and lead to initial specification of epaxial muscle. In contrast, the formation of hypaxial muscles is induced by a combination of signals from ectoderm (Wnt-7a) and lateral plate mesoderm (BMP-4; reviewed in Currie and Ingham, 1998). Additionally, different roles have also been assigned for Myf-5 and MyoD in epaxial and hypaxial myogenesis (Kablar et al., 1997, 1998; Tajbakhsh et al., 1998; Ta-
jbakhsh and Buckingham, 2000). This raises the interesting possibility that different regulators of myogenesis could trigger different effectors, such as the integrins.

Although it is not clear what specific role αβ1 plays in the hypaxial domain, it has been shown to play an important antiapoptotic role in migrating neural crest cells in vivo and in vitro (Haack and Hynes, 2001), as well as in other cell types (Koopman et al., 1994; Garcia-Gilia et al., 2002). Hypaxial MPCs are maintained in an undifferentiated state by BMP4-mediated signals secreted from the lateral plate mesoderm (reviewed in Kablar and Rudnicki, 2000). However, BMP-4 is also a potent inducer of apoptosis (e.g., Schmidt et al., 1998), and it has also been suggested that the dermo-/myotome exhibits a gradient of sensitivity to BMP-4-induced apoptosis from highest epaxially and lowest in the hypaxial domain (Schmidt et al., 1998). In the Pax-3 mutant Splotch, hypaxial MPCs fail to develop properly in vitro (Haack and Hynes, 2001), as well as in different phases of differentiation, including neural crest cells in vivo and in vitro (Haack and Hynes, 2001).

The expression pattern of the αβ1 and αβ1 integrins raises the question of whether they could play a role in promoting the specification and/or separation of the early epaxial and hypaxial lineages. A study of mouse LaacZ/LacZ chimeras demonstrated a clonal separation and regionalization of the medial and lateral lineages of MPCs (Eloy-Trinquet and Nicolas, 2002). The authors suggest that the acquisition of different adhesive properties between the epaxial and hypaxial cells preparing to translocate to the myotome could prevent cell mixing among the cells of the two clonal domains or allow a separation of the epaxial and hypaxial cells through cell sorting. Our results show that the laminin-receptor αβ1 and the fibronectin/VCAM-1 receptor αβ1 are potential candidates for a role in separating epaxial and hypaxial MPCs during early myotome formation in the mouse.

**EXPERIMENTAL PROCEDURES**

**Embryo Collection**

Pregnant females (Charles River CD-1; Harlan Interfauna Iberica, SA) were killed by cervical dislocation and embryos removed from the uterus in cold phosphate buffered saline (PBS). The day of the vaginal plug was designated E0.5. Precise staging of embryos was obtained by counting the number of somites as previously described by Poupin et al. (1995). The most recently formed somite was identified as somite I (Ordahl, 1993).

**In Situ Hybridization**

The mRNA expression patterns of the integrin subunits α1, α4 (probes kindly provided by H. Gardner, J.T. Yang, and R.O. Hynes, respectively) and α6 (Thorsteinsdottir et al., 1999) were determined in whole-mount embryos from E9.0 to E10.5. To help interpret the patterns obtained, we used probes marking myogenic cells in different stages of differentiation, namely Myf-5, MLC1A, and α-cardiac actin (probes kindly provided by M. Buckingham). The transcription factor Myf-5 is the earliest MRF expressed by myotomal cells, and the cytoskeletal proteins MLC1A and α-cardiac actin are specific of differentiated, elongated myocytes (Smith et al., 1993; Buckingham et al., 1992; Kitzmuller et al., 1998).

In situ hybridization (ISH) with digoxigenin-labeled riboprobes was performed essentially as described by Hentique et al. (1995). Embryos processed for whole-mount ISH were fixed in 4% paraformaldehyde (PFA) in PBS, dehydrated in methanol, and stored at −20°C. For ISH on sections, embryos were fixed in 4% PFA and frozen for sectioning. Whole-mounts were hybridized at 55°C and cryostat sections at 65°C. Probe localization was detected with BM Purple (Roche). For a more complete analysis of the distribution pattern of integrin-positive myogenic cells within the myotomes, some whole-mounts were embedded in hydroxyethyl methacrylate (Kulzer Histo-Technik 8100) and sectioned.

