chromogen *O*-phenylenediamine (0.05%) and 0.02% hydrogen peroxide. The absorbance of the reaction product was measured at 490 nm. The wells were then washed extensively with PBS, and MBP-positive cells were visualized by addition of the insoluble chromogen diaminobenzidine (0.05%) and 0.02% hydrogen peroxide. The number of MBP-positive cells per well was counted; the results are expressed as the amount of MBP immunoreactivity (optical density at 490 nm) developed by 1000 MBP-positive cells.
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Inhibition of Neural Crest Cell Attachment by Integrin Antisense Oligonucleotides

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Neural crest cell interactions with extracellular matrix molecules were analyzed with the use of antisense oligonucleotides to block synthesis of integrin subunits. When added to the culture medium of quail neural crest cells, selected antisense phosphorothiol oligonucleotides reduced the amounts of cell surface α_1 or β_1 integrin subunits by up to 95 percent and inhibited neural crest cell attachment to laminin or fibronectin substrata. Differential effects on specific α integrins were noted after treatment with α-specific oligonucleotides. Cells recovered the ability to bind to substrata 8 to 16 hours after treatment with inhibitory oligonucleotides. The operation of at least three distinct α integrin subunits is indicated by substratum-selective inhibition of cell attachment.

The neural crest is a migratory cell population that arises in the dorsal neural tube shortly after tube closure. These cells migrate throughout the embryo along well-defined pathways, then differentiate into a variety of cell types including neurons and glia of the peripheral nervous system, pigment cells, and connective tissue of the face. During migration, neural crest cells contact extracellular matrices containing fibronectin, laminin, collagens, proteoglycans, and tenascin (*1*). Interactions with these molecules are primarily by means of integrins in the β_1 family (*1–3*). Antibodies to the β_1 subunit of chick integrin (*2*, *3*) and several of its ligands in the extracellular matrix (ECM) (*4*, *5*) perturb avian cranial neural crest cell migration both in situ and in vitro. However, relatively little is known about the nature of the integrins on these avian cells.

Integrins are heterodimeric transmembrane glycoproteins composed of noncovalently linked α and β subunits, which mediate interactions between embryonic cells and their surrounding extracellular matrices (*6*). The function of integrins has been studied by antibody inhibition of binding or by transfection of antisense cDNA to block synthesis (*2*, *3*, *7*, *8*). The former method is limited by the paucity of specific blocking antibodies for individual integrin subunits and by possible nonspecific effects. Although transfection with large antisense probes overcomes some of these problems, cDNAs are typically incorporated into only a small percentage of the cells, making the second approach useful for cell lines but not embryonic tissue. An alternative approach is to utilize short antisense oligonucleotides; these are taken up actively by large percentages of cells and decrease the amount of the targeted mRNA or protein (*9*). Phosphorothioate-modified oligonucleotides are particularly useful due to their increased stability: these oligonucleotides have a half-life of greater than 1 hour; the half-life of unmodified oligonucleotides is less than 5 min (*10*).

Here, we have combined molecular, cellular, and biochemical techniques to examine the nature of the integrins involved in quail neural crest cell interactions with ECM molecules. First, we have used antisense phosphorothiol oligonucleotides to perturb integrin synthesis and hence integrin-mediated interactions between quail neural crest cells and the ECM. Second, we have used a functional assay for neural crest cell attachment to specific ECM molecules to test which of several candidate oligonucleotides against integrin α and β subunits inhibit cell attachment in a substratum-specific manner. Finally, we used biochemical analysis to demonstrate decreased amounts of cell surface α and β integrin subunits on neural crest cells after treatment with particular antisense oligonucleotides.

Antisense oligonucleotides (Table 1) were tested for their ability to inhibit the production of the β_1 and various α subunits. The antisense oligonucleotide directed to the β_1 integrin subunit (1B1) encompasses a region (*11*) of high similarity to β_1 subunits from other species but low homology to other β subunits. Because little sequence information is available for avian α subunits, antisense oligonucleotide "guess-mer" sequences were selected from known sequences from mammalian integrins. Oligonucleotides 2aG through 5aG were selected from regions of highest nucleotide sequence identity among integrin α subunits (*12*); for example, 2aG was from the conserved COOH-terminus, a region of sequence identity in mammalian α subunits. Other antisense oligonucleotides (10a1 through 18a5) were selected according to sequence data available for individual mammalian α subunits (α_1, α_4, and α_5). Because these oligonucleotides were determined by sequence information from other species, they do not necessarily correspond to the same α subunits in avian integrins, for which sequence information is currently unavailable. Consequently, we tested the specificity of these antisense oligonucleotides in avian cells with functional and biochemical assays described below.

