Developmentally regulated expression of α6 integrin in avian embryos

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Summary

The distribution pattern of the avian α6 integrin subunit was examined during early stages of development. The results show that this subunit is prevalent in cells of the developing nervous system and muscle. α6 is first observed on neuroepithelial cells of the cranial neural plate and trunk neural tube. With time, immunoreactivity becomes prominent near the lumen and ventrolateral portions of the neural tube, co-distributing with neurons and axons, particularly notable on commissural neurons. The α6 expression pattern is dynamic in the neural tube, with immunoreactivity peaking by embryonic day 6 (stage 30) and decreasing thereafter. The ventral roots and retina exhibit high levels of immunoreactivity throughout development. In the peripheral nervous system, α6 immunoreactivity first appears on a subpopulation of sympathoadrenal cells around the dorsal aorta and later in the dorsal root ganglia shortly after gangliogenesis. Immunoreactivity appears on prospective myotomal cells as the somites delaminate into the dermomyotome and sclerotome, remaining prominent on myoblasts and differentiated muscle at all stages. The mesonephros also has intense immunoreactivity. In the periphery, α6 immunoreactive regions often in proximity to laminin, which is thought to be the ligand of $\alpha_6\beta_1$ integrin.

Key words: cell surface receptors, extracellular matrix, nervous system, myoblasts.

Introduction

During development, interactions between cells and extracellular matrix molecules are thought to play an important role in many morphogenetic processes. Numerous extracellular matrix molecules (ECM) including fibronectin, laminin, collagens, and proteoglycans are present in regions of active cell movement and neurite outgrowth. The best-studied ECM molecules are the glycoproteins, fibronectin and laminin, which are widely distributed and appear to be developmentally regulated. Some of these molecules have established functions in development; for example, fibronectin and/or laminin are required for gastrulation (Boucaut et al., 1984), cranial neural crest cell migration (Poole and Thiery, 1986; Bronner-Fraser and Lallier, 1988) and neurite outgrowth (Lander et al., 1985).

The integrins are a family of cell surface receptors that mediate adhesion of cells to a variety of extracellular matrix molecules including fibronectin, laminin, tenascin and various collagens (Horwitz et al., 1985; Buck et al., 1986; Tomaselli et al., 1988; Bourdon and Ruoslahti, 1989). Integrin receptors are heterodimers of two non-covalently linked transmembrane glycoproteins, one α and one β subunit. Within the integrin family, there are at least fourteen α subunits, each with

significant sequence identity, and at least eight β subunits. Some integrin heterodimers recognize a single ligand specifically whereas others bind to several different extracellular matrix molecules. For example, $\alpha_6\beta_1$ only binds to laminin (Sonnenberg et al., 1988; 1990) whereas $\alpha_3\beta_1$ recognizes fibronectin, laminin and collagens (Wayner et al., 1988; Gehlsen et al., 1988; 1989; Tomaselli et al., 1990).

Although the structural and biochemical characteristics of various integrin heterodimers have been well-studied, little is known about their tissue-specific distribution or function during development. The β_1 subunit is present on most embryonic cell types at early stages of development (Duband et al., 1986; Krotoski et al., 1986) and antibodies against the β_1 subunit interfere with cranial neural crest cell migration (Bronner-Fraser, 1985; 1986a) and myoblast migration (Jaffredo et al., 1988). In avian embryos, this suggests a functional role for integrins during cell migration. However, because the β_1 subunit can pair with multiple α subunits, it remains unclear if single or multiple integrins are involved in these morphogenetic events. To determine which of the possible integrin heterodimers are present in the early embryo, α-specific probes are required. Unfortunately, the numerous antibodies that recognize human α subunits do not cross-react well with other species. Furthermore, few α-

specific probes are available for avian embryos (de Curtis et al., 1991; Muschler and Horwitz, 1991), in which perturbations experiments have indicated a functional significance for integrins. In the present study, we utilize a monoclonal antibody that recognizes the avian α_6 subunit of integrin to describe its distribution in the developing embryo. Our results show that it appears in neuroepithelial cells, subsets of neurons in the developing nervous system, muscle cells and their precursors in a developmentally regulated fashion.

Materials and methods

α_6 antibody production

A monoclonal antibody against the chick α_6 subunit of integrin was prepared by immunizing nine week old female Balb/c mice at 21 day intervals with approximately 50 μg of integrin α subunits purified from adult chicken brain tissue. The chicken integrins were purified using a CSAT antibody (against the β_1 subunit) affinity column, as described by Muschler and Horwitz (1991). The dimeric receptors were denatured in 6 M guanidine isothiocyanate, dialyzed against 200 volumes of extraction buffer and passed several times over a W1B10 (anti-β_1) column to deplete the β subunit. For immunizations, the sample was mixed (1:1) with Freunds adjuvant (Gibco Laboratories; Life Technologies Inc , Grand Island, NY) to a final volume of 0 5 ml/mouse and injected into the peritoneal cavity. The fusion was performed by the methods of Kennett et al. (1982). One hybridoma was identified as specific for a 130 × 10^3 M_r integrin α subunit and was subsequently designated α_6C6. Having been generated against denatured protein, this antibody immunoblots well and immunoprecipitates the monomeric α subunit after the dimeric receptor has been denatured, but does not precipitate the native receptor Verification that the α_6C6 recognizes the chick α_6 subunit of integrin was demonstrated by transfecting NIH3T3 cells with chicken α_6 cDNA (kindly provided by Dr. L. Reichardt; de Curtis et al., 1991) followed by immunoblots with the α_6C6 antibody The methods describing the expression vector, transfection, cloning and protein extraction from NIH 3T3 cells are as described in Hayashi et al. (1990).

α subunit purification

The 130 × 10^3 M_r chicken α subunit was purified from a CSAT affinity column by denaturing in 6 M guanidine isothiocyanate, heating to 60°C for 15 minutes, dialyzing, and then incubating with α_6C6 monoclonal antibody conjugated to Sepharose beads The beads were washed in extraction buffer and eluted with SDS-PAGE sample buffer. CSAT, W1B10 and α_6C6 matrices were constructed by coupling 5 mg/ml of pure monoclonal antibody to CNBr-activated Sepharose 4B (Piscataway, NJ).

Protein electrophoresis

SDS-PAGE was performed by the method of Laemmli (1970). Separating gels were 7% acrylamide and 0.12% bis-acrylamide. Samples were prepared in sample buffer (62.5 mM Tris-HCl, pH 6 8, 2.0% SDS, 10% glycerol). Proteins were visualized by silver staining using Bio-Rad Silver Stain kit (Bio-Rad, Richmond, CA). Immunoblots were performed as described in Muschler and Horwitz (1991). All antibodies were used at an approximate IgG concentration of 25 μg/ml.

Antibodies

The CSAT antibody was prepared as described by Neff et al. (1982). The W1B10 antibody was prepared as described by Hayashi et al. (1990). Rabbit anti-human α_6 and α_5 cytoplasmic domain serum were a gift of Dr. L. Reichardt (UCSF; Tomaselli et al., 1988; de Curtis et al., 1991). The JG22 antibody against the chick β_1 subunit of integrin was purchased from Developmental Studies Hybridoma Bank. An antibody against chick laminin (LM-33) which recognizes the B chains (Krotoski et al , 1986) was kindly provided by Dr. Douglas Fambrough. A polyclonal antibody against mouse laminin was purchased from E-Y laboratories. An antibody against a nonphosphorylated epitope of rat neurofilament was kindly provided by Dr. Virginia Lee (Lee et al., 1987).

Embryos

White Leghorn chick embryos ranging from Hamburger and Hamilton (1951) stage 8 (embryo day 1 5) to stage 35 (embryonic day 9) were obtained by incubating eggs in a forced air incubator at 37°C. Some tissue also were obtained from adult chickens

Fixation and tissue processing

Embryos were fixed in methanol at 4°C overnight, washed in PBS, transferred to 5% sucrose in PBS with azide for 4 - 24 hours, and placed in 15% sucrose in PBS with azide overnight at 4°C Embryos were placed in 7% gelatin (Sigma; 300 Bloom) in 15% sucrose/PBS for 3 hours at 37°C, and embedded in fresh gelatin Embryos were stored for up to two weeks in the refrigerator until the time of sectioning when they were rapidly frozen in liquid nitrogen. 14 μm sections were cut on a Zeiss Microm cryostat and mounted on gelatin subbed slides.

Neural crest cell cultures

Primary neural crest cultures were prepared from the neural tubes of Japanese quail embryos (*Coturnix coturnix japonica*). Embryos were incubated for 48 hours, at which time their developmental age was comparable to that of chick stages 13 - 15. The region of the trunk neural tube adjacent to the six to nine most posterior somites as well as the unsegmented mesenchyme was dissected away from the embryo. The neural tubes were isolated from adjacent tissue by treatment with 160 units/ml of collagenase (Worthington Biochemical, Freehold, NJ), followed by trituration. After stopping the enzymatic reaction with MEM containing 15% horse serum and 10% chick embryo extract, neural tubes were plated onto fibronectin- or laminin-coated culture dishes. After several hours, the neural crest cells migrated away from the explant and the neural tube tissue was removed Neural crest cells were grown for several days prior to fixation in cold methanol for 15 minutes.

