DEVELOPMENTAL BIOLOGY **202,** 29–42 (1998)
ARTICLE NO. DB988985

for Neural Crest Cell Migration

Sung H. Kil,* Catherine E. Krull,* Gordon Cann,†,‡ Dennis Clegg,‡ and Marianne Bronner-Fraser*,[1]

**Division of Biology 139-74, California Institute of Technology, Pasadena, California 91125; †Division of Oncology, Stanford University Medical Center, Stanford, California 94305; and ‡Neuroscience Research Institute, Department of Molecular, Cellular, and Developmental Biology, University of California at Santa Barbara, Santa Barbara, California 93106*

We identify the α_4 subunit of integrin as a predominant integrin expressed by neural crest cells in both avian and murine embryos. Using degenerate primers, we obtained a PCR fragment of the chick integrin α_4 subunit that was subsequently used to clone the full-length subunit with a predicted amino acid sequence 60% identical to human and mouse α_4 subunits. *In situ* hybridization demonstrates that chick integrin α_4 mRNA is expressed at high levels by migrating neural crest cells and neural crest-derived ganglia at both cranial and trunk levels. An antibody against the murine α_4 subunit revealed similar distribution patterns in mouse to chick. In addition to neural crest cells, the integrin α_4 subunit was later observed on the muscle masses of the limb, the apical ectodermal ridge, and the developing liver. To examine the functional role of the integrin α_4 subunit in neural crest cell migration, we used an explant preparation that allows visualization of neural crest cells in their normal environment with or without perturbing reagents. In the presence of a blocking antibody against the mouse integrin α_4 subunit, there was a profound abrogation of neural crest cell migration at trunk and hindbrain levels. Both the numbers of migrating neural crest cells and the total distance traversed were markedly reduced. Similarly, avian embryos injected with synthetic peptides that contain the integrin α_4 binding site in fibronectin displayed abnormal neural crest cell migration. Our results suggest that the integrin α_4 subunit is important for normal neural crest cell migration and may be one of the primary α subunits used for neural crest cell migration *in vivo*. Furthermore, the integrin α_4 subunit represents a useful neural crest marker in the mouse.

INTRODUCTION

Interactions between cells and their surrounding extracellular environment are involved in many aspects of normal development, including gastrulation, neurulation, neurite outgrowth, and organogenesis. The extracellular matrix (ECM) is an organized network composed of glycoproteins including fibronectin and laminin, proteoglycans, and glycosaminoglycans that are secreted locally by cells (Hay, 1989). In addition to serving as a structural support, the ECM plays an important role in several developmental processes, including cell differentiation, cell migration, and axon guidance (Adams and Watt, 1989; Sanes, 1989; Neugebauer, 1991; Chiquet, 1989; Adams, 1989; West, 1979; Spiegelman, 1983; Bronner-Fraser, 1986b; Gong, 1996). For example, fibronectin is involved in gastrulation (Boucaut *et al.*, 1984) and neural crest cell migration (Bronner-Fraser, 1986b; Olsson, 1996), whereas tenascin and laminin play a role in chondrocyte differentiation (Mackie, 1987) and muscle development (Umbhauer, 1994; von der Mark, 1989; Gu, 1994).

Neural crest cells are highly motile cells that interact with the extracellular matrix as they emanate from the neural tube and migrate extensively to their final destinations. Neural crest cells at different axial levels of the embryo follow distinct migratory pathways (Le Douarin, 1982). Cranial neural crest cells migrate ventrally underneath the ectoderm to give rise to cranial sensory ganglia, the ciliary ganglion of the eye, and cartilaginous components of the face (Le Douarin, 1982; Noden, 1983; Nichols, 1986). Trunk neural crest cells migrate dorsolaterally underneath the ectoderm and ventromedially through the rostral half of the somitic sclerotome. The ventromedial

[1] To whom correspondence should be addressed. Fax: (626) 395-7717. E-mail: mbronner@caltech.edu.

0012-1606/98 $25.00

stream gives rise to the dorsal root ganglia, sympathetic ganglia, cells of the adrenal medulla, and aortic plexuses whereas the dorsolaterally migrating cells give rise to melanocytes (Le Douarin, 1982; Rickman *et al.*, 1985; Bronner-Fraser, 1986a; Loring and Erickson, 1987; Serbedzija *et al.*, 1989; Serbedzija *et al.*, 1992). The ECM lining neural crest pathways has been well-characterized and contains fibronectin, laminin, collagen, and vitronectin (reviewed by Erickson and Perris, 1993). A number of these molecules are important for neural crest cell migration both *in vitro* and *in vivo* (Rovasio *et al.*, 1983; Bronner-Fraser and Lallier, 1988; Bronner-Fraser, 1988; Delannet *et al.*, 1994; Perris *et al.*, 1991b; Poole and Thiery, 1986).

Integrins comprise a family of glycoprotein receptors that mediate the adhesion of numerous cell types, including neural crest cells, to ECM molecules (reviewed by Hynes, 1987, 1992). Integrins are composed of α and β subunits that are noncovalently linked. The association of a particular α and β subunits determines the receptor's specificity for a given ligand (Hynes, 1992). Integrins have been shown to be required for normal development. Mice null for integrin α_5 and α_4 are early embryonic lethals, due to defects in mesodermal derivatives, epicardium and coronary vessel development, and failure of chorioallontoic fusion (Yang *et al.*, 1993, 1995). Perturbation experiments using antibodies against the β_1 subunit have demonstrated an important role for β_1 integrins in avian somite development (Jaffredo *et al.*, 1988) and cranial neural crest cell migration *in vivo* and *in vitro* (Bronner-Fraser, 1985, Bronner-Fraser, 1986b; Lallier *et al.*, 1992). Cultured trunk neural crest cells possess several integrin receptors for vitronectin ($\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$; Delannet *et al.*, 1994) and laminin ($\alpha_1\beta_1$; Duband *et al.*, 1992; Lallier and Bronner-Fraser, 1992). Migrating trunk neural crest cells express low levels the α_1 subunit *in vivo* (Duband *et al.*, 1992). Antibodies against the integrin β_1 subunit block trunk neural crest attachment to fibronectin and laminin *in vitro* and perturb cranial neural crest cell migration *in vivo* (Lallier and Bronner-Fraser, 1992; Bronner-Fraser, 1986b). In addition, antisense perturbation experiments show that integrin α subunits are important for neural crest cell migration (Lallier and Bronner-Fraser, 1993; Kil *et al.*, 1996b). However, these studies were unable to identify specific integrin α subunits that are expressed by neural crest cells and play an important role in neural crest cell migration.

Although a subpopulation of neural crest cells (Stepp *et al.*, 1994) and neural crest derivatives (Sheppard *et al.*, 1994) have been shown to express the α_4 subunit of integrin (Stepp *et al.*, 1994; Sheppard, 1994), no function in neural crest cell migration or differentiation has been described. The integrin α_4 subunit heterodimerizes with integrin β_1 and β_7 subunits to form a receptor for fibronectin through a non-RGD site, an EILDV sequence within the type III connecting segment region (CS-1) of the molecule (Mould, 1990). *In vitro* studies showed that a synthetic peptide to CS-1 region of fibronectin promotes neural crest attachment and migration (Dufour *et al.*, 1988). It also binds to a counterreceptor for VCAM-1, a member of the immunoglobulin superfamily (Guan and Hynes, 1990) through an LDV-like sequence (May, 1993). In addition, integrin $\alpha_4\beta_1$ has been shown to be a receptor for thrombospondin-1 (Yabkowitz, 1993).

In the present study, we examined the role of the integrin α_4 subunit in neural crest cell migration in chicken and mouse embryos. Whole mount *in situ* hybridization with an avian α_4 subunit probe and staining with a mouse anti-integrin α_4 antibody revealed similar patterns of expression in avian and mouse neural crest cells. To test the functional importance of the α_4 subunit in neural crest cell migration, we perturbed binding of the α_4 subunits using a blocking mouse antibody in mouse hindbrain and trunk explants. We noted a significant decrease in the numbers of neural crest cells that emigrated from the neural tube in the anti-α_4 antibody-treated versus control preparations. Furthermore, those neural crest cells that left the neural tube traveled a short distance compared to neural crest cells under control conditions. Our results suggest that the α_4 subunit of integrin is important for normal neural crest cell migration *in vivo*. In addition, it represents a useful neural crest marker in the mouse.

EXPERIMENTAL METHODS

Cloning and sequencing of the avian integrin α_4 subunit. To clone the chicken integrin α_4 subunit, we used integrin α subunit degenerate primers (described by Erle, 1991) and reverse transcription–polymerase chain reaction (RT–PCR) to amplify multiple integrin α subunit cDNAs from Embryonic Day 6 chick retina. Using this approach, we obtained partial PCR clones encoding integrin α_2, α_v, α_4, α_6, and α_8 subunits that were approximately 290 bp long. The PCR fragment of integrin α_4 subunit was then subcloned into the Bluescript KS+ vector (Stratagene) and used to screen a chicken stage 12–15 (Hamburger, 1951) cDNA library (kindly provided by Drs. D. Wilkinson and A. Nieto). Using a conventional screening protocol (Sambrook, 1989), we obtained a 3.9-kb integrin α_4 subunit clone. For sequencing, we used both a chain-termination sequencing kit (Sequence version 2.0 DNA kit from Amersham) and an automated PCR sequencing kit (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit from ABI). Sequencing reactions were separated on a polyacrylamide gel or an automated sequencing gel apparatus from ABI at the Caltech sequencing facility. Sequence analysis was accomplished with the aid of Macvector, the AssemblyLGN, the Genework program (Oxford Molecular Group), and the GCG software package from the Wisconsin Genetic Group.