The ability of isolated neural crest cells to attach to individual ECM components was quantitated by the use of a centrifugal attachment assay (*3*, *13*), which provides reproducible results for small numbers (on the order of hundreds) of embryonic cells. Quail neural crest cells were derived from neural tubes removed from the trunk region

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of 16- to 24-somite stage embryos (*3*); after one day in culture, a few hundred neural crest cells migrated away from these explants, the neural tube was scraped away and remaining neural crest cells were incubated with oligonucleotides in serum-free medium for 4 to 6 hours. Antisense oligonucleotides 1B1 and 2aG reduced the attachment of neural crest cells to both fibronectin and laminin substrata in a dose-dependent manner; the greatest response (70% reduction) was observed for both oligonucleotides at a dose of 50 μM, the highest concentration tested (Fig. 1A). This dose-response range is consistent with previous studies, where inhibitory effects of oligonucleotides were observed between 0.02 to 30 μM (*14*). Function-blocking antibodies against β_1 or α_1 integrins result in 100% or 50%, respectively, reduction in attachment (*15*). Antisense oligonucleotide 3aG had no effect on cell attachment at any of the concentrations tested. Sense oligonucleotides or mixtures of sense with corresponding antisense oligonucleotides had no effect (Fig. 1B), suggesting that the observed reductions were specific and not due to general toxicity.

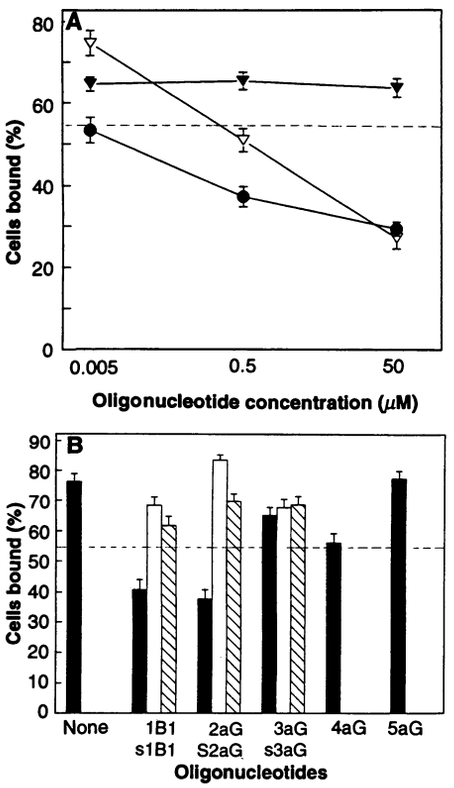

Fig. 1. (**A**) Antisense oligonucleotides reduce neural crest cell attachment to laminin in a dose-dependent manner. The adhesion of neural crest cells to defined substrates was measured as described (*13*) as adapted for neural crest cells (*3, 15*). About 75% (±4) of untreated neural crest cells attach to laminin in this assay. Addition of antibody against the chick β_1 subunit of integrin inhibited neural crest cells attachment by ~75%, such that 11% (±4) of cells bound to substrate. Neural crest cells were also incubated for 4 hours with antisense oligonucleotides 1B1 (●), 2aG (▽), or 3aG (▼). The dashed line at 55% indicates the level below which attachment was judged to be significantly reduced; position of the line relative to control values was determined with the use of a student's one-sided *t* test; $P < 0.05$ was deemed as significant. Points represent the mean of six or more experiments and the error bars indicate standard errors of the mean (SEM). (**B**) Specificity of antisense oligonucleotides. Individual antisense (solid bars), sense (open bars), or mixtures of sense with antisense (hatched bars) oligonucleotides were assayed for ability to reduce neural crest cell attachment to laminin. The five groups of oligonucleotides tested are identified by their sequence location (see Table 1). The dashed line at 55% indicates the level below which attachment was significantly reduced. Bars and SEM represent the mean and standard error for six or more experiments.