Immunocytochemistry

Cryostat sections of embryos were warmed to 25°C and adjacent sections were incubated with either α_6, neurofilament, β_1 or laminin antibodies. For staining neural crest cultures, dishes were incubated with anti-α_6 antibody as described for sectioned tissue. All antibody dilutions were made in 0.1% bovine serum albumen in PBS Anti-α_6 was used at a concentration of 35 μg/ml. Anti-neurofilament was diluted 1:400 from hybridoma supernatant. JG22 and LM-33 were used as straight hybridoma supernatant. The rabbit polyclonal antibody against laminin was diluted 1:50. Following an overnight incubation, sections were rinsed in PBS, and

incubated with a highly fluorescent FITC-goat antibody against mouse Igs (Antibodies Inc., Davis, Ca.; diluted 1:300) for detection of monoclonal antibodies for 1 hour at 25°C. Slides then were rinsed in PBS, coverslipped with glycerol (90% glycerol/10% 0 5 M sodium carbonate, pH 7.8) and viewed with an Olympus Vanox epifluorescence microscope. Data were recorded photographically. For control slides or cultures, the primary antibody was omitted and the sections or dishes were stained with fluorescinated second antibody alone.

Results

The distribution of the chick α_6 subunit of integrin was examined in embryos ranging from 1.5 to 14 days of incubation, as well as some adult tissues. Its expression pattern was compared with that of the β_1 subunit of integrin, neurofilament proteins and laminin, the extracellular ligand of $\alpha_6\beta_1$ integrin.

Characterization of the α_6C6 monoclonal antibody
A monoclonal antibody specific for a $130 \times 10^3 M_r$ chicken integrin α subunit was generated by immunizing mice with purified integrins from chicken brain tissue. Immunofluorescent staining of adult tissue sections using this monoclonal antibody, α_6C6, demonstrated that the antigen was localized on neural tissue and on epithelial cells, where it was concentrated on the basal surface contacting the basal lamina (not shown). This localization is similar to that of the human α_6 subunit. To further test homology of the α_6C6 antigen with human α_6, we utilized an antiserum that recognizes a 35 amino acid polypeptide corresponding to the cytoplasmic domain of the human α_6 subunit (de Curtis et al., 1991). This anti-α_6 antiserum exclusively recognized the $130 \times 10^3 M_r$ α band purified by the α_6C6 antibody (Fig. 1). The $160 \times 10^3 M_r$ band in lane 2 of

Fig. 1 is monoclonal antibody released from the beads used for immunoprecipitation.

To demonstrate that the α_6C6 monoclonal specifically recognizes the chick α_6 subunit of integrin, cDNA encoding for chick α_6 (de Curtis et al., 1991) was expressed in NIH 3T3 cell lines (Hayashi et al., 1990). Extracts of the transfected and untransfected cells were immunoblotted with the α_6C6 antibody. The antibody clearly recognizes a $140 \times 10^3 M_r$ protein product in the α_6 transfected cells which is absent in the untransfected cells (Fig. 2). The gene product in transfected cells migrates slightly larger than α_6 purified from chick tissue (140 compared with $130 \times 10^3 M_r$). This difference is likely to be due to differential glycosylation. These results suggest that by immunological relatedness, similar tissue distribution and recognition of the gene product of chick α_6 cDNA, the $130 \times 10^3 M_r$ α subunit recognized by α_6C6 is the chicken homolog of human α_6. Therefore, this antigen will be referred to as the chicken α_6 subunit.

Onset of α_6 expression in the neural tube and myotome
In the developing head, the α_6 subunit of integrin was observed on neuroepithelial cells of the neural plate in stage 8 (1.5 day) embryos. Its expression continued during neural tube closure and was also observed on premigratory neural crest cells (Fig. 3). Interestingly, the immunoreactivity appeared to drop off gradually caudal to the hindbrain and was not observed in trunk neuroepithelial tissue until after neural tube closure. α_6 expression was not observed outside of the forming nervous system in neurulating embryos.

In the trunk region of a 2 day chick embryo (stage 15), the onset of α_6 immunoreactivity appeared to follow an anterior to posterior sequence, mirroring the developmental progression of the embryo. Because

A

B

anti-human α6   α6C6   anti-human α5

1   2   1   2   1   2   1   2

α

β

*

-200
-116
-92
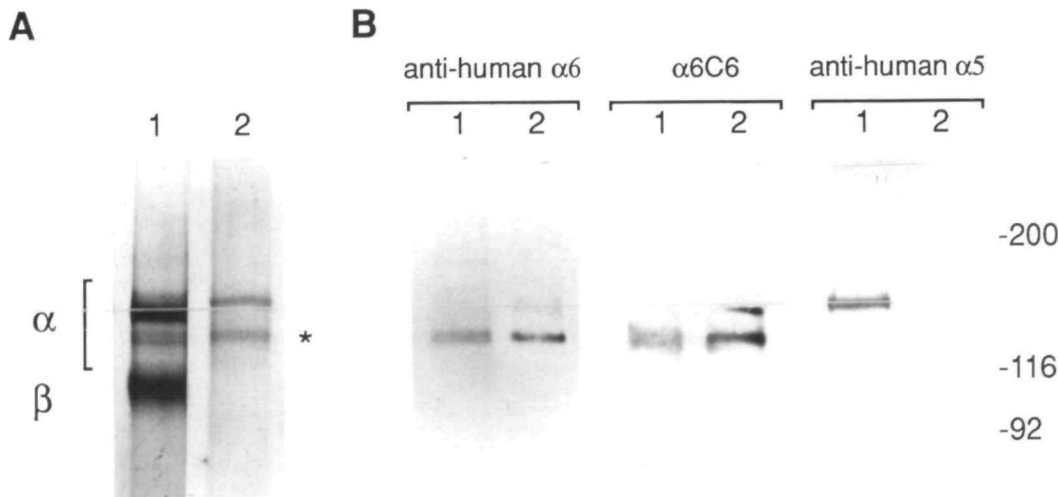
Fig. 1. Purification of the $130 \times 10^3 M_r$ chicken alpha subunit from 11 day chick embryos and identification as the homolog of the human α_6 subunit (A) The $130 \times 10^3 M_r$ α subunit was purified by α_6C6 antibody immunoprecipitation of denatured chick integrin proteins, obtained using a CSAT affinity column. The purified proteins were run on 7% SDS-PAGE gels (non-reduced) and visualized by silver staining. The purified β_1 integrins are shown in lane 1; the purified $130 \times 10^3 M_r$ α subunit is shown in lane 2 (*).

The $160 \times 10^3 M_r$ band in lane 2 is monoclonal antibody released from the beads used for immunoprecipitation (B) Immunoblots of the purified $130 \times 10^3 M_r$ chicken α subunit with α-specific antibodies. The integrin proteins in Fig. 1A were run on 7% SDS-PAGE gels, transferred to nitrocellulose and probed with several α subunit-specific antibodies. The polyclonal anti-human α_6 serum and the anti-chick α_6 subunit antibody α_6C6 showed identical immunoreactivity. The anti-human α_5 serum was used as an additional control to show the selectivity of the antibodies used. The position of marker proteins ($M_r \times 10^3$) is indicated.
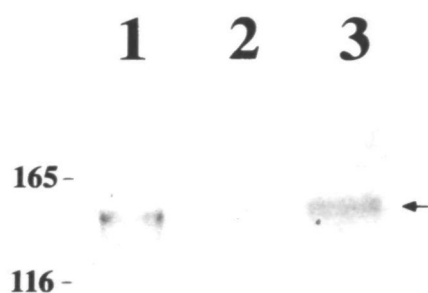
1   2   3

