Cloning and Characterization of Three *Xenopus* Slug Promoters Reveal Direct Regulation by Lef/ß-Catenin Signaling*

**Jérôme Vallin‡, Raphael Thuret, Emiliana Giacomello, Marisa M. Faraldo, Jean P. Thiery, and Florence Broders**

From UMR 144, CNRS/Institut Curie, 26 rue d’Ulm, 75248 Paris Cedex 05, France

In amphibians and birds, one of the first steps of neural crest cell (NCC) determination is expression of the transcription factor Slug. This marker has been used to demonstrate that BMP and Wnt molecules play a major role in NCC induction. However, it is unknown whether Slug expression is directly or indirectly regulated by these signals. We report here the cloning and characterization of three *Xenopus* Slug promoters: that of the *Xenopus tropicalis* slug gene and those of two *Xenopus laevis* Slug pseudoalleles. Although the three genes encode proteins with almost identical amino acid sequences and are expressed with similar spatiotemporal patterns, their 5′-flanking regions are quite different. A striking difference is a deletion in the *X. tropicalis* gene located precisely at the transcription initiation site that results in the *X. tropicalis* promoter being inefficient in *X. laevis*. Additionally, we identified two regions common to the three promoters that are necessary and sufficient to drive specific expression in NCCs. Interestingly, one of the common regulatory regions presents a functional Lef/ß-catenin-binding site necessary for specific expression. As the Lef/ß-catenin complex is a downstream effector of Wnt signaling, these results suggest that *Xenopus* Slug is a direct target of NCC determination signals.

In vertebrates, neural crest cells (NCCs) form a unique population of cells. NCCs are determined during neurulation at the border of the neural plate, delaminate from the neural tube during and after its closure, and migrate extensively before colonizing various tissues in which they differentiate into various neuronal and non-neuronal cell types (1, 2). In several species, the related zinc finger transcription factors Slug and Snail are specific early markers of this population: Slug in chick (3), Snail in mouse (4, 5) and zebrafish (6), and both genes in *Xenopus* (7, 8).

In *Xenopus* laevis embryos, Slug is expressed from the beginning of neurulation in the presumptive neural crest domain, a strip of cells at the border between the neural plate and presumptive epidermis (7). Many studies have used this marker to investigate the molecular basis of NCC induction. Interactions between the neural and non-neural ectoderm seem to be crucial for this induction (9, 10). As it is decisive for neural induction, the role of BMP4 inhibition in NCC determination has been extensively studied. By overexpressing a BMP4 antagonist, noggin or chordin, this inhibition has been shown to be necessary but insufficient for XSSlug expression, which occurs only in synergy with another lateralizing signal (10–12). Both fibroblast growth factor and Wnt pathways have been suggested as sources of this additional signal (7, 11, 13). Recent studies have suggested that Wnt is a direct signal required for NCC determination, whereas fibroblast growth factor-dependent XSSlug induction would be a consequence of an indirect effect mediated by Wnt (10).

These studies have contributed to our understanding of the mechanisms of XSSlug induction, but most are difficult to interpret definitively because it is difficult to determine whether XSSlug is a direct or indirect target of the injected molecules. We cloned the slug promoter to overcome this problem and to analyze precisely the pathways leading to XSSlug induction. In this study, we isolated one promoter from *Xenopus tropicalis* and two different promoters from *X. laevis*. The sequences of the three promoters seemed very different at first glance; but by detailed comparison, we were able to distinguish two conserved regions that appeared to be necessary and sufficient for specific expression in NCCs. This promoter, used with a simple expression system, is a very powerful tool for targeting NCCs. It can thus be used to study the role of several putative candidate molecules in the determination, migration, and differentiation of these cells. We also report here for the first time an extensive comparison between *X. tropicalis* and *X. laevis* promoters, indicating the possibility of differences in transcription mechanisms in these two species, which are assumed to be very closely related. Finally, the two regulatory regions are potential targets for several transcription factors and would be very useful for determining effective XSSlug inducers. We report here the presence in one of these regions of a functional Lef/ß-catenin-binding site that is necessary for expression in NCCs. This suggests that the Wnt pathway is directly involved in *Xenopus* Slug regulation and that Slug is one of the direct targets of NCC determination signals.