To ascertain whether treatment with antisense oligonucleotides caused a decrease in the amount of cell surface integrin proteins, neural crest cells were surface-biotinylated and immunoprecipitated with antibody against the chick 1β1 after exposure to oligonucleotides. Treatment with oligonucleotides 1B1 and 2aG resulted in a 90% decrease in the amounts of detectable β_1 protein compared with untreated controls (Fig. 2A). In contrast, neural crest cells treated with sense oligonucleotides had amounts of β_1 integrins within about 20% of that on untreated cells, indicating that inhibition is not due to some nonspecific effect.

Neural crest cells possess an $\alpha_1\beta_1$ integrin that mediates attachment in the absence of soluble divalent cations to laminin substrata prepared in the presence of Ca^{2+} (*LN-Ca*$^{2+}$); in contrast, attachment to laminin substrata prepared in the presence of EDTA (*LN-EDTA*) is mediated by a different and unidentified Ca^{2+}-dependent integrin (*15*). Unlike 1B1 and 2aG, which inhibited attachment to all substrata tested,

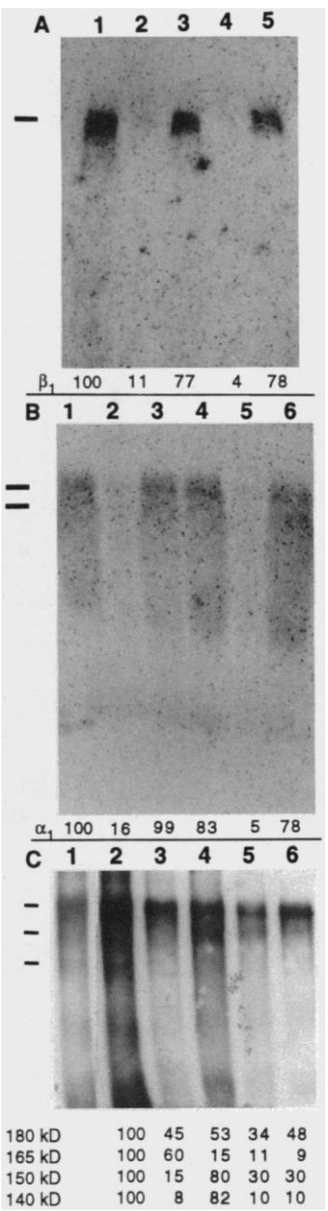

Fig. 2. (**A**) Antisense oligonucleotides reduce protein amounts of the β_1 subunit of integrin. Surface-biotinylated neural crest cells (*3, 15*) were immunoprecipitated with the JG22 antibody (antibody to integrin β_1) in the presence of high concentrations of detergent (0.5% Triton X-100), conditions in which the α and β subunits are dissociated. Bands were detected with iodinated streptavidin. Lane 1, untreated neural crest cells; lanes 2 to 5, neural crest cells treated for 6 hours with 50 μM 1B1 (lane 2), 50 μM sense 1B1 (lane 3), 50 μM 2aG (lane 4), and 50 μM sense 2aG (lane 5). Bar represents molecular mass of 120 kD corresponding to the β_1 integrin subunit. Quantitative differences may reflect differences in the number of cells loaded or toxic effects of the oligonucleotides. β_1, amount of 120-kD protein found in each lane, measured by gel densitometry and presented as percentage of protein in lane 1. (**B**) Antisense oligonucleotides reduce amounts of the α_1 subunit of integrin. Surface-biotinylated neural crest cells were immunoprecipitated with an antibody against the chick α_1 subunit of integrin (*17*). Lane 1, untreated neural crest cells; lanes 2 to 6, neural crest cells treated for 6 hours with 50 μM 2aG (antisense to all α subunits) (lane 2), 50 μM 4aG (lane 3), 50 μM 12a1 (lane 4), 50 μM 14a4 (lane 5), and 50 μM 15a4 (lane 6). Bars represent molecular masses of 165 and 120 kD, corresponding to the α_1 (upper) and β_1 (lower) integrin subunits. α_1, amount of 165-kD protein found in each lane, measured by gel densitometry and presented as percentage of 165-kD protein in lane 1. (**C**) Surface-biotinylated neural crest cells treated with various oligonucleotides (50 μM) for 6 hours and immunoprecipitated with antibodies to β_1 integrin in the presence of 0.1% Triton X-100. Lane 1 and 2, control lane treated with reversed 14a4; lane 1 is a shorter exposure of lane 2 to match the contrast of the other lanes; lane 3, cells treated with 15a4; lane 4, cells treated with 4aG; lane 5, cells treated with 12a1; and lane 6, cells treated with 14a4. Five major bands at 180, 165, 150, 140, and 120 kD were detected in the control lanes 1 and 2. Bars represent molecular masses of 180, 165, and 120 kD. Numbers represent the quantity of each indicated sized protein in lanes 2 through 6, measured by gel densitometry and presented as a percentage of the amount found in lane 2.