In situ hybridization and HNK-1 antibody immunohistochemistry. Fertile White Leghorn chick eggs (HY-line International) were incubated at 38°C for 1.5 to 4 days, collected, rinsed in Howard Ringer's solution and fixed with 4% paraformaldehyde overnight. Embryos were then dehydrated through a series of methanol/phosphate-buffered saline (PBS) with 0.1% Tween 20 solutions (Pwt). To synthesize the integrin α_4 subunit RNA probe, we followed the protocol described by Wilkinson (Wilkinson, 1992). For most *in situs*, we used a 500-bp RNA probe encompassing portion of extracellular domain of integrin α_4 subunit. In other experiments, the entire gene and 700 bp that correspond to another

region of the extracelluar domain were used as probes. All probes gave identical results. The method of Henrique *et al.* (Henrique *et al.*, 1995) was used to perform *in situ* hybridization. After color development, embryos were cleared in 70% methanol at 4°C and photographed. Prior to sectioning, embryos were incubated in 5 and 15% sucrose in PBS for 2 h and overnight, respectively, and embedded in gelatin for 4 h. Embryos were frozen in liquid nitrogen and cryosectioned at 25–30 μm. Sections were affixed to slides precoated with polylysine (Fisher) and coverslipped with Gel Mount (Biomeda). Some embryos were dehydrated in an ethanol series, embedded in paraplast (Oxford Labware), and microsectioned at 20–25 μm. Sections were affixed to slides precoated with polylysine in the presence of 0.2% gelatin in water. Sections were then deparaffinized in a series of histosol and ethanol solutions. For colocalization studies with HNK-1 antibody, the sections (after deparaffinization) were incubated with HNK-1 antibody supernatant overnight at 4°C. After washing, secondary antibody (goat anti-mouse IgM) conjugated to fluorescein was added for 1 h at room temperature. Following washes with PBS, sections were coverslipped with Gel Mount (Biomeda). Sense probe was used as controls and results were negative.

Microinjection of CS-1 peptide into avian embryos. To test the function of the CS-1 region of fibronectin in avian neural crest cell migration, 10 mM CS-1 peptide (DELPQLVTLPHPNLHG-PEILDVPST) or scrambled CS-1 peptide (DELPQLVTLPHPNLHG-PPVISELID) was injected into the mesenchyme lateral to the mesencephalon on the left side of chicken embryos at stage 8–9 (Hamburger, 1951) using a micromanipulator. The peptides were synthesized by the UCSB Advanced Instrumentation Center and HPLC purified. After the injection, embryos were incubated at 37°C for 18–24 h. Embryos were fixed with Carnoy's fixative and embedded in paraplast.

Immunocytohistochemistry with HNK-1 antibody was performed as described above.

PS-2 antibody preparation. The antibodies PS-2 (anti-mouse integrin α_4 antibody ascites from SCID mice prepared by TSD Services of Germantown, NY) and MAB 1997 (non-function-blocking integrin β_1 antibody, Chemicon) were dialyzed extensively in PBS at 4°C for 1 h and once overnight. Dialysis membrane (molecular cutoff of 12,000–14,000 Da, Spectrapor) was boiled in 5 mM EDTA/200 mM sodium bicarbonate for 5 min, rinsed twice, and autoclaved for 10 min in a large volume of deionized water. After dialysis, the antibody was sterilized using a 0.4-μm microcentrifuge filter unit (Fisher) and stored at 4°C.

Immunohistochemistry with PS/2 antibody. E7.5–E10.5 mouse embryos (Kaufman, 1992) from BDF-1 female mice (Caltech) were removed from the mother and placed in sterile PBS. After removal of extraembryonic membranes, the embryos were placed in DMEM–F12 medium (Gibco) containing 10% fetal bovine serum (FBS) and 0.1% penstrep (Gibco). Subsequently, embryos were fixed in 2% paraformaldehyde for 2 h at 4°C, incubated in 5 and 15% sucrose overnight, and embedded in gelatin for 4 h to overnight. Embryos were frozen in liquid nitrogen and cryosectioned at 14 μm. Sections were postfixed with methanol for 5 min at −20°C. After fixation, sections were washed with 0.1 % BSA–PBS, blocked with 10% rabbit serum in PBS/0.1% BSA for 30 min, and incubated with primary antibody, PS/2 (anti-integrin α_4 antibody), overnight. The sections were washed three times with PBS for 5 min and incubated with a biotinylated secondary antibody (anti rat IgG) for three hours at room temperature. Sections were then fixed in methanol containing 0.3% H_2O_2 for 30 min and washed three times for 5 min in PBS. After methanol fixation, sections were incubated with ABC reagent mix (Vector) for 30 min, incubated in DAB substrate (Vector) for 40 min, dehydrated in ethanol and histosol, and mounted with Permount (Fisher). Control experiments were done and the results were negative.

Mouse hindbrain explants. Mouse explants were prepared as described previously (Krull *et al.*, 1995, 1997). To label premigratory neural crest cells, the lumen of the neural tube in E8.5 mouse embryos was injected with DiI (stock concentration of 5 mg/ml diluted 1:10 in 3 M sucrose; Serbedzija *et al.*, 1992). After DiI injection, the region including the hindbrain was excised from the embryo by making two transverse cuts caudal to the forebrain and rostral to the third somite. Tissues ventral to the notochord were removed with fine tungsten needles. These hindbrain explants, including ectoderm, neural tube, and notochord, were then incubated in DMEM–F12 medium containing 10 μg/ml of PS/2 (anti-integrin α_4 subunit antibody). As controls, explants were either incubated with DMEM–F12 medium alone or medium containing 10 μg/ml of MAB 1997 (a non-function-blocking β_1 antibody, Chemicon). Explants were placed on 0.4-μm culture inserts (Millipore) precoated with 20 μg/ml of fibronectin for 1 h at room temperature. One milliliter of medium (DMEM–F12, with 10% FBS, 0.1% penstrep, with or without antibodies) was added to the bottom of the insert. Explants then were incubated at 37°C for 24–48 h and photographed using a Zeiss Axiovert microscope equipped with fluorescence optics or a confocal microscope (Bio-Rad).

Mouse trunk explants. Mouse trunk regions of E9.5 mouse embryos containing the last six somites were prepared in a manner similar to that for the hindbrain explants described above. Premigratory neural crest cells were labeled by filling the lumen of the neural tube with DiI as described previously (Serbedzija *et al.*, 1992). Following antibody treatment as described above, the extent of neural crest cell migration in the explants was analyzed using a confocal microscope (Bio-Rad).

Measurement of neural crest cell migration and distance traveled. To quantify neural crest cell migration, fixed hindbrain and trunk neural crest explants were visualized with a confocal microscope equipped with a 10× objective and images were collected using Comos software. The migration distance (μm) was measured as a straight-line distance between the lateral edge of the neural tube to a given labeled cell using the measurement tool of Photoshop 4.0 software. For the hindbrain explants, the straight-line distance traversed by neural crest cells migrating caudal to the otic vesicle was measured between the lateral edge of the neural tube near the otic vesicle and the cells' endopoint (three explants for a given condition). For trunk explants, the distance from the neural tube of neural crest cells within a 10-somite region were measured (five explants for a given condition). Statistical analyses were done using the χ^2 test for independence between two or more samples.

RESULTS

Cloning of Chicken Integrin α_4 Subunit

Degenerate primers for integrin α subunits (Erle, 1991) were employed to screen the E6 chick retina cDNA library, from which we obtained a 500-bp PCR fragment with a sequence corresponding to the integrin α_4 subunit. The PCR fragment was subcloned into the Bluescript KS+ vector and used as a probe to screen a stage 13–15 avian cDNA library. A 3.9-bp gene was cloned, sequenced, and

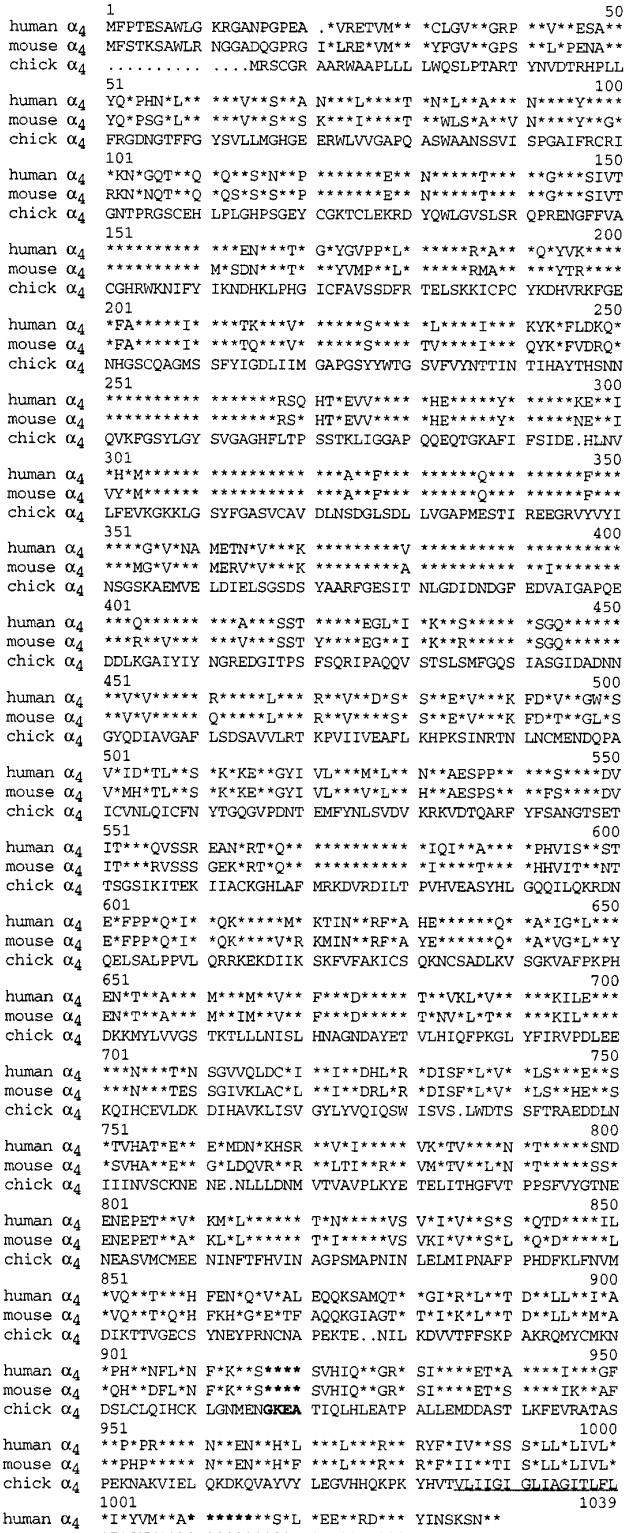

FIG. 1. Comparison of avian integrin α_4 subunit to human and mouse integrin α_4 proteins. A 3.9-bp cDNA was cloned that encodes a protein that is 60% identical to the human and mouse integrin α_4 subunit. The avian integrin α_4 protein contains the GFFKR (in bold) sequence in the cytoplasmic domain of all integrin α subunits and the GKEA (in bold) sequence that is unique to the α_4 subunits. The putative transmembrane domain is underlined. Astericks and periods denote conserved amino acids and missing amino acids, respectively.