165 –

116 –

Fig. 2. Detection of the chicken α_6 cDNA gene product in transfected NIH 3T3 cells by the α_6C6 monoclonal antibody. Purified chicken β_1 integrins (lane 1), a cell extract from untransfected NIH 3T3 cells (lane 2) and a cell extract from an NIH 3T3 cell line expressing the chicken α_6 cDNA gene product (lane 3) were separated on 7% SDS-PAGE gels and immunoblotted using the α_6C6 monoclonal antibody. The antibody recognizes a 140×10^3 M_r protein in transfected cells (arrow) but not in untransfected cells. The α_6 gene product in the mouse cells migrates slightly higher than that purified from chicken tissue, probably as the result of differences in glycosylation in the two species. The position of marker proteins ($M_r \times 10^3$) is indicated.
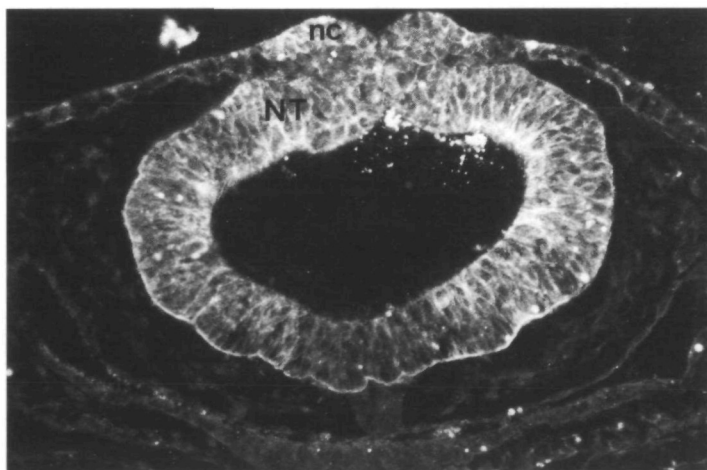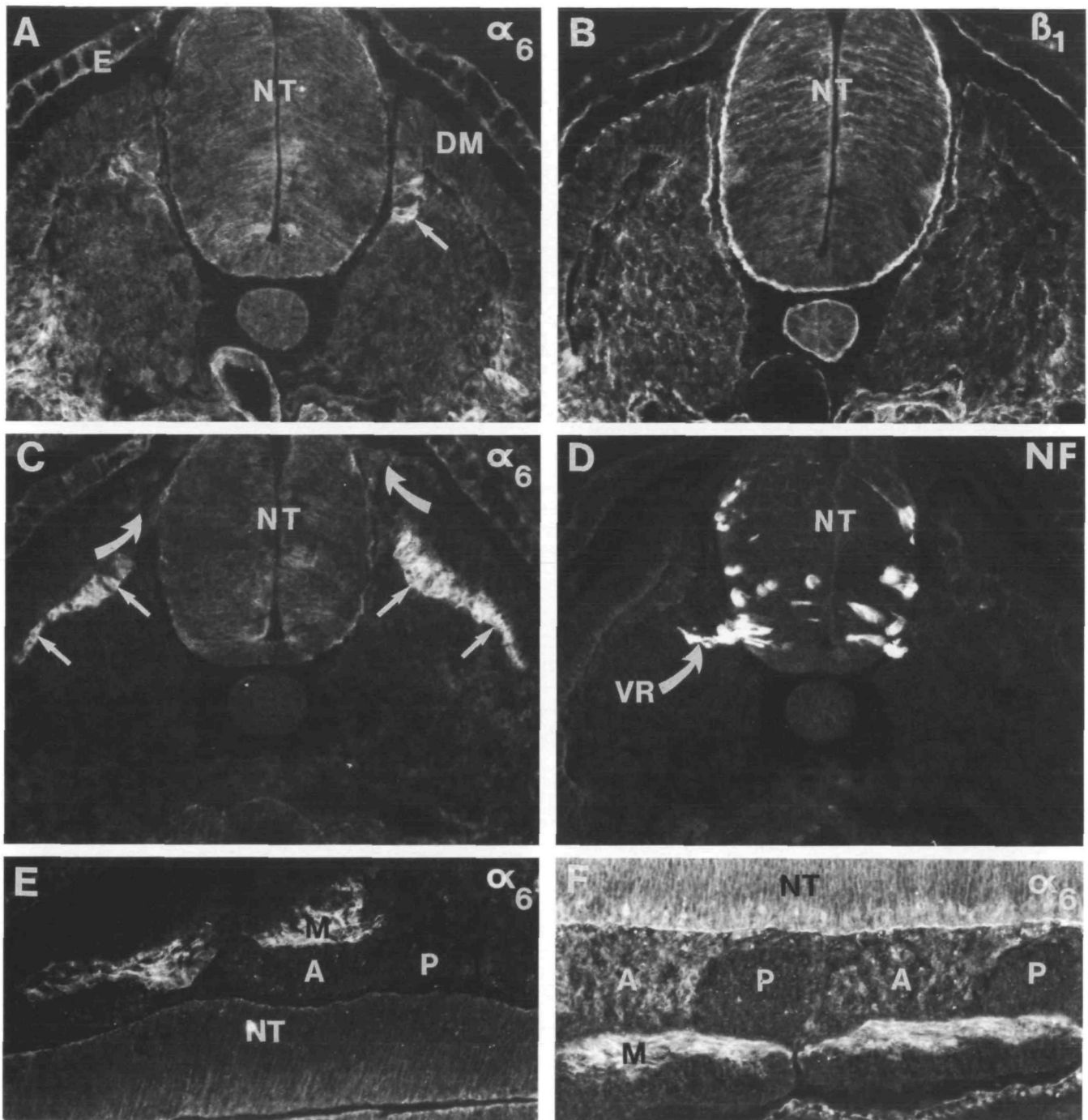

Fig. 3. A transverse section through the midbrain level of a stage 8 embryo (1 5 days). Immunoreactivity for the α_6 subunit of integrin was observed in the neural tube (NT) and premigratory neural crest cells (nc), but was absent from other tissues.

neurulation begins in the head and moves progressively tailward, several stages of α_6 expression could be viewed simultaneously in a single embryo. Within the somites, the posterior extent of its expression was detected approximately five somites above the last somite, at a level where the epithelial somites undergo a transition to form the dermomyotome and sclerotome. In transverse sections, α_6 expression can first be seen on

Fig. 4. The distribution of the α_6 subunit of integrin (A,C,E), the β_1 subunit of integrin (B,F) and neurofilament (D) in sections through the trunk region of avian embryos at stages 15 - 16. (A and B) Adjacent transverse sections at a level approximately 5 somites above the last-formed somite. α_6 (A) is observed in a small populations of cells (arrows) in the mediolateral portion of the somite as it forms the dermomyotome (DM). α_6 is also present at low levels in the neuroepithelial cells of the neural tube (NT), the ectoderm (E) and in ventral regions where the mesonephros will form In contrast, β_1 (B) is expressed on all embryonic cells. (C and D) Adjacent transverse sections through the same embryo pictured in (A and B) in a more anterior region of the trunk, approximately 10 somites above the last-formed somite. The dermomyotome is formed and neural crest cell emigration is ongoing. α_6 (C) expression brightly labels the myotomal cells of the somite (straight arrows) and has increased on the neuroepithelial cells of the neural tube. Neural crest cells (curved arrows) migrating in the extracellular space between the neural tube, ectoderm and somites express low levels of α_6. By this stage, some neurofilament (NF)-immunoreactive cells (D) are present within the neural tube, including motor neurons which extend their axons out the ventral root (VR). (E) A longitudinal section through somites of a stage 15 embryo At this level, approximately 5 somites above the last-formed somite, the dermatome, myotome and sclerotome are forming. α_6 immunoreactivity appears on presumptive myotomal cells (M) in the anterior (A), but not the posterior (P), portion of this somite. Just one somite anterior to this (left), α_6-positive cells are distributed throughout the anterioposterior length of the somite. (F) A longitudinal section through a stage 17 embryo at the level of the ventral neural tube α_6 immunoreactivity is present throughout the myotome (M) and is pronounced within the neural tube, motor axons and/or Schwann cells within the anterior portion of the somite have pronounced α_6 immunoreactivity.

a few presumptive myotomal cells in the portion of the somite closest to the neural tube (Fig. 4A) within the newly formed dermomyotome. Approximately 5 - 6 somites above the last-formed somite, a subpopulation of immunoreactive cells is observed in the anterior portion of the somite just medial to the dorsal neural tube (Fig. 4E). The number of α_6-positive cells increases and these cells appear to progressively fill in the space between the dermatome and sclerotome, in an anterior to posterior sequence (Fig. 4E), such that only 1 - 2 somite lengths contained 'partial' α_6-immunoreactive myotomes. In slightly more mature somites located in more anterior (older) regions of the same embryos, α_6 reactivity within the somite appears restricted to myotomal cells (Fig. 4C,F).

Concomitant with its appearance in the myotome, α_6 becomes apparent in the trunk neural tube and the intensity of immunoreactivity increases in progressively more anterior regions of the embryo. Some α_6 reactivity is detectable in the surface ectoderm and in ventral regions of the embryo where the mesonephros begins to form.

Expression of α_6 in the neural tube

When the neural tube is a single cell layer thick, α_6 appears to outline the columnar neuroepithelial cells of the tube. Its expression pattern in the neural tube overlaps with that of β_1 integrin (Fig. 4B). Though neurons are morphologically distinguishable by neurofilament staining at this stage (Fig. 4D), levels of α_6 are not elevated on neurons compared with undifferentiated neuroepithelial cells until a slightly later stage.