several of our antisense oligonucleotides inhibited neural crest cell attachment specifically to fibronectin, *LN-Ca*$^{2+}$, or *LN-EDTA*. Oligonucleotide 4aG inhibited attachment to fibronectin but not laminin; 14a4 inhibited attachment to *LN-Ca*$^{2+}$ only; 15a4 and 12a1 blocked attachment to *LN-EDTA* only (Fig. 3). Both sense and reversed antisense oligonucleotides (that is, with the sequence reversed in the 3′ to 5′ direction) for 4aG, 12a1, 14a4, and 15a4 had no significant effect on adhesion of neural crest cells to any of the substrata.

Neural crest cells were surface-biotinylated and immunoprecipitated with an antibody to the α_1 subunit of chick integrin after oligonucleotide treatment to ascertain their effects on amounts of α_1 protein. Incubation with oligonucleotides that affect attachment to *LN-Ca*$^{2+}$ (2aG and 14a4) resulted in decreases in the detectable α_1 protein of 85% and 95%, respectively. In contrast, neural crest cells treated with oligonucleotides that affect cell attachment to fibronectin or *LN-EDTA* (4aG, 12a1, and 15a4) did not significantly decrease amounts of α_1 over untreated control levels (<20%; Fig. 2B). Thus, only two of the five oligonucleotides that affect neural crest cell attachment decrease the amounts of the α_1 subunit: 2aG, which blocks attachment to all substrates, and 14a4, which blocks attachment to *LN-Ca*$^{2+}$ substrates only.

Because antibodies that recognize specific avian α subunits are not readily available, we have used an alternative approach for examining whether oligonucleotides 4aG, 12a1, 14a4, and 15a4 differentially reduce specific α subunits. Surface-labeled neural crest cells were treated with these antisense oligonucleotides and immunoprecipitated with antibody to β_1 integrin in the

Table 1. Antisense oligonucleotides.

Type	Name	Sequence	Strand	Sequence location (source)
β_1				
	1B1	5′-GCAGTAAGCATCCATATC-3′	Antisense	1643–1661 (chick β_1)
	s1B1	3′-CGTCATTCGTAGGTATAG-5′	Sense	1643–1661 (chick β_1)
Conserved α sequences				
	2aG	5′-TTGAAGAAGCCAAGCTT-3′	Antisense	3042–3059 (human α_4)
	s2aG	3′-AACTTCTTCGGTTCGAA-5′	Sense	3042–3059 (human α_4)
	3aG	5′-GAATAACCTAAATA-3′	Antisense	779–793 (human α_4)
	s3aG	3′-CTTATTGGATTTAT-5′	Sense	779–793 (human α_4)
	4aG	5′-GGGTGCCCCCATGAGGA-3′	Antisense	1009–1026 (human α_4)
	5aG	5′-CCAAAGTAGGAGCCAA-3′	Antisense	947–962 (human α_4)
Nonconserved α sequences				
	10a1	5′-AAACCTTGTCTGATTCACAGC-3′	Antisense	1500–1521 (rat α_1)
	11a1	5′-GGCAGCGACTGAAATGTGATG-3′	Antisense	2793–2814 (rat α_1)
	12a1	5′-CAGGCTCTTTTCTGTGGTGGA-3′	Antisense	2379–2400 (rat α_1)
	13a4	5′-GCCTTTATATGAGAAACACAG-3′	Antisense	2052–2073 (human α_4)
	14a4	5′-TACAGACTCAGGATGGCTTAA-3′	Antisense	1323–1344 (human α_4)
	15a4	5′-GTCTTTTTCTTTCTTCTGCTG-3′	Antisense	1707–1728 (human α_4)
	16a5	5′-TCCAGAAGCATTGAGGCAGAA-3′	Antisense	1581–1602 (human α_5)
	17a5	5′-GAACATGGCGGGGAAGATGGT-3′	Antisense	1497–1518 (human α_5)
	18a5	5′-AGCCTTGTCCTCTATTGCGCT-3′	Antisense	1899–1920 (human α_5)