analyzed. The cDNA of the integrin α_4 subunit encodes a protein that is 60% identical to the human (Takada, 1989) and mouse (Neuhaus, 1991) integrin α_4 subunit (Fig. 1) but is 19 and 20 amino acids shorter at the N-terminus than human and mouse integrin α_4 proteins. At the nucleotide level, the avian integrin α_4 subunit is 69 and 68% identical to the human and mouse genes, respectively. The protein contains a long extracellular domain, a transmembrane domain, and a short cytoplasmic domain (30 amino acids) that is characteristic of most integrin α subunits (reviewed by Hynes, 1987). In addition, the integrin α_4 protein contains the GFFKR (Fig. 1) sequence in the cytoplasmic domain that is conserved in other integrin α subunits and is thought to play a role in inside-out signaling (reviewed by Schwartz *et al.*, 1995). Importantly, the GKEA sequence (Fig. 1), unique to the α_4 subunit (Takada, 1989; Neuhaus, 1991), is present in the chick gene. These results suggest that this integrin corresponds to the avian homolog of the α_4 subunit.

Integrin α_4 Subunit mRNA Expression in Avian Neural Crest Cells

Whole mount *in situ* hybridization with a digoxygenin-labeled 500-bp integrin α_4 subunit RNA probe was performed on chicken embryos ranging from stage 5 to stage 17 (Hamburger, 1951). Prior to initiation of cranial neural crest cell migration at the eight-somite stage, strong expression was observed in premigratory cranial neural crest cells situated within the dorsal midbrain (Figs. 2A and 2B). Subsequently, integrin α_4 mRNA was expressed by cranial neural crest cells during their migratory phase both at midbrain (Figs. 2D and 2E) and hindbrain (Figs. 2G and 2H) levels. In addition to expression in presumptive neural crest cells, there was intense expression in Hensen's node and in the mesoderm at the level of the open neural plate (Fig. 2C). Integrin α_4 subunit expression was also noted in the nasal and optic placode, the otic vesicle, and the trigeminal ganglia in the cranial region.

In the trunk region, prominent integrin α_4 expression was observed in migrating trunk neural crest cells (Figs. 3A–3D). The myotome and lateral mesoderm displayed high levels of integrin α_4 mRNA expression, as did the developing musculature and apical ectodermal ridge of the limb (data not shown).

To verify that cells expressing the α_4 subunit mRNA were indeed neural crest cells, embryos were processed by *in situ* hybridization with the integrin α_4 probe and subse-

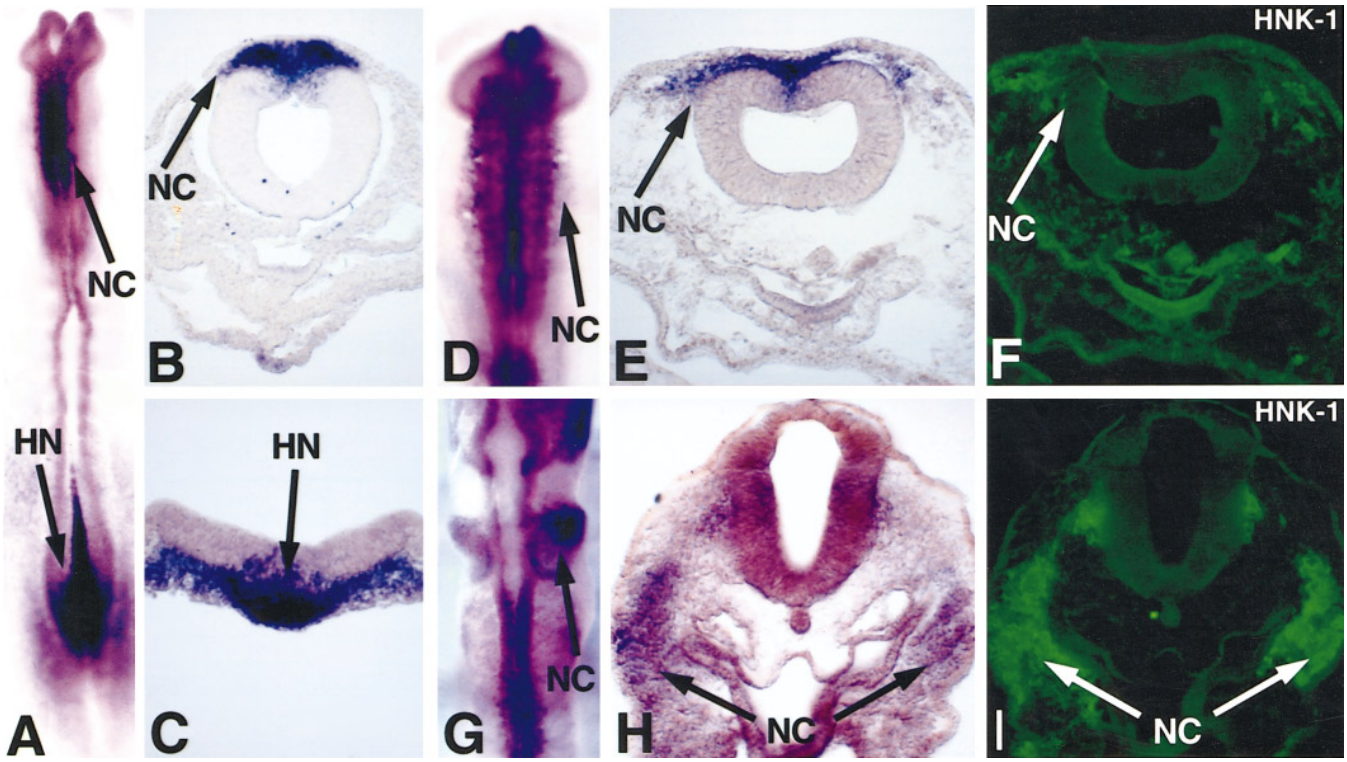

FIG. 2. Integrin α_4 mRNA was detected in cranial regions of 8- to 15-somite chick embryos as seen in sections through embryos after *in situ* hybridization with integrin α_4 mRNA. At the 8-somite stage, strong expression was observed in premigratory cranial neural crest cells (NC) situated within the dorsal midbrain neural tube (A and B). Integrin α_4 mRNA was expressed by cranial neural crest cells during their migratory phase both at midbrain (D and E) and hindbrain (G and H) levels. In addition, there was strong expression in Hensen's node (HN) and in the mesoderm at the level of the open neural plate (A and C). Colocalization of HNK-1 immunoreactivity and integrin α_4 mRNA expression was observed in migrating neural cells between the epidermis and cranial mesenchyme (E and F, H and I). The HNK-1 antibody does not recognize premigratory and early migrating neural crest cells.

quently stained with the HNK-1 antibody, which recognizes a carbohydrate epitope on most migrating neural crest cells (Vincent, 1984; Tucker *et al.*, 1984). In cranial regions, colocalization of HNK-1 immunoreactivity and integrin α_4 mRNA expression was observed in migrating neural cells in the cranial mesenchyme (Fig. 2H and 2I). In contrast, premigratory and early migrating neural crest cells within and above the dorsal neural tube expressed the α_4 subunit (Fig. 2E), but did not yet express the HNK-1 epitope (Fig. 2F), which only becomes detectable following their departure from the neural tube (Teillet, 1987). Trunk neural crest cells migrating ventrally through the rostral portion of the somitic sclerotome also coexpressed the HNK-1 epitope and integrin α_4 mRNA (Figs. 3C and 3D).

Integrin α_4 Subunit Protein Expression in Murine Neural Crest Cells

In the mouse, the PS/2 antibody recognizes the integrin α_4 subunit and has been used to functionally inhibit lymphocyte adhesion to fibronectin (Miyake, 1991). We used this antibody to compare integrin α_4 expression in the chick and mouse. In E8.5 mouse embryos, we noted that cranial neural crest cells displayed strong α_4 integrin immunoreactivity (Figs. 4A–4C) at the level of the forebrain, midbrain, and hindbrain. Migrating neural crest cells that have entered the first branchial arch demonstrated strong α_4 protein expression. At later stages, α_4 immunoreactivity was prevalent in neural crest-derived cranial ganglia including the trigeminal ganglia (Fig. 4D).