In anterior (pharyngeal) regions of the stage 15 - 16 embryo or in the trunk of stage 18 - 19 embryos, the pattern of α_6 immunoreactivity changes within the neural tube. Although low levels of reactivity remain detectable on neuroepithelial cells, some subpopulations of cells within the neural tube express particularly high levels of α_6. The most notable are the cells with the morphological appearance of commissural neurons which extend their axons toward the floorplate (Tessier-Lavigne et al., 1988). The cell soma as well as axonal processes are outlined by α_6 immunoreactivity (Fig. 5A,C). Some of these processes appear to project toward the laminin-rich basement membrane (data not shown) along the lateral margin of the neural tube. Some motor neurons with axons extending into the sclerotome also appear to express α_6 (Figs 4F, 5A, 7A). This is particularly clear when viewed in longitudinal
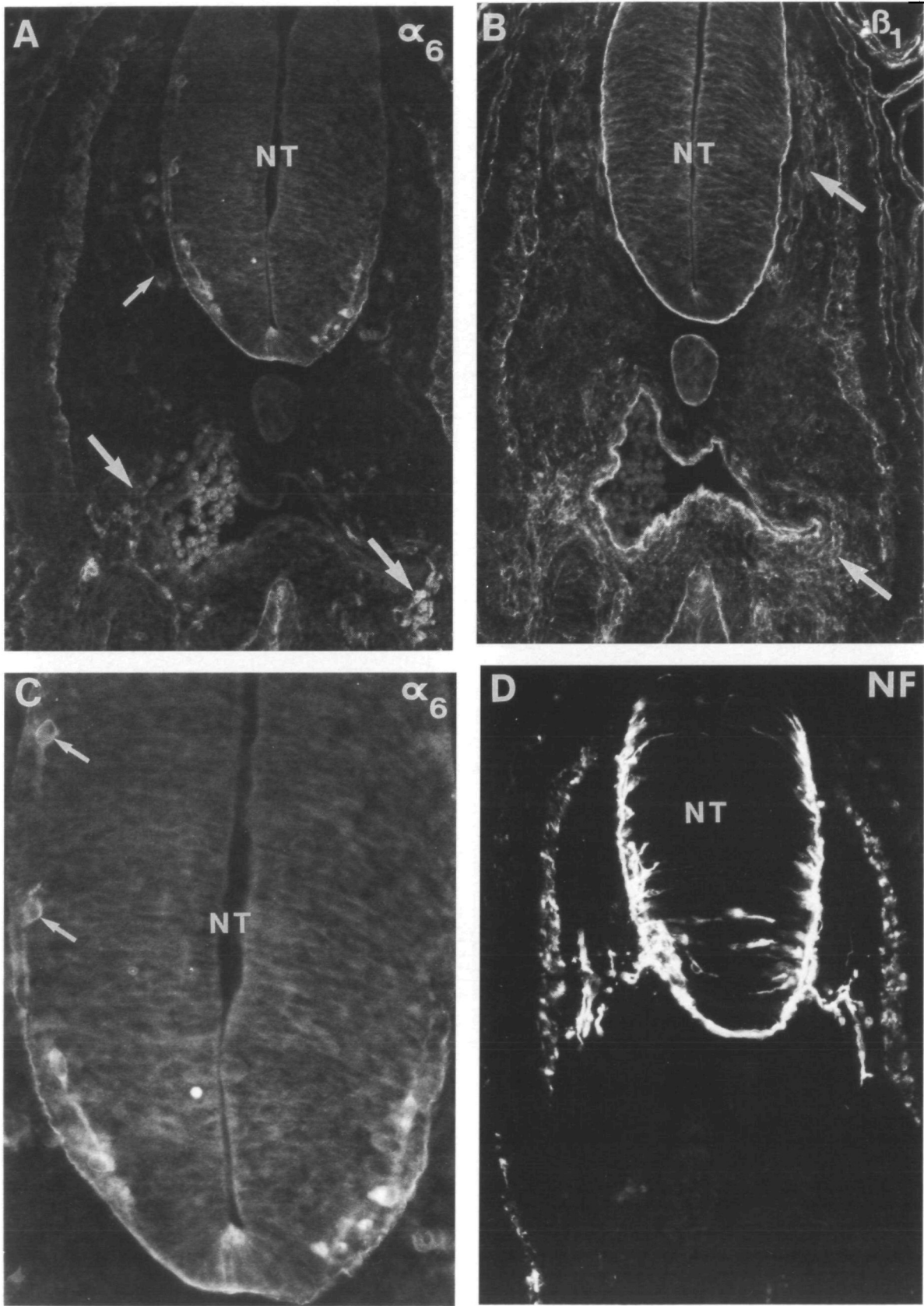
Fig. 5. The distribution of the α_6 subunit of integrin (A,C), the β_1 subunit of integrin (B) and neurofilament (D) in adjacent transverse sections at the level of the developing pharynx in a stage 16 embryo. At low (A) and high (C) magnification, α_6 immunoreactivity was prominent on neuroepithelial cells and on a subpopulation of neurons in the neural tube (NT). Some of these neurons had the characteristic appearance of commissural neurons, as indicated by small arrows in (C), sending axons along the ventrolateral side of the neural tube toward the floorplate. Others were probably motor neurons which extended axons outside of the neural tube (small arrow in A). α_6 reactivity also was present in the myotome and ectoderm. Although absent from neural crest cells within the sclerotome, cells (large arrows in A) at the level of the dorsal aorta (presumably sympathoadrenal cells) expressed α_6 In contrast, β_1 immunoreactivity (B) was present on all embryonic cells and was detectable on neural crest cells both in the sclerotome and around the dorsal aorta (large arrows) Numerous neurofilament (NF)-positive cells were present in the embryo at this stage (D), including motor axons exiting the ventral neural tube.

section (Fig. 4F), where its expression can been seen on processes and/or Schwann cells emerging from the ventral neural tube into the anterior portion of the sclerotome. Laminin is present surrounding the neural tube and in the sclerotome through which the axons project (data not shown; and Krotoski et al., 1986).

The pattern of α_6 expression within the neural tube changes with progressive development. High levels of reactivity continue to be observed on neuroepithelial cells in the germinal region of the neural tube, toward its lumen (Figs 6A, 7A, 8A). Immunoreactivity becomes reduced in the region of the floorplate (Figs 8A, 9A), but remains high in regions that are neurofilament-positive as well is near the lumen (Fig. 8D). By stage 25, immunoreactivity is apparent on cell bodies and processes within both the neural tube and dorsal root ganglia (Fig. 8A,B). In contrast to α_6 expression, β_1 immunoreactivity is only present at low levels in the ventrolateral regions of the neural tube containing neuronal cell bodies and processes, but remains high on neuroepithelial cells. In the developing brain, the pattern of α_6 expression is similar to that observed in the developing spinal cord. It also is observed in the optic cup of the 2 day old embryo, and later on the ganglion cell layer of the neural retina during embryogenesis and into adulthood.

Throughout the nervous system, levels of α_6 immunoreactivity remained relatively unchanged in distribution and intensity through stage 28 (day 6). By stage 30 (day 7), however, the intensity of staining appeared to diminish. In embryos examined at stage 33, the levels of reactivity were markedly lower than those observed at stage 30 (Fig. 9). This trend continued such that by stage 37, the latest stage examined, the neural tube
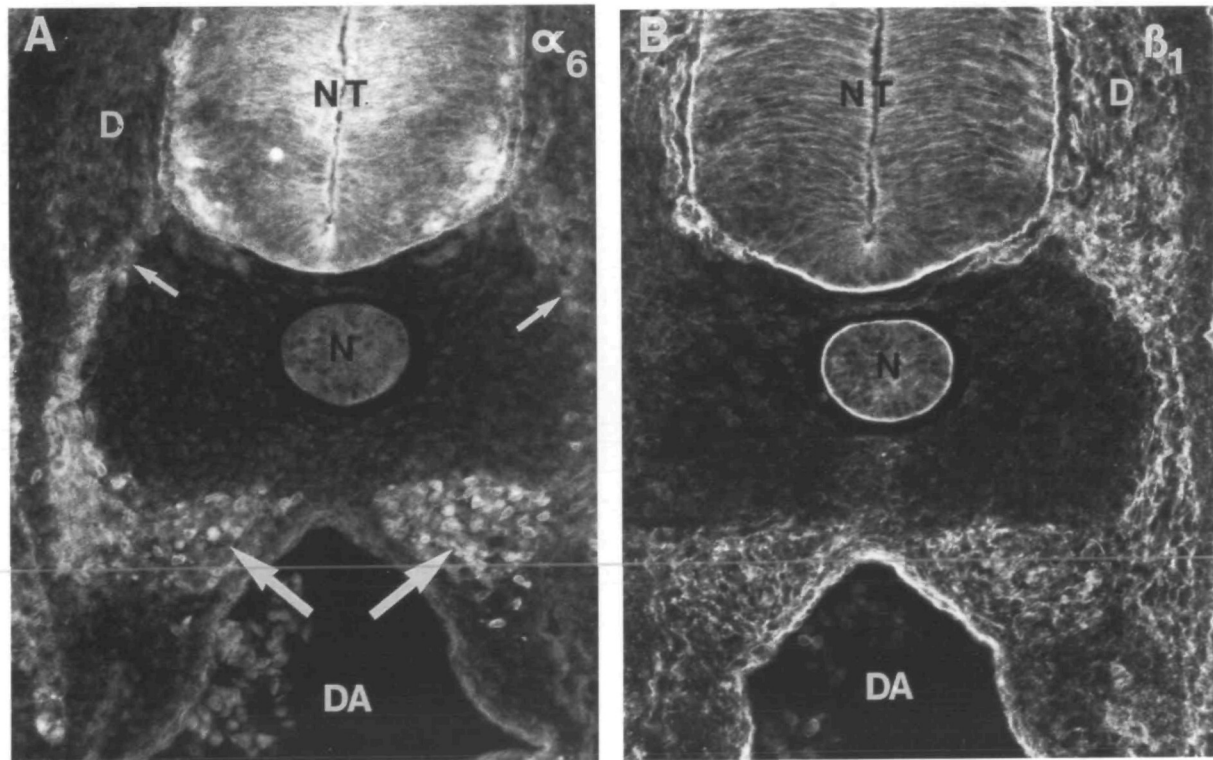

