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Snail-Related Transcriptional Repressors Are Required in *Xenopus* for both the Induction of the Neural Crest and Its Subsequent Migration

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The neural crest is a transient population of precursor cells that arises at the border between the neural plate and prospective epidermis in vertebrate embryos. The earliest known response to neural-crest-inducing signals is the expression of the zinc-finger transcription factors *slug* and *snail*. Although it is widely believed that these transcription factors play an essential role in neural crest development, relatively little is understood about their mechanism of action during this process. We have previously shown that overexpression of XSlug leads to expanded expression of neural crest markers and an excess of at least one neural crest derivative, melanocytes. In order to further investigate XSlug function, we overexpressed mutant constructs in which the DNA-binding domain was fused to either the activation domain from Gal4 or the repressor domain from *Drosophila* Engrailed. The Engrailed repressor fusion was found to mimic the effects of wild-type XSlug, indicating that XSlug functions as a transcriptional repressor during neural crest formation. In contrast, overexpression of either the activation domain fusion or the DNA-binding domain alone was found to inhibit XSlug function. Using a hormone-inducible inhibitory mutant, we show that inhibition of XSlug function at early stages prevents the formation of neural crest precursors, while inhibition at later stages interferes with neural crest migration, demonstrating for the first time that this transcriptional repressor is required during multiple stages of neural crest development.

Key Words: **neural crest; slug; snail; *Xenopus*.**

INTRODUCTION

Neural crest cells, a cell type unique to vertebrates, arise at the juncture between the neural and nonneural ectoderm shortly after induction of the nervous system. These multipotent precursor cells ultimately undergo an epithelial to mesenchymal transition, migrate to diverse regions throughout the embryo, and give rise to a wide range of derivatives (Le Douarin, 1982; Hall and Hörstadius, 1988). The neural crest contributes to cell types as diverse as the elements of the craniofacial skeleton, melanocytes, and the neurons and glia of the peripheral nervous system. Although the precise mechanisms underlying neural crest induction have yet to be elucidated, this process is thought to be dependent upon signals emanating from the nonneural ectoderm, the nonaxial mesoderm, or both (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Bonstein *et al.*, 1998; Marchant *et al.*, 1998).

The earliest known response to neural-crest-inducing signals is the expression of one or more zinc-finger-containing transcription factors related to *Drosophila snail* (reviewed in LaBonne and Bronner-Fraser, 1999). In *Xenopus*, expression of *XSnail* and the closely related gene *XSlug* can be detected in neural-crest-forming regions by late gastrula stages (Essex *et al.*, 1993; Mayor *et al.*, 1995). These factors, which are thought to be functionally homologous, display overlapping patterns of expression at the lateral edges of the open neural plate and the developing neural crest. Overexpression of XSlug in frog embryos leads to expanded expression of early neural crest markers, including *Slug* itself, and to an excess of at least one neural crest derivative, melanocytes (LaBonne and Bronner-Fraser, 1998). This finding suggested that XSlug could play a role in its own regulation and that it might be important for early aspects of neural crest development. Little else was known, however, about its role in this process.

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Some clues to the potential mechanism of XSlug action can be gleaned from related molecules in other species. In *Drosophila, DSnail* is expressed in the mesoderm where it prevents the expression of neurectodermal genes. Indeed, DSnail has been shown to function as a transcriptional repressor (Leptin, 1991; Nibu *et al.,* 1998) that binds to a corepressor, C-terminal-binding protein (CtBP; Nibu *et al.,* 1998). Snail-related factors have also been identified in ascidians, amphioxus, and numerous vertebrates. Ascidians appear to have a single *Snail* homolog that is expressed in the trunk musculature as well as in the developing neural folds (Corbo *et al.,* 1997). Similarly, amphioxus *Snail* is expressed at the lateral margins of the neural plate and subsequently in the dorsal neural tube (Langeland *et al.,* 1998). These findings suggest that the neural crest may have originated as a population of nonmigratory cells in the dorsal neural tube of the ancestral prevertebrate chordate. Presumably due to genomic duplications, vertebrate embryos have multiple *Snail* homologs. Chick and mouse, like *Xenopus,* have one *Snail* homolog and one *Slug* homolog that are expressed in both distinct and overlapping patterns in the developing mesoderm and neural crest (Nieto *et al.,* 1992; 1994; Jiang *et al.,* 1998; Sefton *et al.,* 1998). In contrast, sequence analysis has suggested that zebrafish have two distinct *Snail* homologs, but no *Slug* (Thisse *et al.,* 1993, 1995). Interestingly, the expression patterns of *Slug* and *Snail* appear to be reversed in the mouse compared with patterns in the chick, although the sum of the expression patterns of these genes has been highly conserved across vertebrates (Sefton *et al.,* 1998). This inversion of expression patterns adds further weight to the hypothesis that these highly related genes are functionally homologous.

In contrast to *Drosophila* where the function of Snail is well documented, little is known about the function of Snail or Slug in vertebrate development. Mouse knockouts of MSlug have no apparent neural crest phenotype (Jiang *et al.,* 1998), but this is likely due to functional redundancy with MSnail. Overexpression of MSlug in bladder carcinoma cells, however, has been found to promote desmosome dissociation (Savenger *et al.,* 1997). An essential role for chick Slug during neural crest development has been suggested by antisense oligonucleotide experiments (Nieto *et al.,* 1994), although these experiments provided no information as to its mechanism of action. Overexpression of XSlug in frogs leads to increased expression of early neural crest markers (LaBonne and Bronner-Fraser, 1998), suggesting that it could function either as a transcriptional activator or by repressing a transcriptional repressor(s). Here, we test the function of XSlug and demonstrate that it acts as a repressor during neural crest development, similar to what has been found for *Drosophila* Snail. Furthermore, we demonstrate that XSlug plays an important role in early neural crest development as well as a later role in neural crest migration.