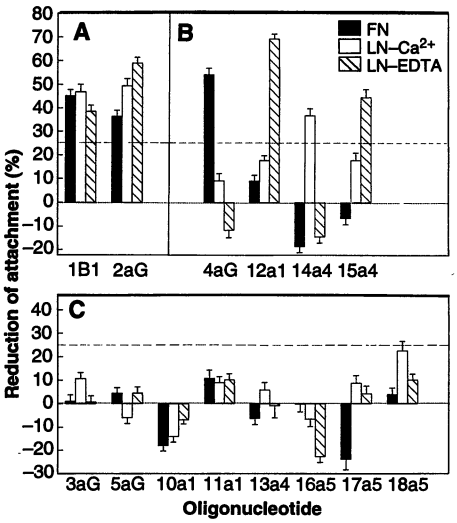

Fig. 3. Antisense oligonucleotides reduced attachment of neural crest cells in a substrate-specific manner. Neural crest cell attachment to fibronectin (solid bars), *LN-Ca*$^{2+}$ (open bars), or *LN-EDTA* (hatched bars) substrates was assayed 6 hours after addition of the indicated antisense oligonucleotides. (**A**) Oligonucleotides that reduced attachment to all substrata. (**B**) Oligonucleotides that affect neural crest cell binding to a single substrate. (**C**) Oligonucleotides that had no significant effects. Dashed lines at 25% indicate the level below which attachment was judged to be significantly reduced. Bars and SEM represent the mean and standard error for six or more experiments.