Fig. 6. Adjacent transverse sections above the limb bud level of a stage 19 embryo. The dorsal root ganglia (D) are beginning to condense by this stage. α_6 expression (A) remains high within the neural tube (NT). Reactivity is also prominent in the ventral roots (small arrows), where individual cell staining is clear Sympathoadrenal neural crest-derived cells (large arrows) at the level of the dorsal aorta (DA) have abundant α_6 reactivity. In addition, low levels of expression are detectable in the forming dorsal root ganglia (D) β_1 expression (B) is present on the same populations of cells, but is expressed at much higher levels than α_6 in the dorsal root ganglia. N, notochord.

contained little α_6 immunoreactivity with the exception of the germinal region adjacent to the lumen (Fig. 10A). Interestingly, the ventral roots continued to express high levels at these late stages, suggesting that

this subunit of integrin is expressed on axons and/or Schwann cells, though only low levels of immunoreactivity are observed on motor neuron cell bodies (Fig. 10A). α_6 is also prevalent on the sciatic nerve from day
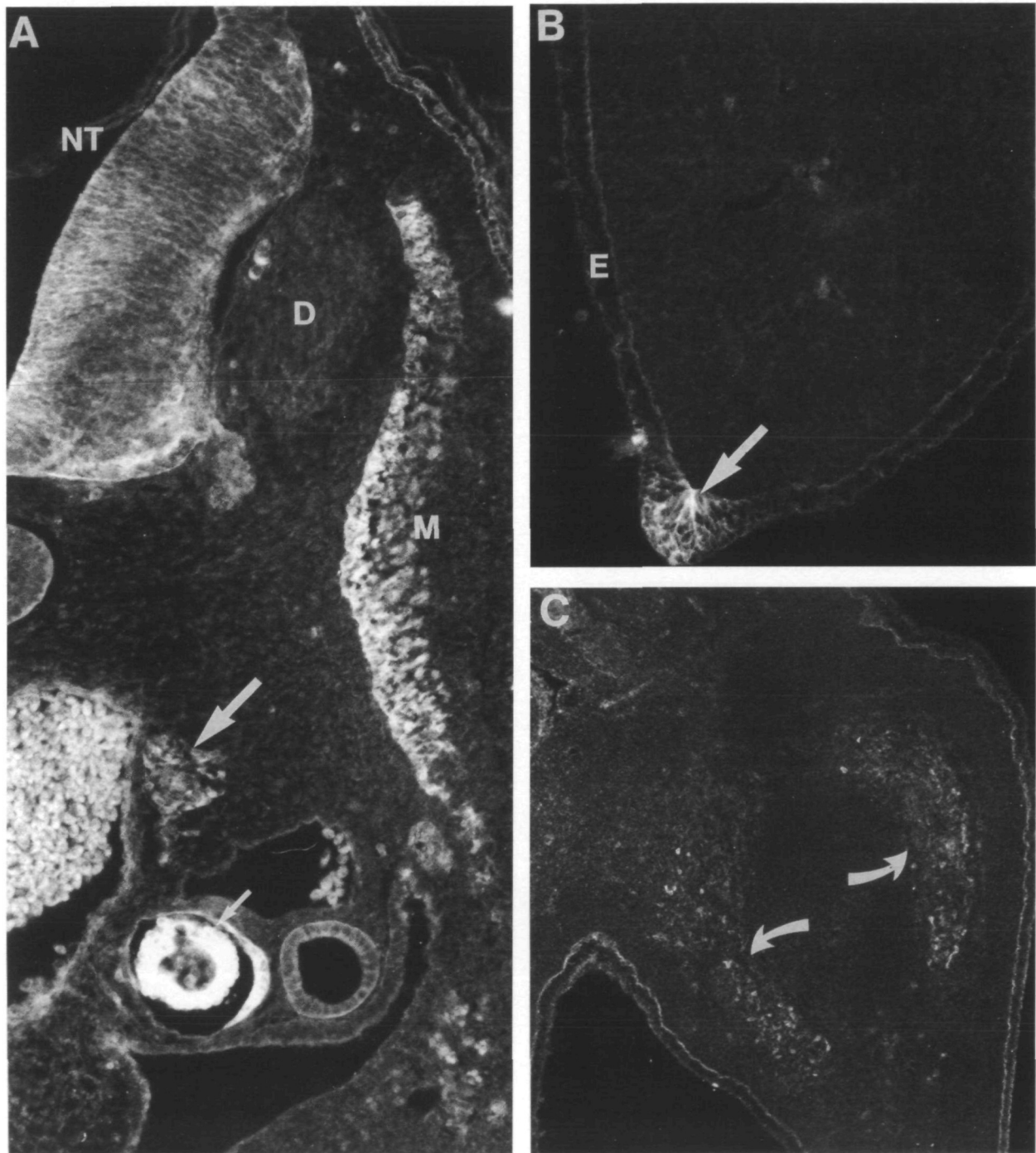

Fig. 7. Transverse sections illustrating the distribution of the α_6 subunit in a stage 21 embryo at the level of the limb bud (A and B) and in the limb bud of a stage 25 embryo (C) α_6 is expressed in the neural tube (NT) in neuroepithelial cells, particularly near the lumen, as well as in neurons and processes in the lateral portion of the neural tube. Immunoreactivity remains high in the myotome (M), from which some cells appear to be leaving to enter the limb. α_6 is present at low levels in the dorsal root ganglion (D) and higher in sympathoadrenal cells near the aorta (large arrow). Prominent immunoreactivity is observed in the mesonephros (small arrow). In the limb of the same embryo (B), α_6 is present in the ectoderm (E) and is particularly elevated in the apical ectodermal ridge (large arrow). α_6 immunoreactive cells are not observed within the distal portion of the limb at this stage By stage 25 (C), however, numerous α_6 immunoreactive cells with the typical distribution of myoblasts are present in the limb (curved arrows).

11 to adulthood. Most of the regions containing immunoreactivity are adjacent to regions containing high levels of laminin (Fig. 9C), though the distributions of the two molecules do not always overlap.

Expression of α_6 on neural crest derivatives

Faint immunoreactivity for the α_6 subunit of integrin is visible on early migrating neural crest cells within the cell-free space between the neural tube, ectoderm and somites in the trunk region (Fig. 4C). Slightly later in development, trunk neural crest cells enter the somitic sclerotome, where they migrate through the anterior but not the posterior half of this tissue (Rickmann et al., 1985; Bronner-Fraser, 1986b). After entering the anterior half of the sclerotome, α_6 immunoreactivity is no longer detectable on neural crest cells (Fig. 5A), identified on the basis of their HNK-1 immunoreactivity though these cells express abundant β_1 integrin immunoreactivity (Fig. 5B). With further development, neural crest cells reach the level of the dorsal aorta, where they will differentiate into catecholamine-containing cells of the sympathetic ganglia, adrenal medulla and aortic plexuses. α_6 immunoreactivity is again detectable on the subpopulation of neural crest cells at the level of the dorsal aorta (Fig. 5A), before these cells express catecholamines.

The dorsal root ganglia begin to aggregate at stage 19 - 20 in the chick embryo (Lallier and Bronner-Fraser, 1988). At the level of the forelimb bud, low levels of α_6 immunoreactivity are visible on the forming dorsal root ganglion cells (Fig. 6A), though the levels are far less prevalent that those of the β_1 subunit (Fig. 6B). In contrast, immunoreactivity for both the α_6 and β_1 subunits are high around the dorsal aorta by stage 19 (Fig. 5A,B), though the sympathetic ganglia do not aggregate until stage 20 - 21 (Lallier and Bronner-Fraser, 1988). By this stage, prominent α_6 and β_1 immunoreactivities are observed in the ventral roots (Fig. 6A,B). This immunoreactivity could be on axons and/or Schwann cells (Fig. 6A).