MATERIALS AND METHODS

Constructs

CS2+ vector derivatives were kindly provided by D. Turner and R. Davis. For ΔSlug, a fragment of the XSlug cDNA spanning the zinc-finger region (amino acids D117–H266) was generated by low-cycle-number PCR using a high-fidelity polymerase (PWO, Boehringer-Mannheim) and primers with terminal *Stu*I sites. Amplified products were cloned into the *Stu*I site in the polylinker of pCS2 or pCS2+NLS (R. Rupp, D. Turner) and sequenced on both strands using an automated DNA sequencer. The pCS2 glucocorticoid receptor hormone-binding domain derivative (pCS2GRR3, R. Davis) contains the 265aa hormone-binding domain from the human glucocorticoid receptor cloned between *Bam*HI and *Eco*RI in pCS2+. For GR-ΔSlug, an *Nco*I/*Xho*I fragment from pCS2+NLS-ΔSlug was filled and blunt end ligated into the *Stu*I site of pSC2GRR3. The pCS2 activation domain derivative (pCS2+NLSMTG4A, R. Davis) contains the Gal4 activation domain (amino acids G766–E881) in the pCS2+NLSMT derivative (D. Turner). To generate G4A-ΔSlug, a *Stu*I fragment from PCS2-ΔSlug was cloned into the *Xba*I site C-terminal to the activation domain. The pCS2 Engrailed repressor derivative (pCS2+MTenR, R. Rupp) contains the *Drosophila* Engrailed repressor domain cloned into pCS2+MT. For EnR-ΔSlug, a *Stu*I fragment from PCS2-ΔSlug was cloned into a blunted *Xba*I site C-terminal to the repressor domain and a *Bam*HI/*Eco*RI fragment was replaced by the corresponding fragment from pCS2+NLSMTG4A. All fusion constructs were sequenced at junction sites using an automated DNA sequencer.

Preparation of Embryos, Microinjection, and Hormone Treatment

Albino and pigmented *Xenopus* eggs were collected and fertilized using established methods and staged according to Nieuwkoop and Faber (1967). Microinjection was carried out in 3% Ficoll/1.0× MMR. One to 2 h postinjection embryos were transferred to 0.1× MMR for culture. Embryos were injected with 100–500 pg of *in vitro* transcribed capped sense mRNA delivered in a volume of approximately 10 nl. Albino embryos were used for experiments to be analyzed by *in situ* hybridization; pigmented embryos were used for grafting experiments. To induce the activity of GR-ΔSlug, embryos were transferred to 0.1× MMR containing 10 μM dexamethasone (Sigma) at the stage noted in the text.

β-Galactosidase Assays and Whole-Mount in Situ Hybridization

Embryos were fixed in MEMFA for 1 h. For β-galactosidase staining, embryos were washed 2× in PBS and 2× in staining solution (10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS). Color reaction was carried out for 5–15 min at 37°C in staining solution containing 1.5 mg/ml X-gal. Embryos were then refixed for 30 min in MEMFA and stored dehydrated in 100% methanol. Antisense digoxigenin-labeled probes were synthesized using Boehringer-Mannheim (Indianapolis, IN) RNA labeling mix according to the manufacturer's instructions. *In situ* hybridization was carried out as previously described (Knecht *et al.,* 1995). Color reactions were developed using NBT/BCIP as a substrate. *In situ* probes were as previously described (LaBonne and Bronner-Fraser, 1998). Only embryos with β-galactosidase activity or green fluores-

cent protein (GFP) expression in neural crest forming regions were scored for phenotype.

Microsurgery and Grafts

Grafting assays were carried out on stage-matched donor and host embryos between stages 16 and 17. This allowed ample time for grafts to heal prior to the onset of neural crest migration (stage 20). Donor embryos were injected at the two-cell stage with mRNA for GFP alone, or coinjected with GFP and ΔSlug. Injected embryos which displayed strong uniform fluorescence in the cranial neural folds were selected for microsurgery. Donor and graft embryos were transferred to shallow depression wells in agar coated petri dishes. Cranial neural folds spanning the region which will give rise to the mandibular, hyoid, and branchial segments of the neural crest were explanted using eyebrow hair knives and fine watchmaker forceps from both donor and host embryos. Fluorescently labeled grafts explanted from donor embryos were positioned over the ablated region in host embryos and held in place using light pressure from a small piece of coverglass for 30 min in 1× MMR. Embryos were then transferred to 0.1× MMR for culture. Neural crest migration was followed using either a fluorescence stereo dissecting microscope fitted with a GFP filter set or an Zeiss Axiophot fitted with a fluorescein filter set.

RESULTS

XSlug Functions as a Transcriptional Repressor

We have previously shown that ectopic expression of XSlug in early *Xenopus* embryos leads to expanded expression of early neural crest markers (LaBonne and Bronner-Fraser, 1998). In order to better understand the molecular mechanisms underlying this induction, we replaced the region N-terminal to the DNA-binding domain with either the activation domain from Gal4 or the repressor domain from *Drosophila* Engrailed (Fig. 1A). mRNA encoding these fusion proteins was injected into one cell of two-cell-stage embryos, and these embryos were examined at neurula stages (stages 17–18) by *in situ* hybridization for expression of *XSlug* itself, an unrelated early neural crest marker, *XTwist*, and the neural plate marker *XSox-2*. Since in *Xenopus*, first cleavage divides the embryo approximately along the left/right axis, this allows gene expression to be manipulated on only one side of the embryo, while the other side acts as an internal control. mRNA encoding either β-galactosidase or GFP was coinjected as a lineage tracer to mark the injected side of the embryo.