In the trunk region of E9.5 embryos, neural crest cells displayed integrin α_4 immunoreactivity both during their migration as well as during condensation into dorsal root ganglia (DRG) and other derivatives (Figs. 4D–4F). In addition to the expression in the neural crest cells and their derivatives, integrin α_4 immunoreactivity was noted in the gut (Fig. 4E) and myotome (Fig. 4F). The expression in the DRG and myotome increased with developmental age and was pronounced by E11.0 (Fig. 4G). In addition, the apical epidermal ridge of the forelimb (Fig. 4H) and the hindlimb (data not shown), the myoblasts of the forelimb (Fig. 4H),

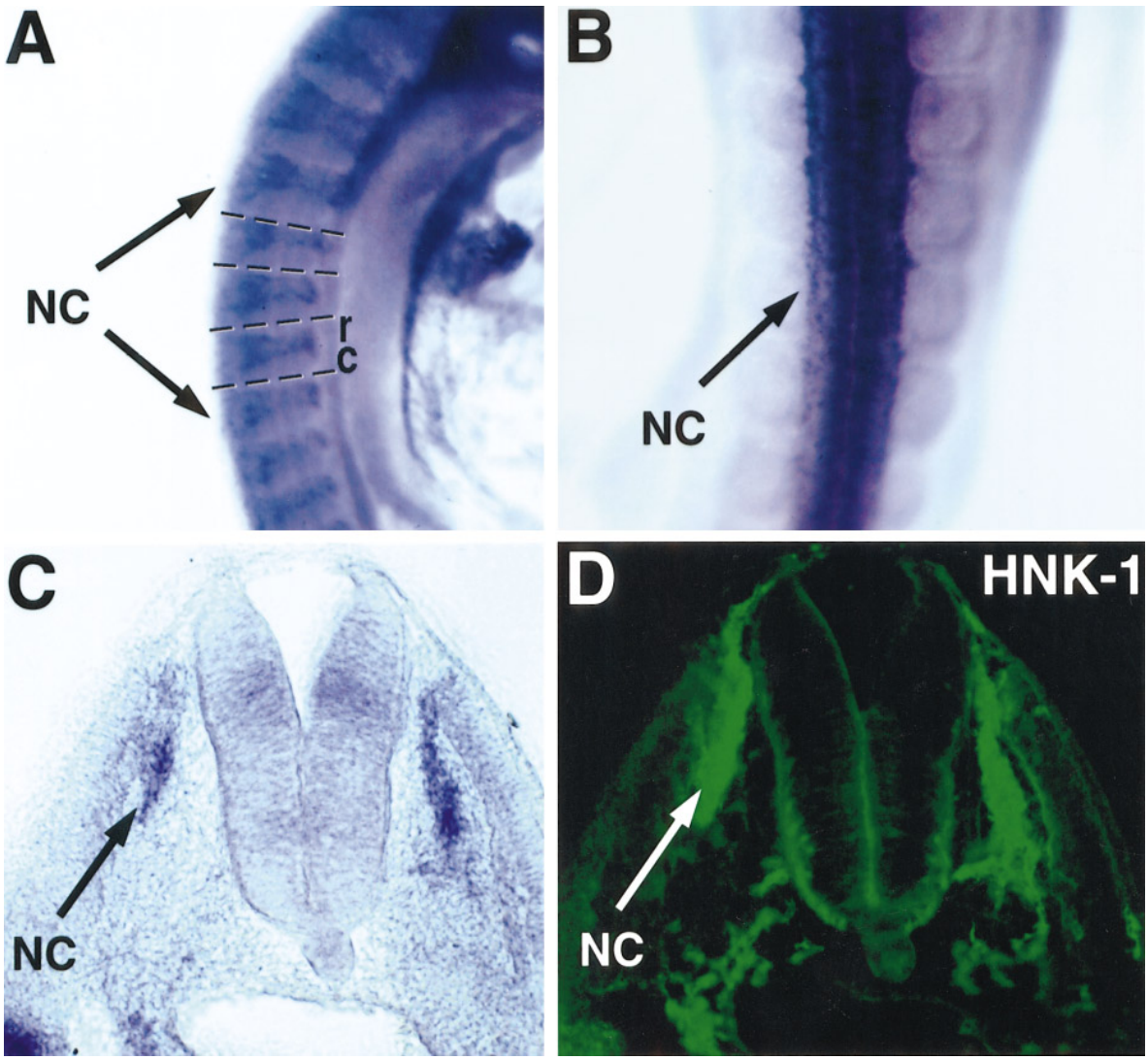

FIG. 3. Integrin α_4 subunit mRNA was detected in trunk regions of 27- to 30-somite chick embryos. Neural crest cells (NC) of a 27-somite-stage embryo express integrin α_4 mRNA as they migrate through the rostral half of the somite (A). Premigratory trunk neural crest cells stain intensely for integrin α_4 mRNA expression in the caudal region of a 30-somite-stage embryo (B). Trunk neural crest cells migrating ventrally through the rostral (r) portion of the somitic sclerotome also coexpress the HNK-1 epitope and integrin α_4 mRNA (C and D). The HNK-1 antibody does not recognize premigratory and early migrating neural crest cells. The dashed lines represent borders of somites. c denotes the caudal portion of the somitic sclerotome.

the liver (Fig. 4I), and the heart (data not shown) showed prominent integrin α_4 immunoreactivity.

Some differences were noted between the expression patterns of avian integrin α_4 mRNA and mouse integrin α_4 protein. For example, there was little or no integrin α_4 immunoreactivity localized to premigratory mouse neural crest cells whereas high levels of α_4 mRNA were noted in premigratory avian neural crest cells in the cranial region. Similarly, in avian embryos, the dorsal half of the neural tube displayed low levels of α_4 expression, whereas no protein expression was noted in the mouse neural tube. It is unclear whether these represent species differences or reflect differences between mRNA and protein expression. With the exception of these differences, the avian and murine patterns overlapped.

Anti-α_4 Antibody Blocks Neural Crest Cell Migration in Mouse Hindbrain and Trunk Explants

We recently developed a novel explant preparation that allows direct visualization of neural crest cell migration in normal living tissue (Krull *et al.*, 1995, 1997) Advantages of

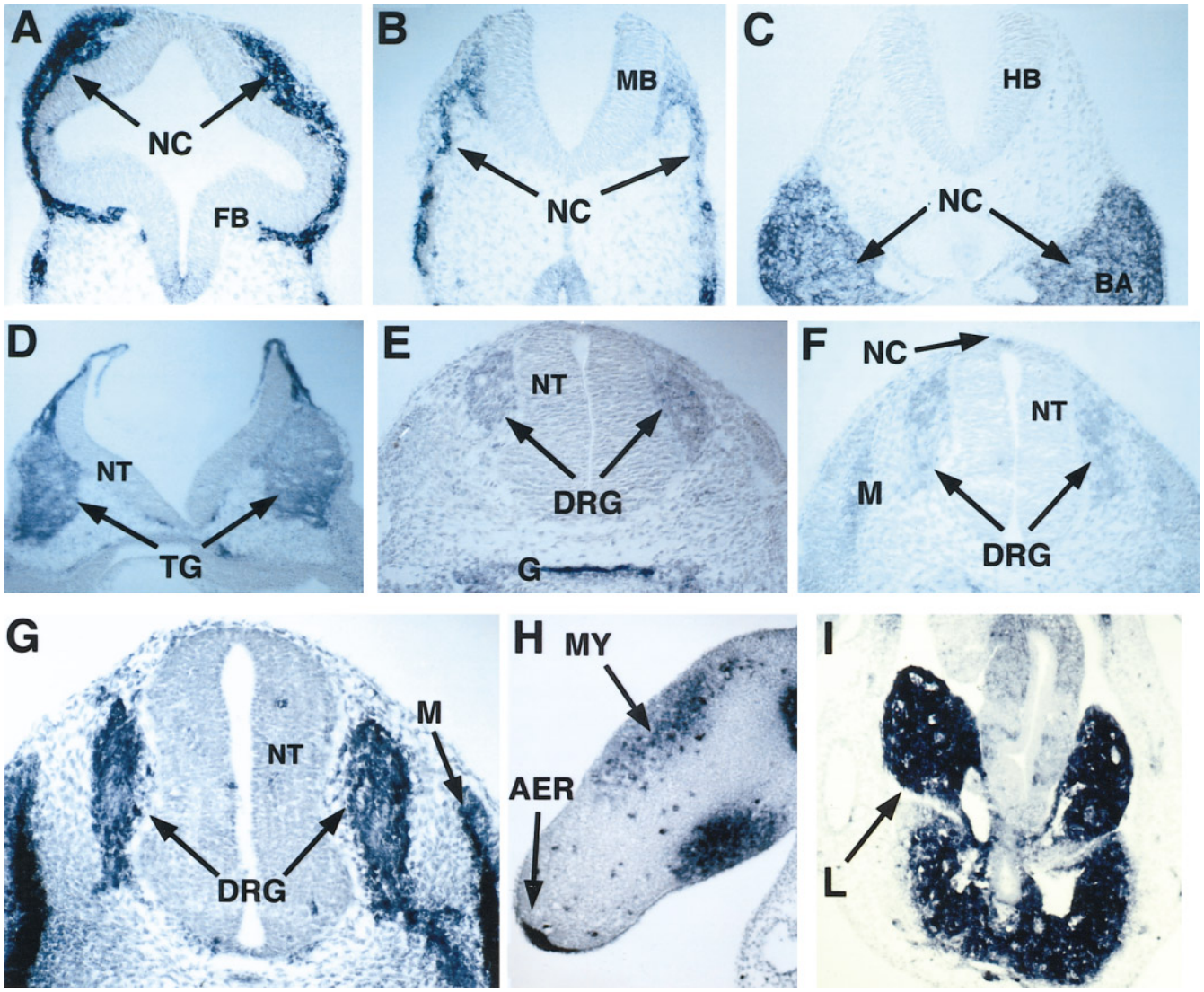

FIG. 4. Integrin α_4 subunit protein was detected in early mouse embryos. In E8.5 mouse embryos, cranial neural crest cells displayed strong PS/2 immunoreactivity (A–C) at the level of the forebrain (FB), the midbrain (MB), and the hindbrain (HB). Migrating neural crest cells that entered the first branchial arch (BA) had strong integrin α_4 protein expression (C). In the trunk region of E9.5 embryos, neural crest cells displayed prominent integrin α_4 immunoreactivity both during their migration as well as during condensation into dorsal root ganglia (DRG) and trigeminal ganglia (TG; see D–F). In addition to expression in neural crest cells and their derivatives, integrin α_4 immunoreactivity was noted on the myotome (M; see F). Expression in the DRG and myotome increased with developmental age and were pronounced by E11.0 (G). In addition, the apical epidermal ridge (AER) and myoblasts (MY) of the forelimb (H), liver (L; see I), and gut (G; see E) showed prominent integrin α_4 immunoreactivity.

these preparations are that they offer high accessibility to perturbing reagents, easy visualization of migrating neural crest cells, and applicability to avian or murine systems. To examine the role of the integrin α_4 subunit in cranial and trunk neural crest cell migration, we added function-blocking PS/2 antibody against the α_4 subunit of integrin as a perturbing reagent to explants removed from the mouse hindbrain and trunk.