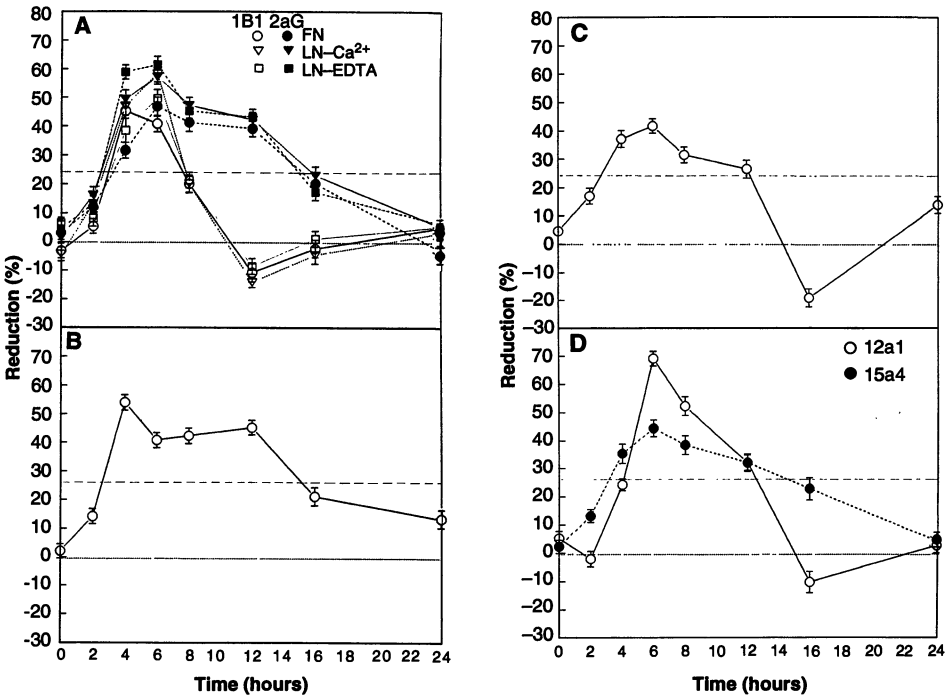

Fig. 4. Time course of recovery of neural crest cells after treatment with antisense oligonucleotides. The ability of neural crest cells to bind to fibronectin, *LN-Ca*$^{2+}$, or *LN-EDTA* was assayed at the indicated times after addition of antisense oligonucleotides. (**A**) Recovery of attachment to all substrates after treatment with 1B1 or 2aG. (**B**) Recovery of attachment to fibronectin after treatment with 4aG. (**C**) Recovery of attachment to *LN-Ca*$^{2+}$ after treatment with 14a4. (**D**) Recovery of attachment to *LN-EDTA* after treatment with 12a1 or 15a4. Neural crest cells showed differential recovery from the different oligonucleotides. Maximum reduction in the percentage of cell attachment was observed by 4 to 6 hours. Dashed lines at 23 to 27% indicate the level below which attachment was judged to be significantly reduced. Bars and SEM represent the mean and standard error for six or more experiments.

presence of low concentrations of detergent, conditions known to coprecipitate multiple α subunits together with the β_1 subunit. Probes that selectively affect particular α subunits would be expected to reduce amounts of or remove individual species from these precipitates. In control immunoprecipitates with reversed antisense oligonucleotides, five major bands were detected at 180, 165, 150, 140, and 120 kD, the latter representing the β_1 subunit. Treatment with different oligonucleotides yielded differential reduction of α protein bands (Fig. 2C). Gel densitometry revealed that the oligonucleotide 4aG selectively reduced the 165-kD band by 85%, whereas the 150- and 140-kD bands were changed less than 20%. In contrast, 15a4 caused 85% reduction in the 140- and 150-kD bands, and the 165-kD band was relatively unchanged. 12a1 and 14a4 reduced the 140- and 165-kD bands by 90%, whereas other α bands were affected to a lesser extent. Although the precise identity of the α subunits involved cannot be determined in these immunoprecipitates, these results support the idea that different oligonucleotides have differential effects on the pattern of α subunits.

The antisense oligonucleotide effects did not necessarily correspond to known properties of the α subunits used as the source of their sequence; for example, although the sequence of 14a4 was derived from human α_4 integrin sequence, the 14a4 oligonucleotide affected the avian α_1 integrin. This suggests that the avian integrin sequences may be somewhat different than those in human and rat, and that integrins may share some short sequence similarity with heterologous α subunits in other species.

Because phosphorothiol oligonucleotides have been reported to have a half-life on the order of 1 hour (*10*), their effects may be transient. To follow the recovery of neural crest cells' ability to attach to the ECM, we assayed neural crest cell attachment to fibronectin, *LN-*Ca^{2+}, or *LN-EDTA* from 2 to 24 hours after addition of blocking oligonucleotides (Fig. 4). Cells regained normal attachment to all substrates 8 to 12 hours after incubation with 1B1 and 16 hours after treatment with 2aG. After treatment with the substrate-selective oligonucleotides 4aG, 14a4, or 12a1/15a4, neural crest cell attachment to fibronectin, *LN-*Ca^{2+}, or *LN-EDTA* returned to normal by 16 hours after addition of oligonucleotides. The data suggest that integrin subunit turnover on neural crest cells occurs in a matter of hours, consistent with the fact that embryonic cells must rapidly make and break their adhesions during migration. The turnover rate of the β_1 subunit on fibroblasts is 3 or 10 hours in the presence or absence, respectively, of TGF-β (*16*). Our observation that cell attachment recovers more slowly after treatment with oligonucleotides antisense for α than β_1 integrin subunits is consistent with the idea that the production of α subunits is the rate-limiting step in integrin assembly (*16*).

Because several oligonucleotides inhibited interactions of neural crest cells with specific ECM components, the results suggest that neural crest cells have at least three functionally different α subunits, all of which pair with the β_1 subunit. Our oligonucleotides, generated without the need for isolation of the receptor complex, sequencing of the genes, or preparation of function-blocking antibodies, offer useful tools for dissecting functional interactions of integrins in complex settings. In addition, these oligonucleotides will provide sequence information useful for cloning the α subunits to which they correspond.

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