At midneurula stages, the neural crest markers *XSlug* and *XTwist* are expressed lateral to the closing neural folds in cranial regions. In embryos injected with the repressor domain fusion (EnR-slug), the size of the region expressing *XSlug* (Fig. 1C; $n = 120$) and *XTwist* (Fig. 1E; $n = 62$) was markedly expanded, phenocopying the effects we reported previously for wild-type XSlug overexpression (LaBonne and Bronner-Fraser, 1998). In contrast, in embryos injected with the activation domain fusion (G4A-Slug), expression of *XSlug* (Fig. 1B; $n = 98$) and *XTwist* (Fig. 1D; $n = 53$) was absent or greatly diminished. The finding that EnR-Slug overexpression mimics the effects of wild-type XSlug overexpression indicates that XSlug functions as a transcriptional repressor during neural crest formation. Thus, the positive effects of XSlug overexpression on neural crest markers are likely to be indirect, perhaps the result of directly or indirectly repressing the transcription of an inhibitor of neural crest formation. As we found for wild-type XSlug, the expanded expression of neural crest markers in embryos injected with EnR-Slug was predominantly into more lateral and posterior regions of the ectoderm normally fated to give rise to epidermis (Figs. 1C and 1E). Nevertheless, these embryos do display a small but reproducible loss of *XSox-2* expression on the injected side (Fig. 1G; $n = 30$). This indicates that a portion of the increased domain of neural crest induction is at the expense of more medial aspects of the developing neural plate. Conversely, expression of *XSox-2* was expanded in embryos injected with G4A-Slug (Fig. 1F; $n = 20$).

Inhibition of XSlug Activity Blocks the Expression of Early Neural Crest Markers

If the results of overexpressing the Gal4 activation domain fusion can be equated with a loss-of-function phenotype, the above findings suggest that XSlug function is required during early stages of neural crest formation for the expression of neural crest markers at the lateral edges of the neural plate. It is possible, however, that the observed loss of neural crest precursors results not from interfering with XSlug's function as a transcriptional repressor, but instead from endowing it with novel transcriptional activation abilities. In order to better determine the loss-of-function phenotype, we overexpressed a truncated form of XSlug, containing only the DNA-binding domain (ΔSlug). When the DNA-binding domain of a transcription factor is sufficient for DNA binding, overexpressing it can saturate binding sites, preventing binding by the functional holoprotein and thus dominantly interfering with its ability to regulate transcription. In the case of XSlug, this approach has an important added advantage. As XSlug and XSnail are highly related (over 92% identical in the DNA-binding domain) and thought to be functionally redundant (Sefton *et al.*, 1998) this construct should inhibit both XSlug and XSnail activity, thus bypassing the problem of redundancy that has complicated the interpretation of mouse knockouts (Jiang *et al.*, 1998).

As was found for G4A-Slug, overexpression of ΔSlug resulted in a loss of *XSlug* (Fig. 2A; $n = 48$) and *XTwist* (Fig. 2C; $n = 65$) expression on the injected side of the embryo. These results confirm that the activity of XSlug-related transcriptional repressors is required for the expression of neural crest markers at early stages, when neural crest precursors are forming. Importantly, expression of these markers could be rescued by coinjecting mRNA encoding full-length XSlug (Figs. 2B and 2D; $n = 42$). This demonstrates both the specificity of the effects of the truncation mutant and that XSlug function alone is sufficient to rescue those effects.