The neural tube at hindbrain levels in E8.5 mouse embryos was labeled with DiI to mark premigratory neural crest cells (Serbedzija *et al.*, 1992). Hindbrains plus adjacent tissue (including the otic placode and three rostral-most somites) were excised from labeled mouse embryos and incubated for three hours in (1) control medium without antibody (n = 10; Fig. 5A), (2) control medium with a non-function-blocking antibody to integrin β_1 subunit (n = 5, Fig. 5B), or (3) medium containing a function-blocking anti-integrin α_4 antibody (n = 11 at 1 μg/ml and n = 14 at

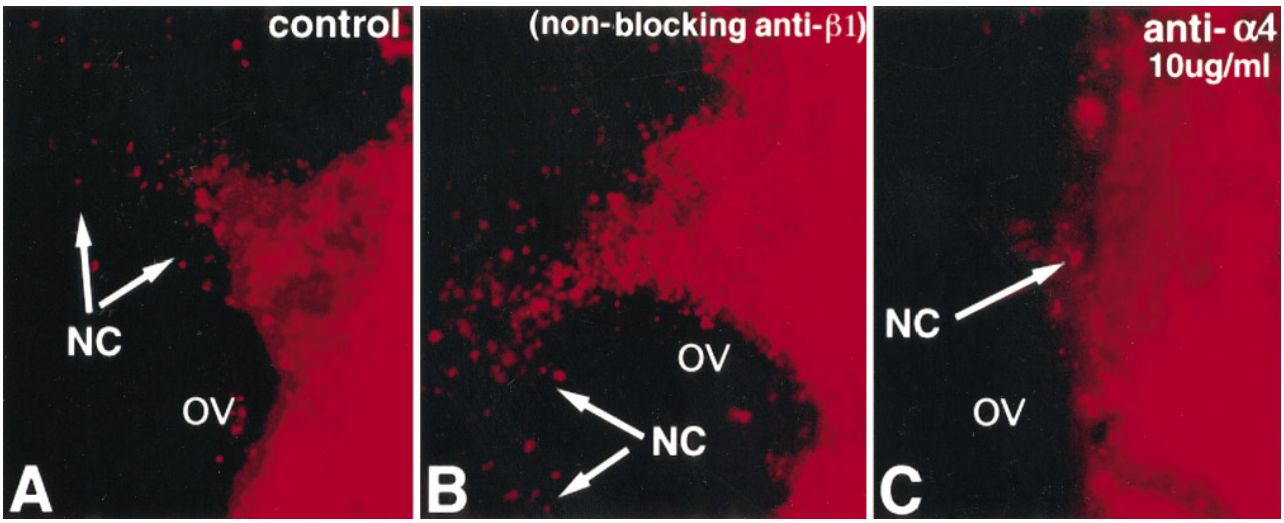

FIG. 5. To test the function of the integrin α_4 subunit in cranial neural crest cell migration, hindbrain explants were treated with anti-integrin α_4 (PS/2) antibody. The neural tube at hindbrain levels in E8.5 mouse embryos was labeled with DiI to mark premigratory neural crest cells. Labeled hindbrains plus adjacent tissue (including the otic placode and three rostral-most somites) were incubated for 3 h in (A) control medium without antibody, (B) control medium with a non-function-blocking antibody to integrin, or (C) medium containing a function-blocking anti-integrin α_4 antibody. Numerous migrating neural crest cells (NC) were observed in characteristic streams around the otic vesicle (OV) in all control explants. In the presence of 10 μg/ml of PS/2 antibody, hindbrain neural crest cell migration was markedly reduced.

10 μg/ml; Fig. 5C). The explants were positioned on Millicell inserts and incubated for an additional 24–48 h.

In the presence of 10 μg/ml of anti-integrin α_4 antibody, hindbrain neural crest cell migration was markedly reduced. Only three explants (n = 14) exhibited detectable neural crest cell migration (Fig. 7A). There was a 57% reduction in number of neural crest cells (n = 23) that emigrated from the neural tube when compared to the number of cells emigrated in control explants (n = 77 with medium alone and n = 81 with control antibody). Neural crest cells in the anti-integrin α_4 antibody-treated explants were not found at distances greater than 200 μm away from the neural tube, whereas 30–35% neural crest cells in the control antibody-treated and nontreated explants migrated distances greater than 200 μm. Numerous migrating neural crest cells were observed in characteristic streams around the otic vesicle in all control explants. For hindbrain explants, the P value for explants treated with the control anti-β_1 antibody compared with no antibody was $>$0.25. The P value for explants in the presence of the PS/2 anti-α_4 antibody compared with controls was $\leq$0.05.

To examine the potential function of the integrin α_4 in trunk neural crest cell migration, the neural tubes of E9.5 mouse embryos were prelabeled with DiI. Trunk regions containing the last 6 somites plus the neural tube of 20- to 25-somite-stage embryos were explanted as described (Krull *et al.*, 1997; Studer, 1996). In control explants treated without antibody (n = 16; Fig. 6A) and in the presence of the non-function-blocking anti-integrin β1 antibody (n = 13; Fig. 6B), neural crest cells migrate in their typical metameric pattern through the rostral but not caudal half of each somitic sclerotome. In presence of the anti-integrin α_4 antibody (n = 15; Fig. 6C), we observed that 7 of 15 explants showed nearly complete inhibition of neural crest cell emigration. Analysis of explants showed a 70% reduction in the numbers of neural crest cells (n = 98) that emigrated from the neural tube compared with control explants (n = 323 with medium alone and n = 262 with control antibody). Furthermore, those neural crest cells in treated explants that left the neural tube only traveled a short distance compared to neural crest cells under control conditions. Neural crest cells in the anti-integrin α_4 antibody-treated explants were not found at distances greater than 150 μm from the neural tube, whereas 10–17% of the neural crest cells in the control antibody-treated and nontreated explants migrated to distances greater than 150 μm (Fig. 7B). For trunk explants, the P values were $\leq$0.005 for explants treated with the control anti-β_1 compared with blocking anti-α_4 antibodies. However, the segmental pattern of migration was unaffected by addition of the anti-α_4 antibody. These results indicate that the integrin α_4 subunit is required for neural crest cell emigration and migration in both the head and trunk.

CS Peptide Injections into Avian Embryos

Integrin α_4 subunit is a receptor for fibronectin and VCAM-1 (Mould, 1990; Guan and Hynes, 1990). The CS-1

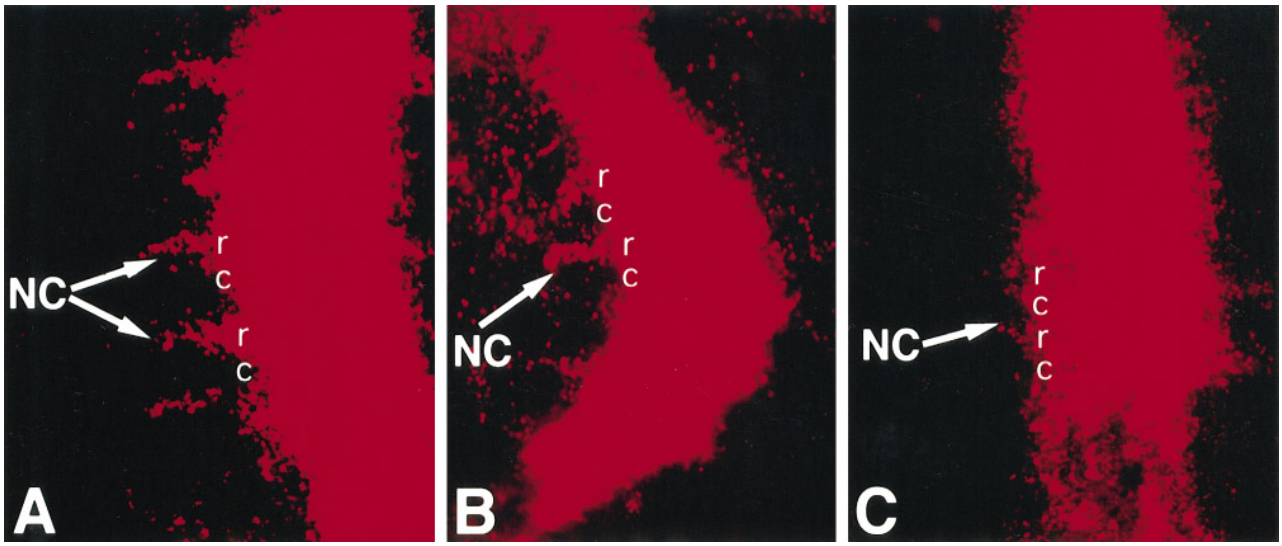

FIG. 6. To test the function of the integrin α_4 subunit in trunk neural crest cell migration, trunk explants were treated with anti-α_4 integrin (PS/2) antibody. The neural tubes of E9.5 mouse embryos were prelabeled with DiI and trunk regions containing the last six somites plus the neural tube were treated with antibodies. In control explants in the absence of antibody (A) or in the presence of the non-function-blocking anti-integrin β_1 antibody (B), neural crest cells (NC) migrate in their typical metameric pattern through the rostral (r) but not caudal (c) half of each somitic sclerotome. In the presence of the anti-integrin α_4 antibody (C), there was a nearly complete inhibition of neural crest cell emigration in 7of 15 explants tested.

peptide contains the LDV sequence of fibronectin that represents one of the binding sites for the integrin α_4 subunit (Wayner *et al.*, 1989; Guan and Hynes, 1990; Mould, 1990; Ruegg, 1992). CS-1 also inhbits adhesion to VCAM-1 (May, 1993), which contains an LDV-like sequence. To competitively inhibit cell interactions with the CS-1 domain, we injected CS-1 peptide into the mesenchyme adjacent to the mesencephalon of chick embryos. In injected embryos, we noted both neural tube defects and neural crest cells in ectopic locations (17 of a total of 23 embryos). Some neural crest cells were found in the lumen of the neural tube or situated on the dorsal portion of the neural tube (Fig. 8B), atypical of migratory neural crest. In addition, some neural tube cells detached from each other, appearing rounded rather than as columnar epithelial cells (data not shown). The control embryos injected with a scrambled version of CS-1 appeared normal with respect to cranial neural crest cell migration and neural tube morphology (7 of a total of 8 embryos; Fig. 8A). These results suggest that a molecule containing an LDV-like sequence is necessary for cranial neural crest cell migration.