The relative levels of α_6 expression remain constant on forming sympathetic ganglia from stage 19 to stage 28. However, the levels of expression of dorsal root ganglia increase dramatically after ganglion formation. While α_6 immunoreactivity is faint at stage 21 (Fig. 7A), it increases to become comparable in intensity to that observed on the sympathetic ganglia by stage 24 to 25 (Fig. 8A). The expression of α_6 appears to be associated with both cell bodies and processes within the ganglia (Fig. 8D). At these times, there is prominent laminin immunoreactivity surrounding the ganglia and their processes (data not shown).

In embryos observed between stages 30 and 37, α_6 expression remains detectable on dorsal root and sympathetic ganglion cells. However, the levels of immunoreactivity decline relative to those observed at earlier stages. For example, the relative level of immunoreactivity on dorsal root ganglion cells at stage 33 (Fig. 9A,B) is less than that observed at stage 25 (Fig. 8A). In the stage 37 embryo, α_6 immunoreactivity is present on cells of the dorsal root ganglion (Fig.

10A), but of lower intensity than that observed either in the ventral roots or on dorsal root ganglion cells of stage 25 embryos.

Expression of α_6 on cultured neural crest cells

Neural crest cells were grown in tissue culture on fibronectin- or laminin-coated dishes for 1 - 7 days prior to fixation and staining. Similar results were found for both substrates and the results will be presented collectively. In 1 to 2 day old cultures, little or no α_6 immunoreactivity above background was observed on neural crest cells. By 3 days *in vitro*, expression was observed in a subpopulation of neural crest cells. The immunoreactivity appeared to be punctate in the cytoplasm, as well as outlining the cell periphery, suggesting that the integrin is present in cytoplasmic pools as well as on the cell surface. A similar pattern was maintained throughout the culture period examined. The α_6 immunoreactive cells represented a significant subpopulation of cells in these neural crest cultures, comprising approximately one quarter of the total cell number.

Expression of α_6 on myoblasts

After the differentiation of the somite into dermatome, myotome and sclerotome, α_6 is only detectable on myotomal cells (Fig. 4C). In the trunk, immunoreactivity remains on myotomal cells as long as the myotome is visible (Figs 5A,7A,8A). In the limb of a stage 21 embryo, no α_6 reactivity is detectable (Fig. 7B) with the exception of a few positive cells beginning to enter the limb from the base of the dermomyotome (Fig. 7A). The numbers of immunoreactive cells in the limb increase with time. By stage 25, the limb is populated by numerous α_6-reactive cells in regions where myogenesis will occur (Fig. 7C). One interpretation of these results is that the presumptive α_6-bearing myoblast cells derived from the myotome invade the limb and proliferate, while continuing to express the antigen.

As the myotubes differentiate, α_6 is expressed at high levels on early myotubes. Its is expressed in skeletal muscle of the limb and body wall and in intervertebral muscle (Fig. 10B) throughout the embryonic stages examined, but does not appear to be concentrated at the neuromuscular junction. In contrast, the presence of α_6 on adult muscle is ambiguous. Laminin immunoreactivity is prevalent in regions containing myotomal cells, myoblasts and differentiated muscle at all times examined (data not shown).

Expression of α_6 on other embryonic tissues

Although low levels of immunoreactivity can be detected on ectodermal cells as early as stage 15-16, staining becomes more intense at approximately stage 21 compared with earlier times. Particularly bright reactivity is observed on the apical ectodermal ridge (Fig. 7B) and in the developing mesonephric rudiment. By stage 21, intense reactivity is present in the mesonephric region (Fig. 7A). The developing gonads have high levels of expression, most prominent in the
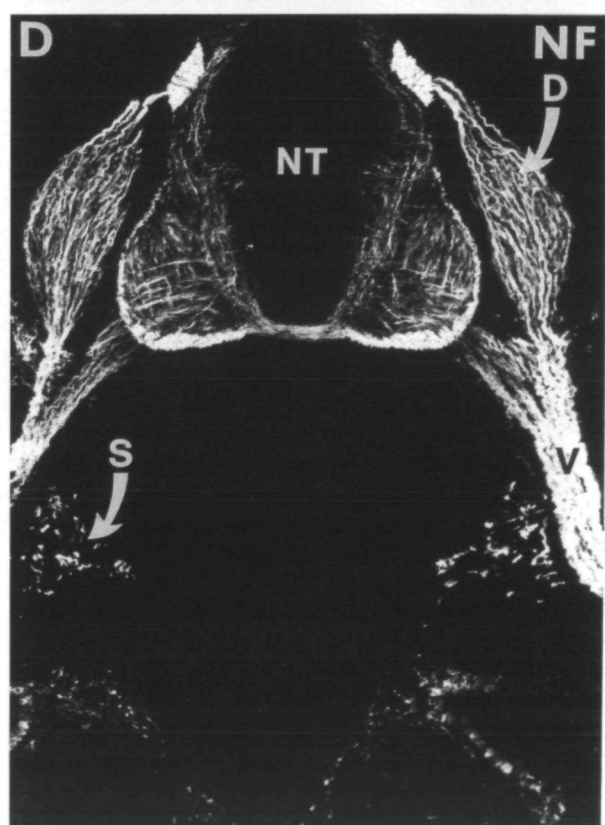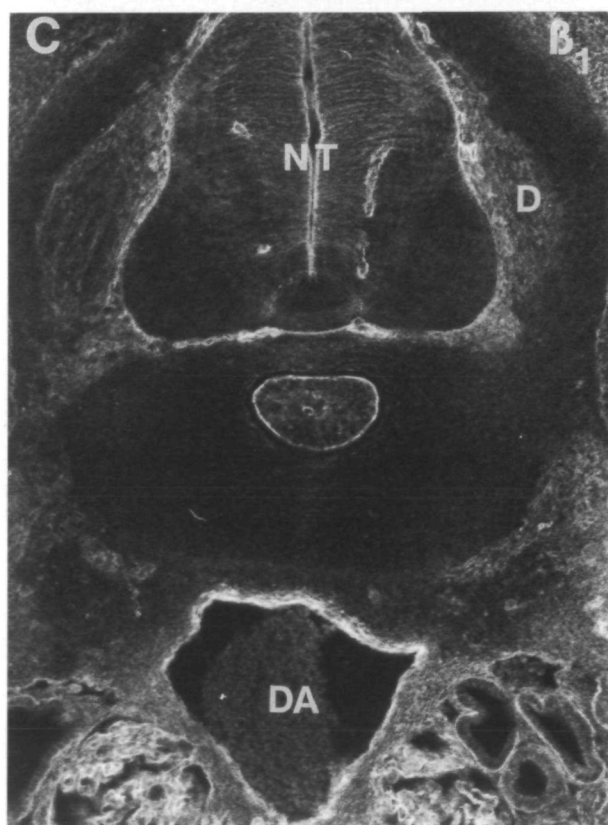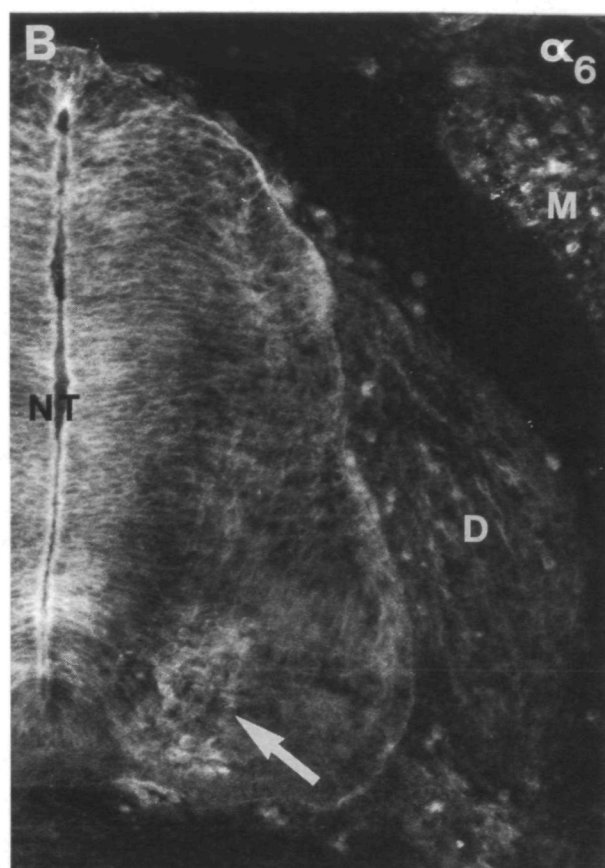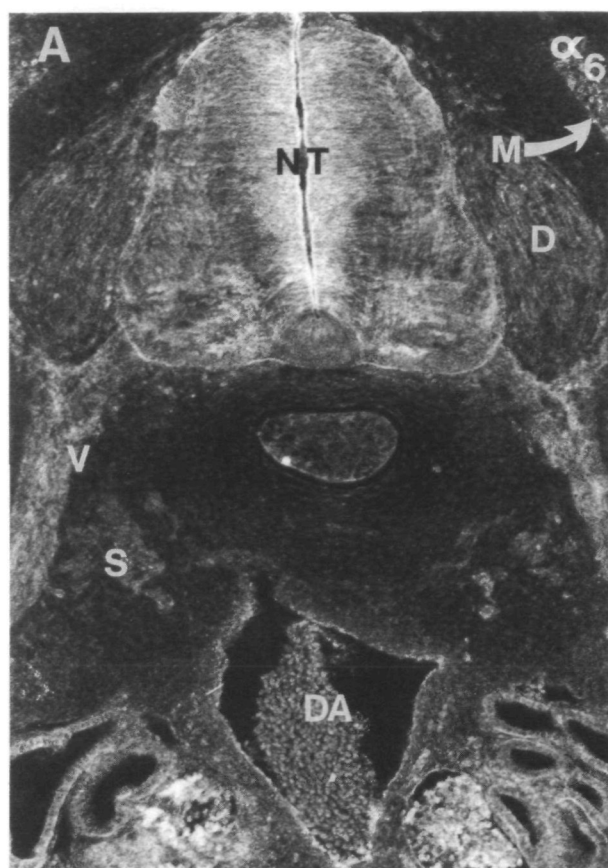
Fig. 8. Adjacent transverse sections through a stage 25 embryo, by which time α_6 immunoreactivity has reached its peak. α_6 immunoreactivity (A and B) is present at high levels in the neural tube (NT), myotome (M), dorsal root ganglia (D), ventral root (V) and sympathetic ganglia (S). At higher magnification (B), α_6 immunoreactivity appears reduced in the region of the floorplate. α_6 is expressed at high levels in ventral (arrow) and lateral regions of the neural tube and in the dorsal root ganglion, where it appears to be present on both cell bodies and processes. These areas correspond to regions that are neurofilament (NF)-positive (D). In contrast to α_6 expression, β_1 immunoreactivity (C) is present at low levels in the ventrolateral regions of the neural tube containing neuronal cell bodies and processes, but remains high on neuroepithelial cells. In other regions, β_1 is found in all tissues containing α_6.