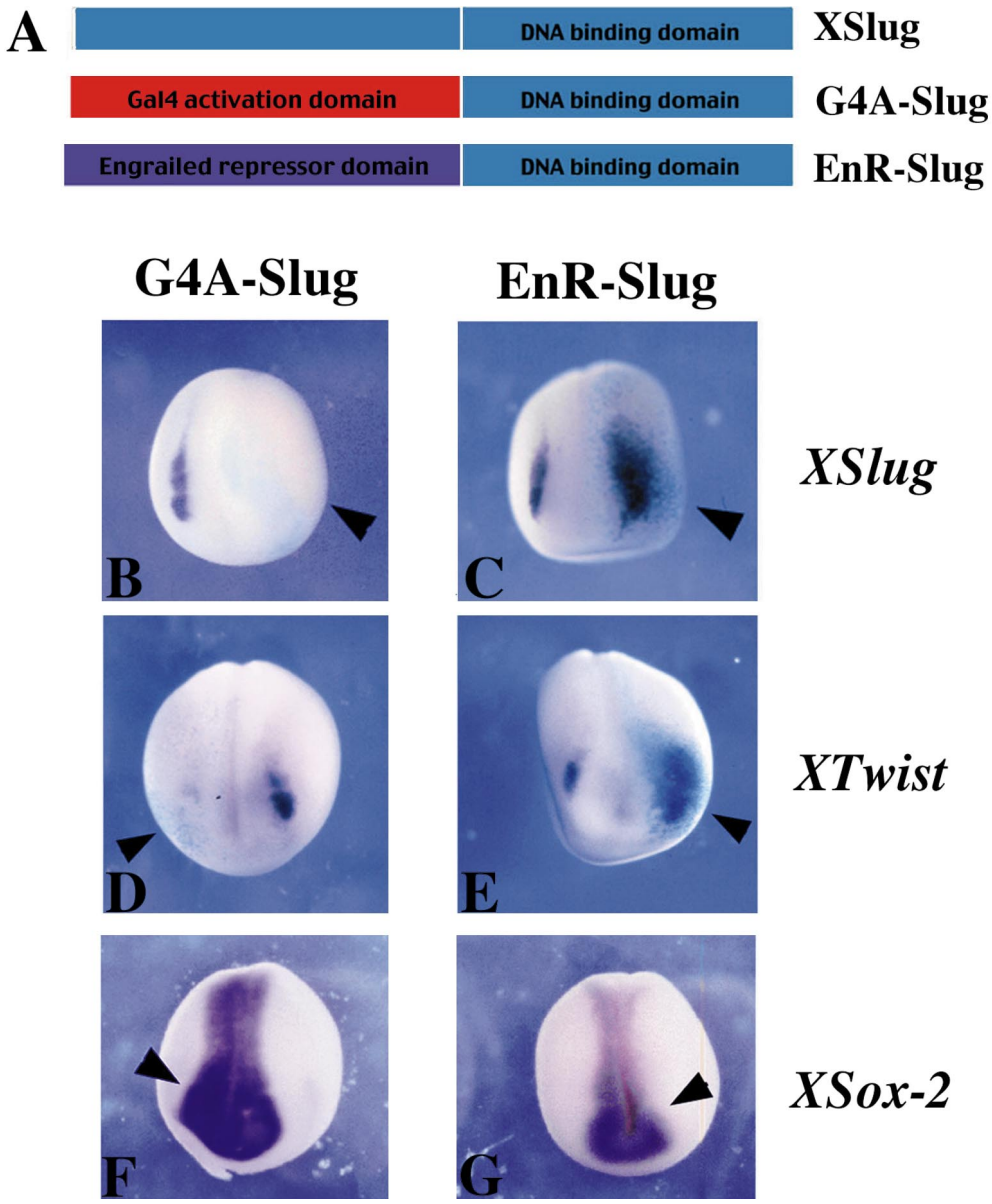


FIG. 1. XSlug functions as a transcriptional repressor. (A) Diagram of the constructs used for overexpression experiments. The zinc-finger region of XSlug was fused in frame to either the activation domain of Gal4 (G4A-Slug) or the repressor domain from *Drosophila* Engrailed (EnR-Slug). (B–G) Whole-mount *in situ* hybridization of embryos injected in one cell with mRNA encoding either G4A-Slug (B, D, F) or EnR-Slug (C, E, G). Embryos were examined at midneurula stages (stage 17–18) for the expression of *XSlug* (B, C), *XTwist* (D, E), or *XSox-2* (F, G). Overexpression of the activation domain fusion caused a loss of neural crest marker expression and an increase in neural plate marker expression on the injected side (black arrowheads), while overexpression of the repressor fusion had the opposite effects.

Inhibition of XSlug Function Prevents Neural Crest Formation

The loss of early neural crest marker expression in ΔSlug injected embryos suggests that neural crest precursors fail to form in the absence of Slug/Snail function. However, as the function of these genes in neural crest development is not fully understood, it remains possible that neural crest cells that do not express *XSlug* and *XTwist* nevertheless form and migrate in these embryos. Therefore, in order to better investigate whether neural crest cells do form in embryos expressing ΔSlug, we utilized an assay that uncouples neural crest production from marker gene expression.

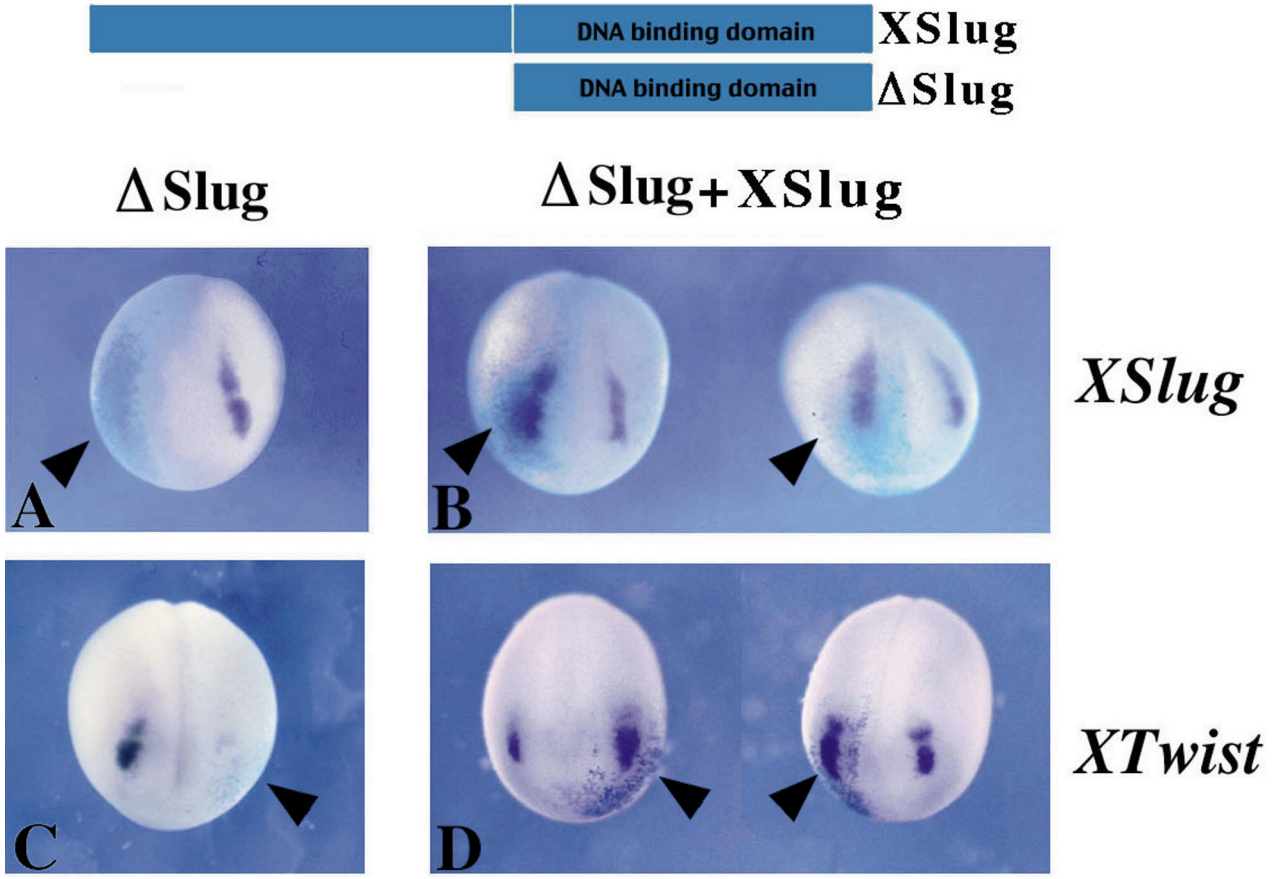


FIG. 2. Inhibition of XSlug activity blocks the expression of early neural crest markers. Whole-mount *in situ* hybridization of embryos injected in one cell with a truncation mutant encoding the DNA-binding domain of XSlug (ΔSlug; A, C) or coinjected with full-length XSlug (B, D). Embryos were examined at midneurula stages (stage 17–18) for the expression of the neural crest markers *XSlug* (A, B) and *XTwist* (C, D). Overexpression of the truncation mutant inhibited the expression of early neural crest markers on the injected side (black arrowheads). These effects could be rescued by coexpression of the full-length protein.