DISCUSSION

We have taken advantages of both chick and mouse systems to show that the integrin α_4 subunit plays a functionally important role in neural crest cell migration. Both cranial and trunk neural crest cells display high levels of integrin α_4 subunit expression. Based on the finding that a blocking antibody significantly abrogates neural crest cell migration *in vivo*, we propose that the α_4 subunit is an important integrin α receptor on neural crest cells and is likely to be the major receptor utilized by the neural crest cell to emigrate from the neural tube. At the immunocytochemical level, its expression appears higher than that of other α subunits (α_v and α_1; Kil *et al.*, 1996a; Lallier and Bronner-Fraser, 1992), although no direct quantitation has been performed. Thus, this subunit can serve as a useful marker for migrating neural crest cells in both species. This may be particularly useful in mice, where few good markers for neural crest cells have been described.

Sequence Analysis of Avian Integrin α_4 Subunit

Sequence comparison of avian integrin α_4 subunit with human and mouse integrins α_4 subunits reveals some unusual properties. At the nucleotide level, avian integrin α_4 subunit is 69 and 68% identical to the human and mouse genes, respectively. However, the avian integrin α_4 shows lower amino acid identity to human and mouse integrin α_4 protein (60% to human and mouse) but a high nucleotide identity (69%; data not shown). The avian integrin α_4 is 19 and 20 amino acids shorter at the N-terminus than human and mouse integrin α_4 proteins, respectively. We believe the methionine indicates the N-terminus since the AUG is embedded in a consensus initiation sequence ((GC-C)GCCA/GCC**AUG**G; Kozak, 1987). The predicted protein

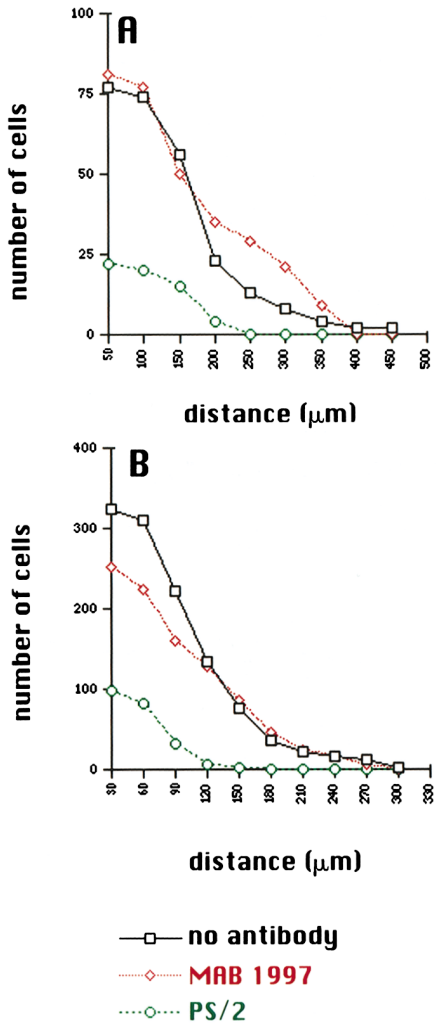

FIG. 7. To quantify neural crest cell migration observed in hindbrain and trunk explants treated with anti-α_4 integrin antibody, the migration distance (μm) was measured as a straightline distance from the lateral edge of the neural tube to a given cell's final position. For the hindbrain explants (A), the cell number and distance traversed by neural crest cells of the caudal stream around the otic vesicle were measured (three explants for a given condition). For trunk explants (B), the cell number and distance traversed of neural crest cells within 10-somite explants were measured (three explants for a given condition). The number of cells (y-axis) was plotted against distance migrated (x-axis). Statistical analyses were done using χ^2 test for independence between two or more samples. For hindbrain explants, the P value for explants treated with the control anti-β_1 antibody was >0.25. The P value for explants in the presence of the anti-α_4 integrin antibody was ≤ 0.05. For trunk explants, the P values were ≤ 0.005 for explants treated with the anti-β_1 and anti-α_4 antibodies.

sequence of our chick cDNA clone is 98% identical to a C-terminal fragment of ~100 amino acids in length previously reported by Stepp *et al.* (1994).

The Integrin α_4 Subunit is Expressed by Neural Crest Cells

Colocalization studies using the HNK-1 antibody, which recognizes migrating avian neural crest cells, and integrin α_4 mRNA show that many HNK-1-immunoreactive neural crest cells are also positive for integrin α_4 mRNA expression. The integrin α_4 subunit is expressed in premigratory cranial neural crest cells within the dorsal neural tube and, therefore, precedes both their emigration and the onset of HNK-1 epitope production. At the level of the trunk, we noted many neural crest cells that were positive for integrin α_4 mRNA during their migratory phase. Our colocalization study shows that many, but not all HNK-1-immunoreactive neural crest cells express the integrin α_4 subunit. Since not all HNK-1-positive cells express the α_4 subunit, we cannot rule out the possibility that there are other α subunits expressed by some of the migrating avian neural crest cell population.

Previous studies have alluded to the expression of the integrin α_4 subunit on neural crest cells and their derivatives. Our results concur with those of Sheppard *et al.* (1994), who reported the presence of the α_4 subunit on neural crest-derived dorsal root ganglia. However, our results differ markedly from those reported by Stepp *et al.* (1994), who noted a complex pattern of integrin α_4 mRNA and protein expression in the mesenchyme, somites, neural tube, ectoderm, and a subpopulation of trunk neural crest cells. In contrast, we find that the integrin α_4 subunit is expressed predominantly on neural crest cells, with little or no expression in the ectoderm and early somites, although there is later expression in the dermomyotome. Although the reasons for these differences are unclear, these previous authors used, for immunolabeling, an antibody against the human integrin α_4 cytoplasmic domain whose specificity is unknown in chick. We used several different RNA probes encompassing the entire coding region of integrin α_4 subunit for whole-mount *in situ* hybridization which gave identical results. In contrast, they performed radioactive *in situs* on sections using a different probe. Therefore, it is likely that the apparent differences are technical and that recent advances in *in situ* hybridization and antibody labeling technology have greatly increased the resolution and decreased the signal to noise problems associated with detection of mRNA.

Role of Integrin α_4 Subunit in Neural Crest Cell Migration

In the presence of a function-blocking integrin α_4 antibody, there was a significant reduction (57–70%) in the number of neural crest cells that migrated. Importantly, more than half of the explants tested did not show any neural crest cell emigration from the neural tube. In contrast, explants treated with a nonblocking integrin antibody were unaffected. These results suggest that integrin α_4 subunit is involved in both emigration and migration of neural crest cells. Our colocalization studies with HNK-1

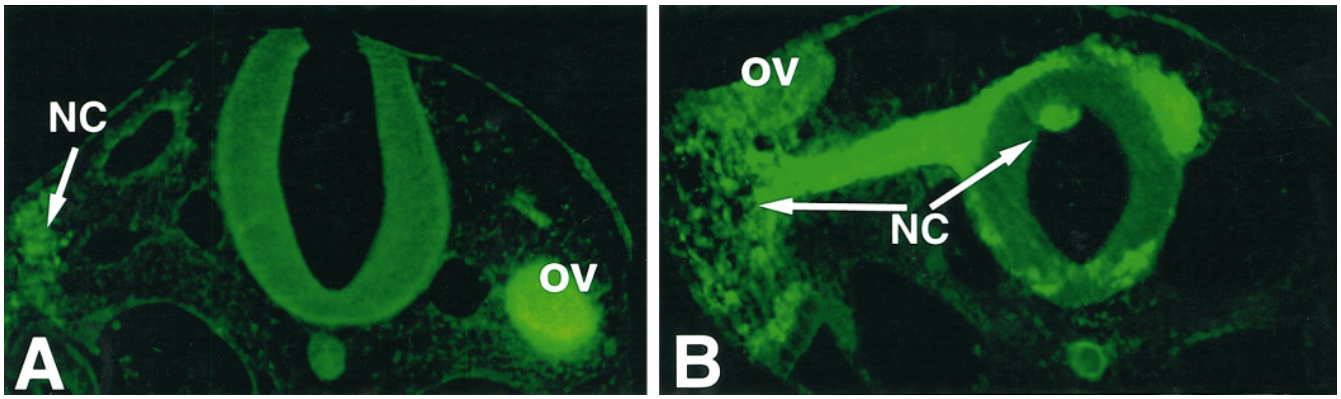

FIG. 8. Fluorescence photomicrographs of transverse paraffin sections showing the effects of the CS-1 peptide on cranial neural crest cell migration in embryos fixed 18–24 h after injection as observed by HNK-1 staining. Embryos injected with scrambled CS-1 peptides (A) have normal cranial neural crest (NC) migration at the level of the otic vesicle (OV) and neural tube; neural crest cells emigrate from the dorsal neural tube and migrate ventrally underneath the ectoderm. Embryos injected with CS-1 peptides (B) have neural crest cells (NC) in the lumen of the neural tube and on top of the neural tube.

antibody and integrin mRNA probe show that many but not all HNK-1-positive neural crest cells express integrin α_4 mRNA. HNK-1-positive/integrin α_4-negative neural crest cells may account for the neural crest cell migration observed in some treated explants. However, it is possible that some of the apparently nonexpressing cells express sufficient levels of α_4 to be functionally active without being detectable by *in situ* hybridization. It should be noted that the colocalization experiments were done in the chick, where a good neural crest marker is available, whereas the perturbation experiments were done in mice and the two systems may not be entirely equivalent.