Discussion

We have examined the distribution of the α_6 subunit of chick integrin in the early embryo. Because the expression of this subunit overlaps with that of the β_1 subunit in most tissues examined, it is likely that $\alpha_6\beta_1$ heterodimers are formed in the majority of the tissues expressing α_6. $\alpha_6\beta_1$ heterodimers function as laminin receptors (Sonnenberg et al., 1988; 1990; Hall et al., 1990) and laminin is prominent in the basal lamina and in some interstitial matrices such as in the sclerotome at the stages examined in this study (Krotoski et al., 1986). Thus, it appears likely that the $\alpha_6\beta_1$ receptor complex is expressed in numerous sites in the vicinity of its natural ligand. However, α_6 can pair with the β_4 subunit of integrin (Sonnenberg et al., 1988). The ligand for $\alpha_6\beta_4$ has not been defined, nor is the distribution of this β subunit known in the embryo. Therefore, we cannot rule out the possibility that α_6 also forms heterodimers with the β_4 subunit. In the neural tube, laminin is absent, levels of β_1 are low and levels of the α_6 subunit are high; therefore, it is tempting to speculate that α_6 may pair with a different β subunit there. At the stages examined in our study, immunoreactivity for the β_1 subunit of integrin is present in most parts of the embryo. An exception is in the germinal ridge of the gonads. In the cortical region, α_6 immunoreactivity is

cortical region. However, the germ cells themselves are α_6 negative. β_1 and laminin immunoreactivities are observed in the medulla of the gonads but not the cortex (data not shown). α_6 immunoreactivity remains associated with the developing kidney tubules through development and into adulthood. It is also observed at low levels on the lung and some other endodermal organs. For example, it is found on intestinal epithelium from embryonic day 14 through adulthood, where it is concentrated on the basal cell surface.
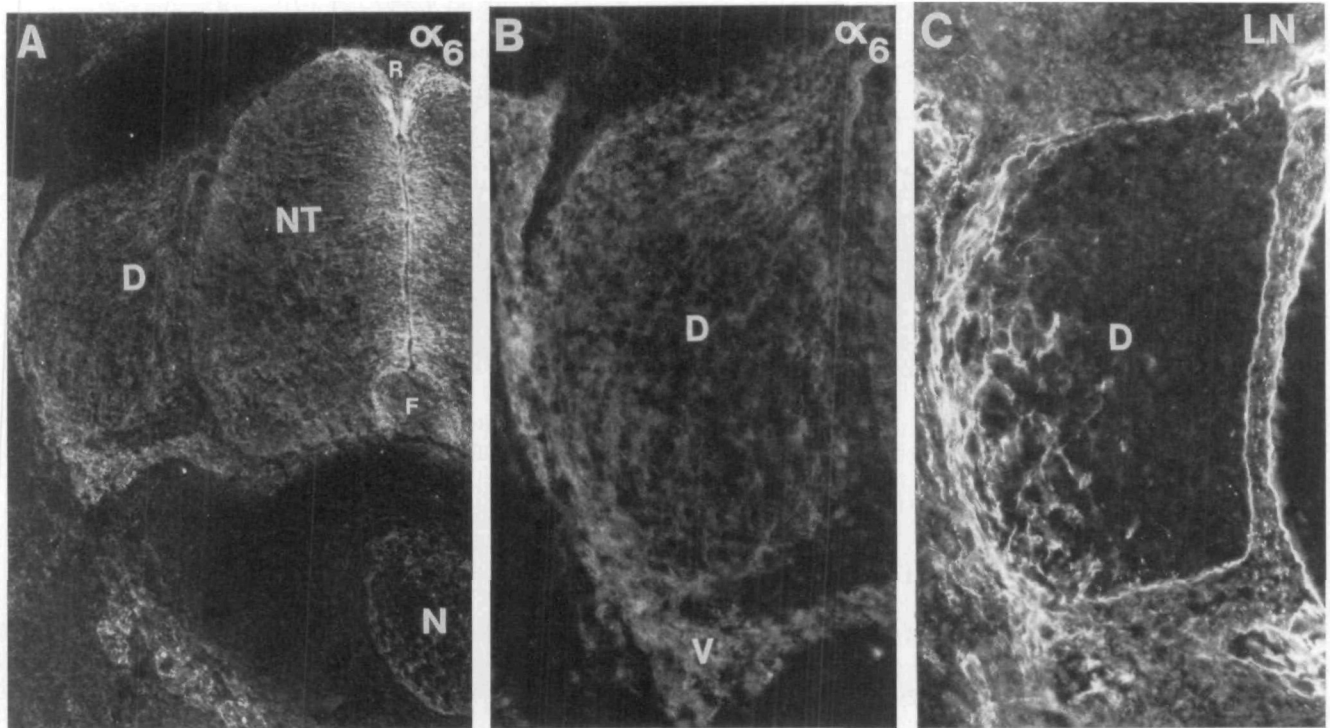