In *Xenopus* embryos, cranial neural crest migration commences at late neurula stages (stage 21) and proceeds in characteristic streams into prospective mandibular, hyoid, and branchial arch regions. If neural fold regions are fluorescently labeled and grafted into unlabeled hosts, the neural crest cells produced from them can be easily recognized and followed as they migrate along these pathways. In order to determine whether such migration occurs in the absence of Slug/Snail function, embryos were injected at the two-cell stage with GFP mRNA alone or were coinjected with mRNA-encoding GFP and ΔSlug. These embryos were allowed to develop until midneurula stages, when labeled neural folds were explanted and grafted into the corresponding region of unlabeled host embryos (Figs. 3A and 3B). Embryos receiving grafts were allowed to further develop until late neural stages, when they were examined for neural crest migration using fluorescence microscopy.

Embryos receiving grafts from donors injected with GFP alone had fluorescently labeled cells migrating along all characteristic neural crest migratory pathways (Fig. 3C; $n = 14$). These cells are easily detected because the only fluorescently labeled cells present in these embryos are derived from the graft. In contrast, GFP-labeled grafts from embryos coinjected with ΔSlug failed to produce migratory neural crest cells (Fig. 3D; $n = 18$). Instead, labeled cells remained closely associated with the closed neural tube/developing brain through swimming tadpole stages (Fig. 3D and not shown). These results indicate that migratory neural crest cells do not form in embryos injected with ΔSlug. This finding, together with the marked absence of expression of known early neural crest markers in these embryos, strongly suggests that neural crest precursor cells fail to form in the absence of Slug/Snail function.

Inducible Inhibition of XSlug Function

The above experiments indicate that in *Xenopus*, Snail-family-mediated transcriptional repression is required dur-

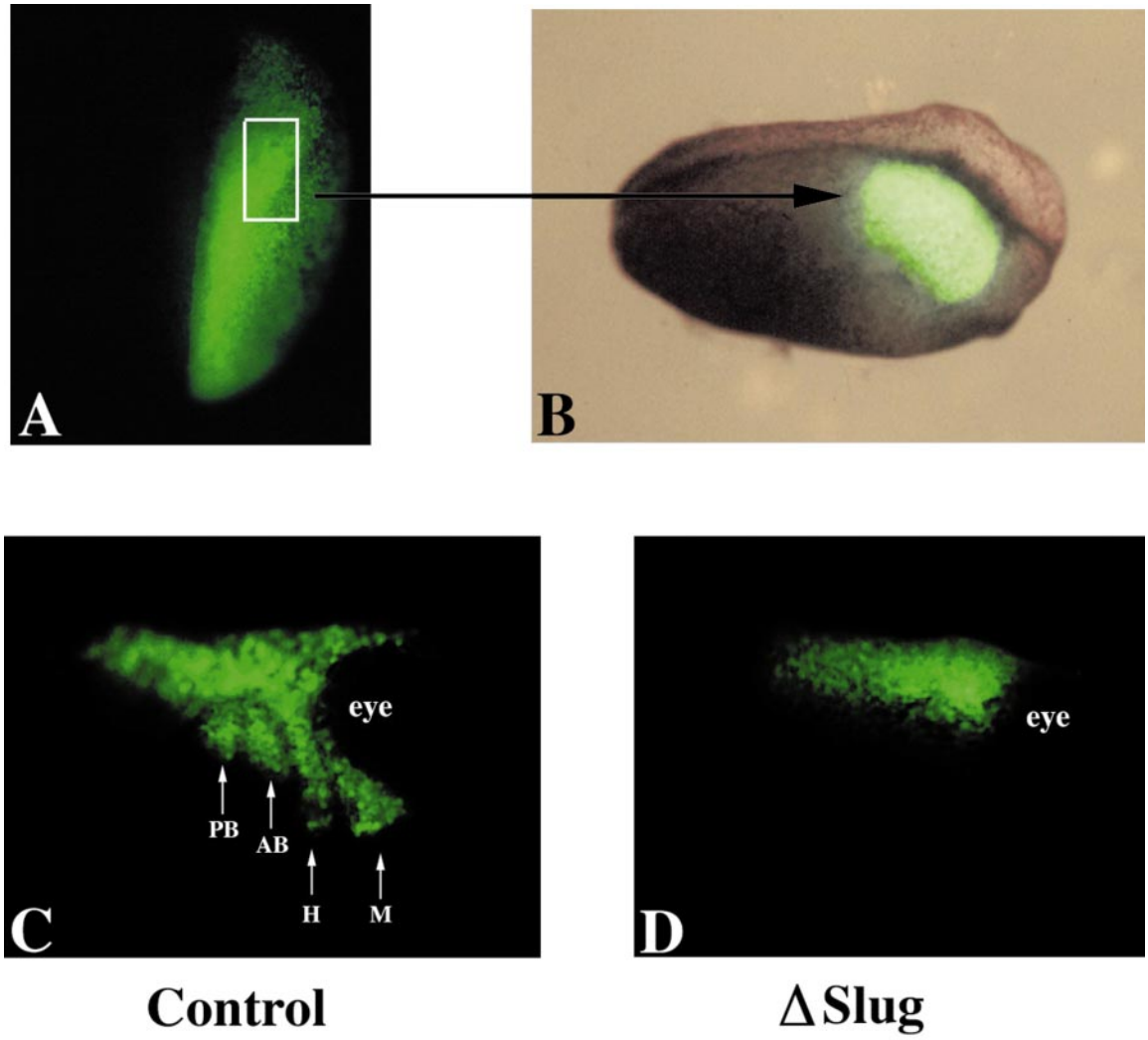


FIG. 3. Inhibition of XSlug function prevents neural crest formation. Donor embryos expressing GFP alone or coexpressing GFP and ΔSlug were cultured to midneurula stages (stage 16–17). Cranial neural folds were explanted from donor embryos displaying strong GFP expression in this region (A) and grafted into the same location on stage-matched unlabeled host embryos (B). Host embryos were cultured through late neurula stages and migration of neural crest cells was followed using fluorescence microscopy. Grafts from donor embryos injected with GFP alone produced copious numbers of neural crest cells that migrated in well-characterized mandibular (M), hyoid (H), anterior branchial (AB), and posterior branchial (PB) arch streams (C). In contrast, grafts from embryos coinjected with GFP and ΔSlug failed to produce migratory neural crest cells (D).