Our transient perturbation experiments demonstrate a functional role for the integrin α_4 subunit at both cranial and trunk levels. However, disruption of the integrin α_4 subunit by homologous recombination in mice (Hynes *et al.*, 1995) did not produce an obvious neural crest phenotype. In homozygous α_4-null mice, neural crest-derived tissues such as the dorsal root and trigeminal ganglia are present. One possibility is that the subpopulation of neural crest cells that normally lack α_4 subunits contributes to the formation of dorsal root ganglia in integrin α_4-deficient mice. Alternatively, it is possible that other integrin subunits may functionally compensate in neural crest cells after inactivation of the α_4 subunit, accounting for the relatively normal ganglion formation. For example, Yang *et al.* showed that α_v integrin subunit can replace fibronectin receptor function in embryonic cells deficient in $\alpha_5\beta_1$ integrin (Yang *et al.*, 1996). Caution must be used, however, in interpreting apparently negative results from a genetic loss-of-function analysis. More thorough characterization of the null mice may reveal subtle defects in the numbers of emigrating neural crest cells and their distances of migration. In addition, targeted disruptions of the integrin α_4 subunit at the time of neural crest cell migration may yield results different than those obtained in mice that are α_4-null from conception. Thus, further experiments are necessary to compare and contrast the phenotypes of antibody perturbation and genetic loss-of-function of the integrin α_4 subunit.

In some cases, integrin antibodies have been shown to induce signaling through integrin receptors; for example, in the immune system, an antibody against $\alpha_4\beta_7$ can trigger T-cell blastogenesis (Teague *et al.*, 1994). Similarly, the TASC antibody against β_1 integrin has been shown to induce signal transduction in retinal neurons binding responding to laminin (Neugebauer and Reichardt, 1991). Although there is no evidence that the PS/2 antibody used in this study can induce cell signaling, we cannot formally rule out the possibility that addition of this antibody may influence neural crest cell behavior by activating an intracellular signaling cascade.

What Types of Neural Crest Cell–ECM Interactions Are Mediated by Integrin α_4?

Integrin α_4 associate with integrin β_1 and β_7 subunits. Both integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ bind to an LDV-like sequence found in the CS-1 region of fibronectin (Wayner *et al.*, 1989; Guan and Hynes, 1990; Mould, 1990; Ruegg, 1992) and VCAM-1, a member of the immunoglobulin superfamily (Elices *et al.*, 1990). Neural crest cells have been shown to express the integrin β_1 subunit (Krotoski *et al.*, 1986). In addition, fibronectin is abundant along neural crest cell pathways (Newgreen and Theiry, 1980; Thiery *et al.*, 1982; Krotoski *et al.*, 1986). Therefore, integrin α_4 likely associates with the β_1 subunit to form a receptor that mediates neural crest cell interaction with fibronectin. Results of our perturbation experiments using the CS-1 peptide, containing the LDV sequence (Mould *et al.*, 1990), suggest that the

CS-1 region of fibronectin may be important for neural crest cell migration. *In vitro* studies by Dufour *et al.* (1988) have shown that both the RGD and the CS-1 regions of the fibronectin molecule are required for neural crest cell migration. Some differences in peptide effects were observed: whereas the RGD region supported both attachment and spreading of neural crest cells, the CS-1 region only supported cell attachment (Dufour *et al.*, 1988). However, it is formally possible that CS-1 peptides inhibit interactions with unknown molecules. Although the integrin β_7 subunit has not yet been shown to be expressed by neural crest cells, $\alpha_4\beta_7$ may also be present on neural crest cells. VCAM-1 has been shown to be expressed on the neural crest-derived outflow tract of the embryonic heart, where it was colocalized with integrin α_4 in mouse (Sheppard, 1994) but was not detectable along the migratory pathways of mouse neural crest cells (Kwee, 1995). Hence, neural crest cell interaction with VCAM-1 by integrin α_4 subunit may not be relevant to neural crest cell migration. These findings suggest that integrin α_4 coupled with integrin β_1 may mediate the interaction of neural crest cells with fibronectin.

In summary, our findings show that the integrin α_4 subunit displays high levels of expression on both cranial and trunk neural crest cells in birds and mice. Furthermore, the α_4 subunit of integrin is functionally important for neural crest cell migration, such that blocking its function significantly abrogates neural crest cell migration *in vivo*. The results suggest that the α_4 subunit may be a predominant integrin α receptor on neural crest cells. It may play a particularly important role in neural crest cell emigration from the neural tube.

ACKNOWLEDGMENTS

We thank Drs. Arthur Lander, Diane O'Dowd, Ben Murray, and Martin Garcia-Castro for critical comments on the manuscript and constructive discussion during the course of this work. This study was supported by USPHS HD15527 and a grant from the Muscular Dystrophy Foundation to M.B.F.

REFERENCES

Adams, J. C., and Watt, F. M. (1989). Fibronectin inhibits the terminal differentiation of human keratinocytes. *Nature* **340,** 307–309.

Boucaut, J. C., Darribere, T., Boulekbache, H., and Thiery, J. P. (1984). Prevention of gastrulation but not neurulation by antibodies to fibronectin in amphibian embryos. *Nature* **307,** 364–367.

Bronner-Fraser, M. (1985). Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* **101,** 610–617.

Bronner-Fraser, M. (1986a). Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. *Dev. Biol.* **115,** 44–55.

Bronner-Fraser, M. (1986b). An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development. *Dev. Biol.* **117,** 528–536.

Bronner-Fraser, M. (1988). Distribution and function of tenascin during cranial neural crest development in the chick. *J. Neurosci. Res.* **21,** 135–147.

Bronner-Fraser, M., and Lallier, T. (1988). A monoclonal antibody against a laminin–heparan sulfate proteoglycan complex perturbs cranial neural crest migration *in vivo*. *J. Cell Biol.* **106,** 1321–1330.

Chiquet, M. (1989). Tenascin/J1/cytotactin: The potential function of hexabrachion proteins in neural development. *Dev. Neurosci.* **11,** 266–275.

Delannet, M., Martin, F., Bossy, B., Cheresh, D. A., Reichardt, L. F., and Duband, J. L. (1994). Specific roles of the αVβ1, αVβ3 and αVβ5 integrins in avian neural crest cell adhesion and migration on vitronectin. *Development* **120,** 2687–2702.

Duband, J.-L., Belkin, A. M., Syfrig, J., Thiery, J. P., and Koteliansky, V. E. (1992). Expression of alpha 1 integrin, a laminin–collagen receptor, during myogenesis and neurogenesis in the avian embryo. *Development* **116,** 585–600.

Dufour, S., Duband, J. L., Humphries, M. J., Obara, M., Yamada, K. M., and Thiery, J. P. (1988). Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. *EMBO J.* **7,** 2661–2671.

Elices, M. J., Osborn, Y., Takada, C., Crouse, S., Luhowsky, M., Helmer, E., and Lobb, R. R. (1990). VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* **60,** 577–584.

Erickson, C. A., and Perris, R. (1993). The role of cell–cell and cell–matrix interactions in the morphogenesis of the neural crest. *Dev. Biol.* **159,** 60–74.

Erle, D. J., Sheppard, D., Breuss, J., Ruegg, C., and Pytela, R. (1991). Novel integrin alpha and beta subunit cDNAs identified in airway epithelial cells and lung leukocytes using the polymerase chain reaction. *Am. J. Respir. Cell Mol. Biol.* **5,** 170–177.

Gong, Q., and Shipley, M. T. (1996). Expression of extracellular matrix molecules and cell surface molecules in the olfactory nerve pathway during early development. *Comp. Neurol.* **26,** 1–14.

Gu, M., Wang, W., Song, W. K., Cooper D. N., and Kaufman, S. J. (1994). Selective modulation of the interaction of alpha 7 beta 1 integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. *J. Cell Sci.* **107,** 175–181.

Guan, J.-L., and Hynes, R. O. (1990). Lymphoid cells recognise an alternatively spliced segment of fibronectin via the integrin receptor α4β1. *Cell* **60,** 53–61.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

Hay, E. D. (1989). Extracellular matrix, cell skeletons, and embryonic development. *Am. J. Med. Genet.* **34,** 14–29.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375,** 787–790.

Hynes, R. O. (1987). Integrins: A family of cell surface receptors. *Cell* **4,** 549–554.

Hynes, R. O. (1992). Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **69,** 11–25.

Jaffredo, T., Horwitz, A. F., Buck, C. A., Rong, P. M., and Dieterlen-Lievre, F. (1988). Myoblast migration specifically inhibited in the chick embryo by grafted CSAT hybridoma cells secreting an anti-integrin antibody. *Development* **103,** 431–446.

Kaufman, M. H. (1992). "The Atlas of Mouse Development." Academic Press, San Diego, CA.

Kil, S. H., and Bronner-Fraser, M. (1996a). Expression of the avian a7-integrin in developing nervous system and myotome. *Int. J. Dev. Neurosci.* **14,** 181–190.

Kil, S. H., Lallier, T., Bronner-Fraser, M. (1996b). Inhibition of cranial neural crest adhesion *in vitro* and migration *in vivo* using integrin antisense oligonucleotides. *Dev. Biol.* **179,** 91–101.

Kozak, M. (1987). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J. Mol. Biol.* **196,** 947–950.