**Immunohistochemistry**

Primary antibodies used were rat anti-α6 (GoH3; Serotec), rat anti-α7 (CA5; a gift from A. Sutherland), rabbit anti-laminin (Sigma), and rabbit anti-MyoD (a gift from A.J. Harris), all diluted 1:100; rat anti-α4 (PS2; Serotec), rat anti-VCAM-1 (M/K-2; Serotec), both diluted 1:50; mouse anti-myosin (F-59; a gift from F. Stockdale) diluted 1:200; rabbit anti-Myf-5 (C-20; Santa Cruz Biotechnology) diluted 1:2,000 and mouse anti-tau-Pax-3 (Venters et al., 2004; a gift from C. Marcelle), diluted 1:200. Secondary antibodies used were Alexa Fluor 350–conjugated goat anti-mouse immunoglobulin G (IgG), Alexa Fluor 568–conjugated goat anti-mouse IgG, Alexa Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes), all F(ab’)2 fragments and diluted 1:1,000; tetradecahemidamine isothiocyanate (TRITC)–conjugated goat anti-rabbit IgG, fluorescein isothiocyanate (FITC)–conjugated goat anti-rat IgG, FITC–conjugated rabbit anti-rat (Sigma), all diluted 1:100 and TRITC-conju-
gated rabbit anti-rat IgG (Sigma), diluted 1:160. Some sections were exposed to 5 µg/ml 4',6-diamidine-2-phenylindole-dihydrochloride (DAPI) in 0.1% Triton X-100 in PBS for 2 min. For IHC on frozen sections, E9.0–E11.5 embryos were fixed in 0.2% paraformaldehyde in 0.1 M phosphate buffer (PB) with 0.12 mM CaCl₂ and 4% sucrose overnight at 4°C, washed in buffer only, and then in PB with 15% sucrose, both over-night at 4°C. Embryos were then incubated in PB with 15% sucrose and 7.5% gelatin for 1 hr at 30°C, immediately frozen in liquid nitrogen-chilled isopentane, and stored at −80°C until sectioned. Approximately 10-µm-thick serial sections were collected on Super Frost slides, permeabilized in 0.2% Triton X-100 in PBS for 20 min and blocked for 30 min with 10% normal goat serum in PBS containing 1% bovine serum albumin (except for anti-fibronectin antibody). Primary antibody incubations were overnight at 4°C, followed by washing in PBS and secondary antibody incubations were for 1 hr at room temperature, followed by PBS washes. When secondary antibodies were against mouse IgGs, a final 30-min washing step in 4× PBS was included to reduce nonspecific background labeling. Slides were mounted in 2.5% 1,4-diazabicyclo-2,2,2-octane in 9:1 glycerol/PBS or Vectashield (Vector Laboratories), and sealed after cover-slipping.

Whole-mount IHC was performed essentially as described in Ordahl et al. (2001). Embryos were fixed in 0.2% PFA in PBS and permeabilized with 0.2% Triton X-100 for 20 min. Antibody staining was performed as on sections, except secondary antibody incubations were done overnight.

**Dissociation of Somites**

Groups of three to five somites caudal to forelimb level of E9.5 embryos were dissected out and placed onto Superfrost Plus slides. They were then treated with collagenase II (Sigma) at 37°C for 30 min to dissociate cells and fixed in 0.2% PFA for 30 min (Venters et al., 1999). IHC was carried out as described for sections.

**Imaging**

Embryos processed for whole-mount ISH were photographed by using an Olympus Camedia C-4040 digital camera coupled to a Wild M8 stereomicroscope. Sections processed for ISH and IHC were photographed by using an Olympus DP50 digital camera coupled to an Olympus BX60 microscope equipped with Nor-marski optics and epifluorescence. Optical Z-series of whole-mount embryos were obtained in a Leica SP2 confocal microscope. Images were edited in Adobe Photoshop 7.0.

**NOTE ADDED IN PROOF**

While this article was in its proofing stage, a two-step mechanism for myotome formation in the chick was described (Gros et al. 2004 Dev Cell 6:875–882). Our results on integri n expression patterns in the mouse appear to be compatible with this model.

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