ing early stages of neural crest formation. In contrast, antisense experiments in the chick have suggested that CSlug is required for neural crest emigration. While neural crest fails to migrate in *Xenopus* embryos injected with ΔSlug, this is not surprising if neural crest precursors are absent in these embryos. To investigate whether XSlug function is also required at later stages, in order for neural crest cells to become migratory, it is necessary to inhibit its activity only after neural crest precursor cells have formed. To accomplish this, the XSlug DNA-binding domain was fused in frame to the hormone-binding domain of the glucocorticoid receptor (GR-ΔSlug). It has previously been demonstrated that when mRNA encoding such fusion proteins is injected into *Xenopus* embryos, the fusion protein can translocate to the nucleus and become transcriptionally active only in the presence of an appropriate hormone such as dexamethasone (Kolm and Sive, 1995).

In order to verify the activity of this construct, one cell of two-cell embryos was injected with mRNA-encoding GR-ΔSlug. Injected embryos were cultured in the presence or absence of dexamethasone from midgastrula stages (stage 11.5) until midneurula stages (stage 16–18), when they were

fixed for *in situ* hybridization. As expected, in the absence of dexamethasone, embryos injected with the GR-ΔSlug fusion showed normal expression of neural crest markers (Figs. 4B and 4D; $n = 39$). In contrast, when GR-ΔSlug-injected embryos were cultured in the presence of dexamethasone, expression of *XSlug* (Fig.4C; $n = 32$) and *XTwist* (Fig. 4E; $n = 24$) was inhibited on the injected side, as had been found for ΔSlug. Control mRNA encoding the hormone-binding domain of the glucocorticoid receptor alone had little or no effect on the expression of these markers in either the presence or the absence of dexamethasone (not shown). These results indicate that this construct can be used to inducibly inhibit Slug/Snail function during neural crest development.

A Later Role for XSlug in Neural Crest Migration

In order to determine whether XSlug function is required for neural crest migration, embryos injected with GR-ΔSlug were allowed to develop in the absence of dexamethasone until midneurula stages (stage 17–18). At these stages, *XSlug* expression in neural-crest-forming regions has been well established, as has the anterior-most portion of the *XTwist* expression domain. It is important to note that expression of *XTwist* in neural crest cells originating adjacent to the posterior portion of the rhombencephalon is not observed until around the time neural crest cells become migratory. In *Xenopus*, condensation of the neural crest masses (mandibular, hyoid, and branchial) commences at approximately stage 19, while migrating neural crest can be observed by stage 21 (Sadaghiani and Thiebaud, 1987). When GR-ΔSlug-injected embryos were treated with dexamethasone starting at midneurula stages and examined for the expression of neural crest markers at stage 23, the noted effects were predominantly on neural crest migration. In these embryos, *XSlug*- (Figs. 5A–5C; $n = 29$) and *XTwist*- (Figs. 5D and 5E; $n = 23$) expressing cells on the injected side often remained as a mass of cells adjacent to the neural tube. This is in clear contrast to the migrating streams of neural crest cells observed on the uninjected side of these embryos (Figs. 5A–5E). The observed inhibition of neural crest migration was dependent upon ΔSlug function, since in the absence of dexamethasone neural crest migration was unaffected (Fig. 5F and not shown). These findings indicate that in addition to playing an essential role in neural crest induction, Slug/Snail function is also required for the initiation of neural crest migration. Not surprisingly, cranial neural crest cells that did not yet express *XTwist* prior to dexamethasone treatment (i.e., in posterior hyoid and branchial crest segments) failed to do so in these embryos.

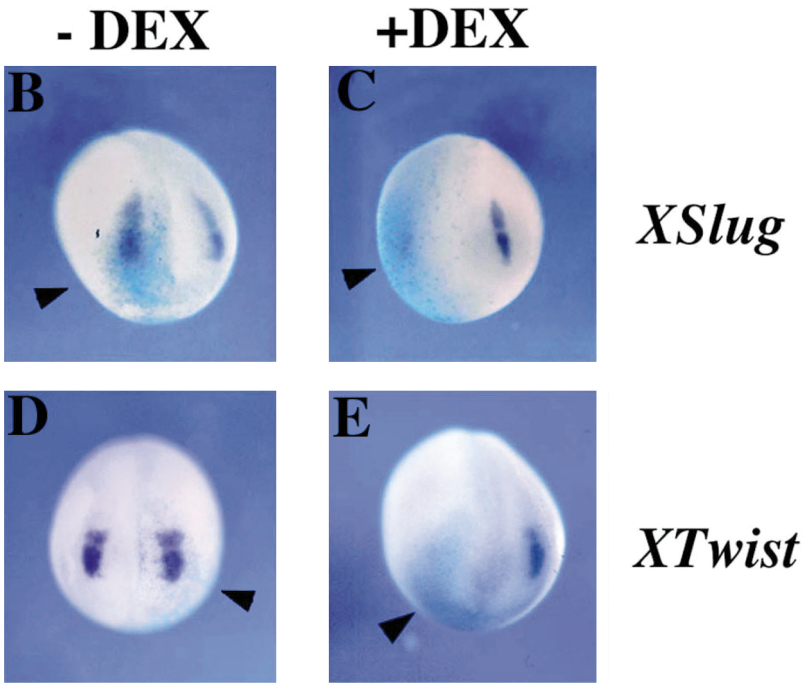
DISCUSSION

Accumulating evidence suggests that the zinc-finger DNA-binding protein Slug and the closely related gene Snail play an important role in neural crest development. In *Xenopus, XSlug* expression is the earliest known marker of neural crest forming regions at the lateral edges of the newly induced neural plate (Mayor *et al.*, 1995). Recently, we demonstrated that overexpression of XSlug in early *Xenopus* embryos results in an expansion of neural-crest-forming regions (LaBonne and Bronner-Fraser, 1998). We found that in embryos that have been injected with mRNA-encoding XSlug, the expression domain of early neural crest markers is markedly increased. Here we expand upon this work and show that a fusion protein in which the DNA-binding domain of XSlug is linked to the repressor domain from *Drosophila* Engrailed (EnR-Slug) has similar effects when overexpressed. This finding indicates that during neural crest formation, XSlug is functioning as a transcriptional repressor.