Fig. 9. Adjacent transverse sections through a stage 33 embryo, illustrating α_6 and laminin (LN) immunoreactivity. (A and B) Low and high magnifications, respectively, of the same transverse section. In the neural tube (NT), α_6 expression is significantly reduced compared to earlier stages, except within the germinal epithelium adjacent to the lumen. Immunoreactivity is absent from the floorplate (F) and roof plate (R) regions. α_6 immunoreactivity is present in the dorsal root ganglion (D), but is less prominent than that observed in the ventral root (V) or in the dorsal root ganglia of younger embryos. (C) An adjacent section showing that laminin immunoreactivity is prominent around the neural tube, dorsal root ganglion, and the surrounding tissue. Some laminin also is observed within the lateral portion of the ganglion. N, notochord.
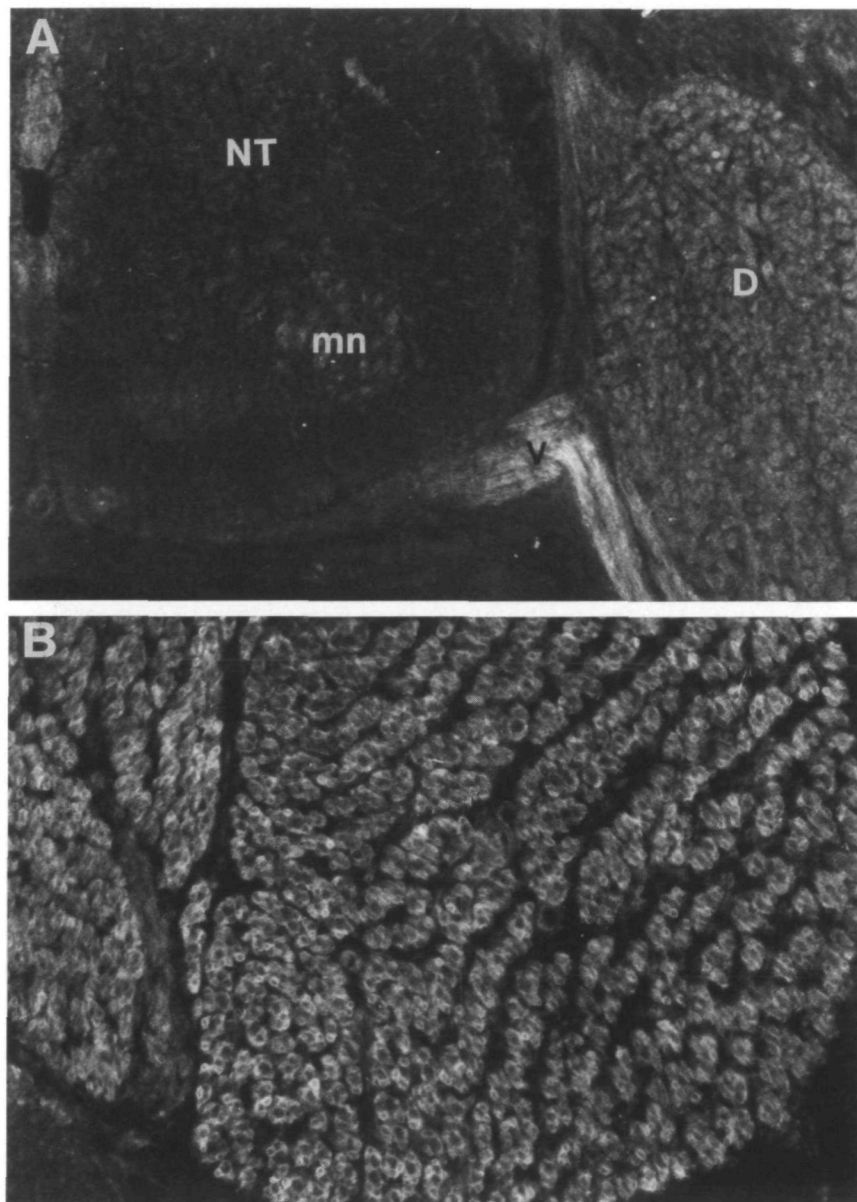
Fig. 10. α_6 immunoreactivity in sections through stage 37 embryos. (A) Within the neural tube (NT), α_6 expression is low except in the luminal region. Motor neurons (mn) exhibit low levels of staining. The ventral root (V) and dorsal root ganglion (D) contain α_6 immunoreactivity, though the levels in the ganglion are comparatively low. (B) Transverse section through the intervertebral muscle showing intense α_6 immunoreactivity.

high whereas β_1 is absent. In the medullary region of the gonads, both subunits are present. Interestingly, laminin is present in the medullary but not cortical portion of the gonad. Thus, it is conceivable that α_6 pairs with a different β subunit, such as β_4, in this organ.

As expected, β_1 immunoreactivity is present in numerous regions which lack α_6 reactivity. These include dermatomal cells, sclerotomal cells, migrating neural crest cells and notochordal cells. Interestingly, all of these cells are present in regions containing high concentrations of laminin, fibronectin, collagens and proteoglycan (Krotoski et al., 1986; Duband and Thiery, 1987; Perris et al., 1991a,b). For example, laminin is prominent around the neural tube, dermo-myotome, under the ectoderm and throughout the somitic sclerotome (Krotoski et al. 1986). Therefore, these tissues are likely to express other β_1 integrin heterodimers for recognition of extracellular ligands. Numerous integrins that interact with laminin have

been described. In addition to $\alpha_6\beta_1$, laminin is recognized by $\alpha_1\beta_1$ (Ignatius and Reichardt, 1988), $\alpha_2\beta_1$ (Languino et al., 1989) and $\alpha_3\beta_1$ (Wayner et al., 1988). Multiple integrins that bind to fibronectin and collagens have been described as well (Pytela et al., 1985; Turner et al., 1987; Wayner and Carter, 1987; Edwards et al., 1987; 1988; Gailit and Ruoslahti, 1988; Kirchhofer et al., 1990; Elices et al., 1991; Guan and Hynes, 1990; Mould et al., 1990; Dedhar and Gray, 1990; Vogel et al., 1990; Charo et al., 1990; Gullberg et al., 1990; Staatz et al., 1989; 1990).

Due to the paucity of available markers for α subunits, little is known about the distribution of other integrin heterodimers in the early embryo. In a recent study examining the distribution of the α_5 subunit of integrin, Muschler and Horwitz (1991) found that α_5 has a widespread distribution in the early embryo, being associated with endothelial and mesenchymal cells at stages when α_6 reactivity is highest. α_5

immunoreactivity is persistent at later developmental stages (E10 and beyond), with a widespread distribution on a myriad of cell types. In contrast, it appears to be absent from most cells in the adult. Taken together with the present study, these results suggest that integrins are developmentally regulated with different α subunits being expressed at different times and on selected populations. In contrast to the α subunits, expression of β_1 appears to be more uniform both spatially and temporally. It is likely that regulation of heterodimers occurs by altering α expression with the β expression remaining constitutive.

α_6 expression on presumptive myotomal cells begins just as the epithelial somite undergoes an epithelial/mesenchymal transition to form the dermomyotome and sclerotome. The appearance of α_6 is coincident with that of earliest myotomal markers, such as acetylcholinesterase (Layer et al., 1988; Kaehn et al., 1988). By examining its expression as a function of time, one can infer the pattern of myotome formation. A subpopulation of immunoreactive cells first appears in the anterior and medial portion of the somite. The number of immunoreactive cells increases to rapidly fill in the space between the dermatome and sclerotome, in an anterior to posterior sequence. Partially formed α_6-reactive myotomes only were observed along 1 - 2 somite lengths and each somite takes approximately 110 minutes to form. This suggests that myotomal precursors arise and/or migrate between the dermatome and myotome during a 4 hour time interval. This migration pattern is consistent with that inferred from observations of acetylcholinesterase staining of the forming myotome (Layer et al., 1988; Kaehn et al., 1988). Interesting, antibodies against the β_1 subunit of integrin have been shown to disrupt trunk myoblast migration into the body walls and limbs (Jaffredo et al., 1988). Because α_6 codistributes with β_1 in the myotome, the receptor disrupted by the injected antibody is likely to be the $\alpha_6\beta_1$ heterodimer. α_6 expression persists on myoblasts as they populate the limb. It is present on myotubes and differentiated muscle throughout the embryonic stages examined. In turn, the muscles are surrounded by a laminin-rich matrix.

In the neural tube, α_6 is expressed on subpopulations of neurons, including commissural neurons and motor neurons. These may extend their axons along the laminin-rich basal lamina on the lateral margin of the neural tube and, in the case of motor neurons, out into the periphery containing interstitial laminin. This raises the possibility that laminin may serve as a permissive substrate for commissural and motor axons. Although α_6 is expressed prominently on numerous neurons and their cell bodies, it is not clear that the expression correlates with initial axon outgrowth. For example, neurofilament-positive cells are observed within cell bodies in the neural tube and on some processes extending out the forming ventral root prior to the expression of high levels of α_6 on their processes.

The α_6 subunit is observed on neural crest cells during the initial stages of their emigration from the neural tube. At this time, neural crest cells enter a cell-free space rich in laminin, fibronectin and other matrix molecules (Krotoski et al. 1986; Perris et al., 1991a,b). After migrating further ventrally and entering the cellular environment of the sclerotome, which contains lower levels of ECM molecules, α_6 immunoreactivity is no longer detectable on neural crest cells. It reappears on a subpopulation of neural crest cells at the level of the dorsal aorta. These cells belong to the sympathoadrenal sublineage (Doupe et al., 1985) and will form sympathetic neurons, chromaffin cells and cells of the aortic plexuses. α_6 expression apparently preceeds that of catecholamines which are first observed about a half day later (unpublished observation) and expression is maintained on both primary and secondary sympathetic chains. In contrast, α_6 is expressed on dorsal root ganglion cells only after gangliogenesis.

In a recent report by de Curtis et al. (1991), the expression, regulation and sequence of the avian α_6 subunit of integrin was described. The antibody used in our study recognizes this same subunit. Our immunocytochemical study suggests that α_6 integrins are high in the retina as early as the optic cup stage. Furthermore, we observed α_6 expression in the retina through adulthood.

In conclusion, our results show that the α_6 subunit of integrin has a developmentally regulated pattern of expression in the embryo. Notably, it appears in the developing nervous system and in presumptive myoblasts. In many places, its distribution overlaps with that of the β_1 subunit of integrin as well as with laminin, the natural ligand for $\alpha_6\beta_1$ heterodimers. Its expression pattern is dynamic and is down-regulated in the neural tube, but not on muscle, by embryonic day 7. The distribution pattern of this subunit is consistent with a possible role for $\alpha_6\beta_1$ heterodimers in numerous morphogenetic processes such as axon guidance and myoblast migration.

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