Krotoski, D., Domingo, C., and Bronner-Fraser, M. (1986). Distribution of a putative cell surface receptor for fibronectin and laminin in the avian embryo. *J. Cell Biol.* **103,** 1061–1072.

Krull, C. E., Collazo, A., Fraser, S. E., and Bronner-Fraser, M. (1995). Segmental migration of trunk neural crest: Time-lapse analysis reveals a role for PNA-binding molecules. *Development* **121,** 3733–3743.

Krull, C. E., Lansford, R., Gale, N. W., Collazo, A., Marcelle, C., Yancopoulos, G., Fraser, S. E., and Bronner-Fraser, M. (1997). Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. *Curr. Biol.* **7,** 571–580.

Kwee, L., Baldwin, H. S., Shen, H. M., Stewart, C. L., Buck, C., Buck, C. A., and Labow, M. A. (1995). Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. *Development* **121,** 489–503.

Lallier, T., and Bronner-Fraser, M. (1992). $\alpha 1\beta 1$ integrin on neural crest cells recognizes some laminin substrata in a Ca^{2+}-dependent manner. *J. Cell Biol.* **119,** 1335–1345.

Lallier, T., and Bronner-Fraser, M. (1993). Inhibition of neural crest cell attachment by integrin antisense oligonucleotides. *Science* **259,** 692–695.

Lallier, T., Leblanc, G., Artinger, K. B., and Bronner-Fraser, M. (1992). Cranial and trunk neural crest cells use different mechanisms for attachment to extracellular matrices. *Development* **116,** 531–541.

Le Douarin, N. (1982). "The Neural Crest." Cambridge Univ. Press, Cambridge.

Loring, J. F., and Erickson, C. A. (1987). Neural crest cell migratory pathways in the trunk of the chick embryo. *Dev. Biol.* **121,** 220–236.

Mackie, E. J., Thesleff, I., and Chiquet-Ehrismann, R. (1987). Tenascin is associated with chondrogenic and osteogenic differentiation *in vivo* and promotes chondrogenesis *in vitro. J. Cell Biol* **105,** 2569–2579.

May, M. J., Entwistle, G., Humphries, M. J., and Ager, A. (1993). VCAM-1 is a CS1 peptide-inhibitable adhesion molecule expressed by lymph node high endothelium. *J. Cell Sci.* **106,** 109–119.

Miyake, K., Weissman, I. L., Greenberger, J. S., and Kincade, P. W. (1991). Evidence for a role of the integrin VLA-4 in lymphohemopoiesis. *J. Exp. Med.* **173,** 599–607.

Mould, A. P., Wheldon, L. A., Komoriya, A., Wayner, E. A., Yamada, K. M., and Humphries, M. J. (1990). Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin alpha 4 beta 1. *J. Biol. Chem.* **265,** 4020–4024.

Neugebauer, K. M., Emmett, C. J., Venstrom, K. A., and Reichardt, L. F. (1991a). Vitronectin and thrombospondin promote retinal neurite outgrowth: Developmental regulation and role of integrins. *Neuron* **6,** 345–358.

Neugebauer, K. M., and Reichardt, L. F. (1991b). Cell-surface regulation of β_1-integrin activity on developing retinal neurons. *Nature* **350,** 68–71.

Neuhaus, H., Hu, M. C., Hemler, M. E., Takada, Y., Holzmann, B., and Weissman, I. L. (1991). Cloning and expression of cDNAs for the alpha subunit of the murine lymphocyte–Peyer's patch adhesion molecule. *J. Cell Biol.* **115,** 1149–1158.

Newgreen, D. F., and Theiry, J. P. (1980). Fibronectin in early avian embryos: Synthesis and distribution along the migration pathways of neural crest cells. *Cell Tissue Res.* **211,** 269–291.

Nichols, D. H. (1986). Formation and distribution of neural crest mesenchyme to the first pharyngeal arch region of the mouse embryo. *Am. J. Anat.* **176,** 221–231.

Noden, D. M. (1983). The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Dev. Biol.* **96,** 144–165.

Olsson, L., Svensson, K., and Perris, R. (1996). Effects of extracellular matrix molecules on subepidermal neural crest cell migration in wild type and white mutant (dd) axolotl embryos. *Pigment Cell Res.* **9,** 18–27.

Perris, R., Krotoski, D., and Bronner-Fraser, M. (1991b). Collagens in avian neural crest development: Distribution *in vivo* and migration-promoting ability *in vitro. Development* **113,** 969–984.

Poole, T. J., and Thiery, J. P. (1986). Antibodies and synthetic peptides that block cell–fibronectin adhesion arrest neural crest migration in vivo. *In* "Progress in Developmental Biology." Al. R. Liss, New York.

Rickman, T. P., Le Douarin, N. M., and Keynes, R. J. (1985). The migration of neural crest cells and the growth cones of motor axons through the rostral half of the chick somite. *J. Embryol. Exp. Morphol.* **90,** 437–455.

Rovasio, R., Delouvee, A., Timpl, R., Yamada, K., and Thiery, J. P. (1983). In vitro avian neural crest cell adhesion and migration: Requirement for fibronectin in the extracellular matrix. *J. Cell Biol.* **96,** 462–473.

Ruegg, C., Postigo, A. A., Sikorsk, E. E., Butcher, E. C., Pytela, R., and Erle, D. J. (1992). Role of integrin alpha 4 beta 7/alpha 4 beta P in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J. Cell Biol* **117,** 179–189.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanes, J. R. (1989). Extracellular matrix molecules that influence neural development. *Annu. Rev. Neurosci.* **12,** 491–516.

Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995). INTEGRINS: Emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* **11,** 549–599.

Serbedzija, G. N., Bronner-Fraser, M., and Fraser, S. E. (1992). Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development* **116,** 297–307.

Serbedzija, G. N., Fraser, S. E., and Bronner-Fraser, M. (1989). A vital dye analysis of the timing and pathways of avian trunk neural crest cell migration. *Development* **106,** 809–816.

Sheppard, A. M., Onken, M. D., Rosen, G. D., Noakes, P. G., and Dean, D. C. (1994). Expanding roles for alpha 4 integrin and its ligands in development. *Cell Adhes. Commun.* **2,** 27–43.

Spiegelman, B. M., and Ginty, C. A. (1983). Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell* **35,** 657–666.

Stepp, M. A., Urry, L. A., and Hynes, R. O. (1994). Expression of alpha 4 integrin mRNA and protein and fibronectin in the early chicken embryo. *Cell Adhes. Commun.* **2,** 359–375.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A., and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb-1. *Nature* 630–634.

Takada, Y., Elices, M. J., Crouse, C., and Hemler, M. E. (1989). The primary structure of the alpha 4 subunit of VLA-4: Homology to other integrins and a possible cell–cell adhesion function. *EMBO J.* **8,** 1361–1368.

Teague, T. K., Lazarovits, A. T., and Mcintyre, B. W. (1994). Integrin $\alpha_4\beta_7$ co-stimulation of human peripheral blood T cell proliferation. *Cell Adhes. Commun.* **2,** 539–547.

Teillet, M. A., Kalcheim, C., and Le Douarin, N. M. (1987). Formation of the dorsal root ganglia in the avian embryo: Segmental origin and migratory behavior of neural crest progenitor cells. *Dev. Biol.* **120,** 329–347.

Thiery, J. P., Duband, J. L., Rutishauser, U., and Edelman, G. M. (1982). Cell adhesion molecules in early chicken embryogenesis. *Proc. Natl. Acad. Sci. USA* **79,** 6737–6741.

Tucker, G. C., Aoyama, H., Lipinski, M., Turz, T., and Thiery, J. P. (1984). Identical reactivity of monoclonal antibodies HNK-1 and NC-1: Conservation in vertebrates on cells derived from neural primordium and on some leukocytes. *Cell Differ.* **14,** 223–230.

Umbhauer, M., Riou, J. F., Smith, J., C., and Boucaut, J. C. (1994). Control of somitic expression of tenascin in *Xenopus* embryos by myogenic factors and Brachyury. *Dev. Dyn.* **200,** 269–277.

Vincent, M., and Thiery, J. P. (1984). A cell surface marker for neural crest and placodal cells: Further evolution in peripheral and central nervous system. *Dev. Biol.* **103,** 468–481.

von der Mark, K., and Ocalan, M. (1989). Antagonistic effects of laminin and fibronectin on the expression of the myogenic phenotype. *Differentiation* 150–157.

Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., and Carter, W. G. (1989). Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* **109,** 1321–30.

West, C. M., Lanza, R., Rosenbloom, J., Lowe, M., Holtzer, H., and Avdalovic, N. (1979). Fibronectin alters the phenotypic properties of cultured chick embryo chondroblasts. *Cell* **17,** 491–501.

Wilkinson, D. (Ed.) (1992). "In Situ Hybridisation: A Practical Approach." IRL Press, Oxford.

Yabkowitz, R., Dixit, V. M., Guo, N., Roberts, D. D., and Shimizu, Y. (1993). Activated T-cell adhesion to thrombospondin is mediated by the alpha 4 beta 1 (VLA-4) and alpha 5 beta 1 (VLA-5) integrins. *J. Immunol.* **151,** 1–14.

Yang, J. T., Rayburn, H., and Hynes, R. O. (1993). Embryonic mesodermal defects in α5 integrin-deficient mice. *Development* **119,** 1093–1105.

Yang, J. T., Rayburn, H., and Hynes, R. O. (1995). Cell adhesion events mediated by α4 integrins are essential in placental and cardiac development. *Development* **121,** 549–560.

Yang J. T., Cardarelli, P. M., Lehnert, K., Rowland, S., and Krissansen, G. W. (1996) Fibronectin receptor functions in embryonic cells deficient in alpha 5 beta 1 integrin can be replaced by alpha V integrins. *Mol. Biol. Cell.* **7,** 1737–1748.

Received for publication April 20, 1998
Revised June 11, 1998
Accepted June 12, 1998