As XSlug is functioning as a transcriptional repressor, its ability to induce ectopic expression of neural crest markers in whole embryos must be indirect. This finding sheds light on why, despite its effects when overexpressed *in vivo*, XSlug overexpression is insufficient to mediate neural crest formation in ectodermal explants (LaBonne and Bronner-Fraser, 1998). Expression of neural crest markers such as *XSnail* and *XTwist*, or *XSlug* itself, are not activated in XSlug-injected ectoderm. XSlug can cooperate with Wnt- or FGF-mediated signals to activate expression of these markers, however. Taken together, these findings suggest that in addition to Slug/Snail-mediated repression of inhibitors of this process, neural crest formation also requires positively acting factors, and these factors can be activated downstream of Wnts and FGFs.

In embryos injected with either XSlug or the repressor domain fusion (EnR-Slug), ectopic expression of neural crest markers occurs predominantly in regions of the ectoderm normally fated to give rise to epidermis. It is therefore possible that during early stages of neural crest formation, XSlug acts to repress the expression of genes that mediate epidermal fate. In *Xenopus*, BMP signaling is thought to be largely responsible for instructing ectoderm to form epidermis (Wilson and Hemmati-Brivanlou, 1995), making BMPs or their downstream effectors attractive candidates for targets of XSlug repression. Indeed, overexpression of either XSlug or EnR-Slug in ectodermal explants inhibits the expression of epidermal keratin (C. LaBonne, unpublished observation). Work is currently under way to determine if these effects are direct or indirect. It should be noted, however, that since XSlug overexpression can cause ectopic expression of neural crest markers in more medial regions of the neural plate, epidermal genes are unlikely to be the only targets of XSlug repression.

Despite the positive effects of XSlug overexpression on neural crest marker expression, the finding that XSlug acts as a transcriptional repressor during neural crest formation was not entirely unexpected. In *Drosophila*, Snail has been shown to repress the expression of neurectodermal genes in the mesoderm (Leptin, 1991). In addition, mouse Snail has recently been found to repress trophoblast giant cell differ-



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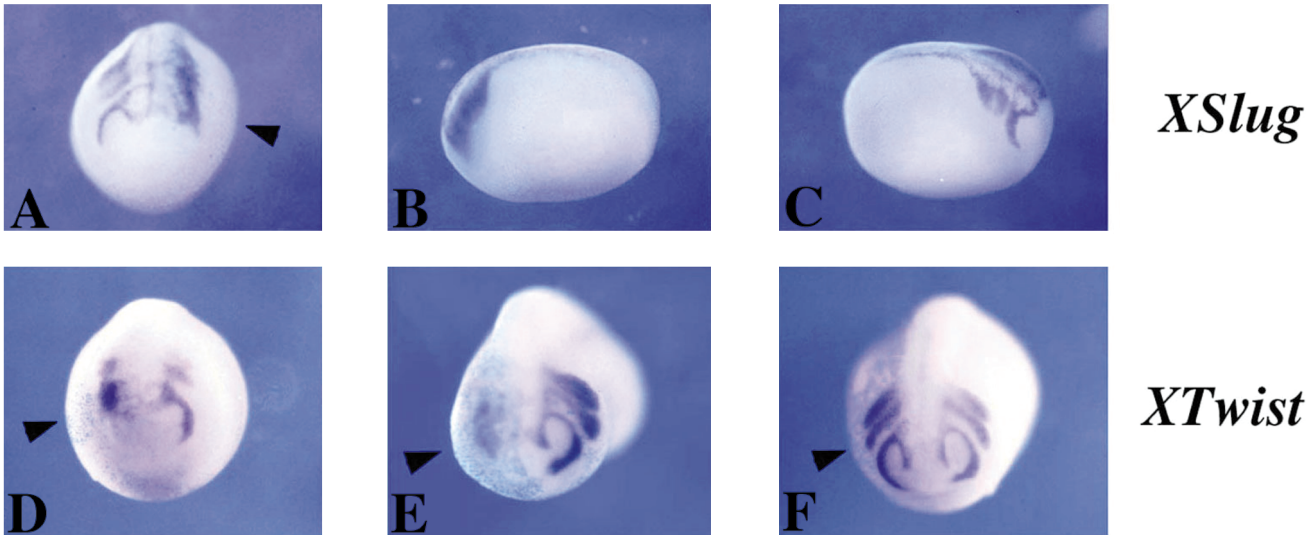

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FIG. 4. Inducible inhibition of XSlug function. The zinc-finger region of XSlug was fused in frame to the hormone-binding domain of the glucocorticoid receptor (GR-ΔSlug; A). Embryos injected in one cell with mRNA encoding this fusion were cultured in the absence of dexamethasone (B, D) or treated with dexamethasone from stage 11.5 onward (C, E). Embryos were examined by *in situ* hybridization at midneurula stages (stage 17–18) for expression of *XSlug* (B, C) or *XTwist* (D, E). In embryos treated with dexamethasone, the expression of neural crest markers was lost or diminished on the injected side (black arrowheads). In the absence of hormone, the expression of neural crest markers was unchanged.

FIG. 5. A later role for XSlug in neural crest migration. Embryos injected in one cell with mRNA encoding the GR-ΔSlug were treated with dexamethasone from stage 17/18 onward (A–E) or left untreated (F). Embryos were examined by *in situ* hybridization for expression of *XSlug* (A–C) or *XTwist* (D–F) at late neurula stages, when cranial neural crest cells are migrating. In embryos treated with hormone, cells expressing neural crest markers on the injected side of the embryo (black arrowheads) remained as a mass of cells adjacent to the neural tube. Normal migration patterns were observed on the uninjected side of hormone-treated embryos and in injected embryos reared in the absence of hormone.

entiation (Nakayama *et al.,* 1998). However, a growing number of transcription factors have been shown to be capable of acting as either transcriptional activators or repressors, and this could be true of Slug and Snail as well. Indeed two other DNA-binding proteins implicated in neural crest development, Pax-3 and Lef-1, can mediate either transcriptional activation or repression (Chalepakis *et al.,* 1994; Eastman and Grosschedl, 1999). Since the role played by such factors during development can be context dependent, it is important to test the function of transcription factors in the system of interest before drawing conclusions about their mode of action.

In contrast to the results obtain with the Engrailed repressor/XSlug fusion, overexpression of a Gal4 activation domain/XSlug fusion (G4A-Slug) resulted in a loss of *XSlug* and *XTwist* expression in neural-crest-forming regions. This result suggested that Slug and/or Snail repressor activity is required for the expression of these genes from early stages of neural crest development. Importantly, similar results were obtained when a truncated form of XSlug (ΔSlug) was overexpressed. Thus, the ability of G4A-Slug to inhibit neural crest formation reflects a requirement for XSlug and/or XSnail function during this process and is not an artifact of endowing the Slug DNA-binding domain with transcriptional activation activities.

The inhibition of early neural crest marker expression following overexpression of either G4A-Slug or ΔSlug suggests that neural crest precursors fail to form in these embryos. An alternative interpretation of these findings could be that neural crest cells do form and migrate in these embryos, but that these neural crest cells do not express early neural crest markers such as *XSlug* or *XTwist*. In order to differentiate between these possibilities, we utilized an assay that uncouples neural crest production from marker gene expression. In these experiments, we grafted cranial neural folds expressing GFP alone or coexpressing GFP and ΔSlug, into the corresponding region of unlabeled host embryos and followed the fate of the grafted cells using fluorescence microscopy. In embryos receiving grafts from donors injected with GFP alone, fluorescently labeled cells were observed migrating along all characteristic cranial neural crest migratory pathways. In contrast, grafts coexpressing ΔSlug failed to produce migratory neural crest cells. These results provide evidence that the absence of early neural crest marker expression in ΔSlug-injected embryos reflects a loss of neural crest precursor cells in these embryos.

Because antisense experiments in the chick have suggested that in these embryos Slug is required for neural crest cell emigration or migration (Nieto *et al.,* 1994), we wanted to ask whether this was also the case in *Xenopus*. However, because mRNA injection of ΔSlmug interferes with early stages of neural crest formation, it was necessary to employ a strategy which would allow us to bypass this early requirement and inhibit XSlug activity only after neural crest precursors had already formed. In order to accomplish this, we created a hormone-inducible version of ΔSlug by fusing the XSlug DNA-binding domain to the hormone-binding domain of the glucocorticoid receptor (GR-ΔSlug). Overexpression of GR-ΔSlug had no effect on neural crest development when embryos were reared in the absence of hormone. However, when GR-ΔSlug-injected embryos were treated with dexamethasone from late gastrula stages onward, the expression of early neural crest markers was blocked, indicating that this construct can be used to inducibly inhibit XSlug/XSnail function. Dexamethasone-mediated induction of this protein at late neurula stages, after expression of neural crest markers had been established, inhibited neural crest migration. These results demonstrate, for the first time, that Slug-related transcriptional repressors are required at multiple steps during neural crest formation.

While this work was being prepared for publication, it was reported that inhibition of *XSlug* by overexpressing antisense mRNA interferes with neural crest migration (Carl *et al.,* 1999). In contrast to the findings we report here, these authors reported effects on neural crest migration but not on neural crest precursor formation. The likely reason for this disparity is that the antisense experiments in that study were designed to specifically eliminate *XSlug* but not *XSnail* transcripts. Functional redundancy between these genes, which these authors demonstrate, would thus mask any role they play in establishing the early expression of neural crest markers such as *XTwist*. Although the authors do not examine whether *XSlug* antisense mRNA interferes with *XSnail* expression in neural crest precursors at early neurula stages, a loss of both *XTwist* and *XSnail* expression was seen at later stages, consistent with our findings.

In order to better understand the role played by these factors during neural crest precursor formation and neural crest migration, it will be important to determine the mechanisms by which they mediate transcriptional repression. The region N-terminal to the DNA-binding domain that is deleted in ΔSlug contains two putative repressor domains: a SNAG domain (Nakayama *et al.,* 1998) and a possible binding site for CtBP, a transcriptional corepressor known to interact with DSnail (Nibu *et al.,* 1998). Interestingly, although the SNAG domain is conserved among all vertebrate Snail family members, it is absent in both *Drosophila* and ascidian Snail. Thus, it is tempting to speculate that the acquisition of this domain may represent a significant event in the evolution of the neural crest. Nonvertebrate chordates such as ascidians and amphioxus have a single *Snail* homologue that is expressed in the developing neural folds and the dorsal neural tube (Corbo *et al.,* 1997; Langeland *et al.,* 1998). The conservation of this expression pattern is of particular interest given that the neural crest is a cell type unique to vertebrates. It suggests that neural crest could have originated as a population of *Snail*-expressing cells within the neural tube of the common ancestor of modern vertebrates (see Baker and Bronner-Fraser 1997). The requirement for Slug/Snail function during neural crest precursor formation may therefore reflect the original role played by Snail in the dorsal neural tube of

the ancestral prevertebrate chordate. These cells may then have acquired migratory ability, perhaps as a consequence of acquiring additional targets of Snail-mediated transcriptional repression. Migratory ability would have allowed these cells to disperse throughout the embryo and ultimately give rise to new derivatives, including those which form the "new head" of vertebrates (Northcutt and Gans, 1983).

In summary, our results demonstrate that in *Xenopus*, DSnail-related transcription factors function as transcriptional repressors and play multiple distinct roles during neural crest formation. Slug/Snail activity is required at early stages for the establishment of neural crest precursors at the lateral aspect of the newly induced neural plate and again at later stages for the production of migratory neural crest cells. An important challenge for the future is to identify and characterize the transcriptional targets of these repressors in order to better understand the mechanisms by which they mediate neural crest induction and migration.

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