**EXPERIMENTAL PROCEDURES**

*Slug Gene Isolation and Sequence Analyses—*The *X. tropicalis* gene was isolated from a genomic DNA library (kind gift of R. Grainger, University of Virginia, Charlottesville, VA) by high stringency nonradioactive screening. A 500-bp fragment of previously described *X. laevis* Slug cDNA (7) was used as a probe. *X. laevis* genes were obtained by PCR. Primers were derived from a 20-bp region common to human, mouse, and *X. tropicalis* Slug promoters. Combined with several oligo-
nucleotides derived from the \textit{X. laevis} cDNA, these primers allowed us to amplify two complete \textit{X. laevis} Slug genes. PCR was carried out with the high fidelity Bio-X-Act polymerase (Bioline, London, United Kingdom), and various clones were sequenced with an ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA). 5'- and 3'-ends of mRNAs were precisely located with the GeneRacer kit (Stratagene, La Jolla, CA). In silico sequence analyses were performed at the Infobiogen facilities (Evry, France).

\textbf{Embryos and Injections}—Wild-type \textit{X. laevis} embryos were used for all injection experiments. Each construct was injected in the Sleeping Beauty plasmid between the two inverted terminal repeats (14). These inverted terminal repeats enable genomic integration if transposase is co-injected, which was not the case here. Since this plasmid is remarkably stable and nontoxic, it can also be used alone for ectopic expression in \textit{Xenopus} embryos. In this study, each construct was injected, in a volume of 15 nl, into one blastomere of a two-cell stage embryo. To compare the effects of different constructs, all the injections in a series were performed at the same molarity. If not otherwise stated below, 2 \times 10^{-7} mol of plasmid was injected in 15 nl; for example, this corresponded to 100 pg of the a3000-GFP construct. Injections were performed in 0.33× Marc’s modified ringers and 4% Ficoll (15). Embryos were then transferred to 0.33× Marc’s modified ringers and cultured at 18 °C until the desired stage of development. Stages were determined as described by Nieuwkoop and Faber (16).

\textbf{RT-PCR Experiments}—Radioactive semiquantitative RT-PCR, with histone H4 used as an internal control, was performed with a pool of 10 embryos as previously described (17). To detect the GFP transcript, we used the following specific primers: U, 5'-GATCCACAGGAGGACCAT for GFP a, with 5'-AGCCGGGGAGGAGGAC for GFP b, and with 5'-CTGT-GCTACCCAGGGCTGAC for the amplification of both Slug genes. PCR products were subjected to electrophoresis on 8% native acrylamide gels and analyzed with a PhosphorImager (Amersham Pharmacia Biotech).

\textbf{In Situ Hybridization}—Whole-mount \textit{in situ} hybridization experiments were performed as described by Harland (18), but with minor modifications (7). For each \textit{X. laevis} Slug gene, the 5'-UTR was used as a short specific probe.

\textbf{Electrophoretic Mobility Shift Assays}—Lef-1 was produced by transcription and translation in vitro from a \textit{pcDNA} construct (kindly provided by A. Ben \textit{z}ev, Weisman Institute, Rehovot, Israel) with a coupled transcription and translation kit (Promega, Madison, WI). The recombinant GST-beta-catenin fusion protein was purified from \textit{Escherichia coli} transformed with \textit{pEGX5-beta-cut} (a gift from A. Cano, Instituto de Investigaciones Biomedicas, Madrid, Spain). For protein-DNA interaction, Lef-1 and beta-catenin produced in vitro were incubated for 30 min with 20,000 cpm of a 32P-labeled duplex oligonucleotide probe containing the predicted Lef-1-binding site of the \textit{slug} promoter, respectively) gave 80% maximum score and centered on the TATA box (70% score and centered on -28), a putative CCAAT box, and two GC boxes. These results suggest that the cloned flanking region is a good candidate for the \textit{X. tropicalis} slug promoter.

\textbf{Characterization of Xenopus Slug Promoters}—To test the activity of the \textit{X. tropicalis} slug promoter in \textit{vivo}, deletion constructs were produced with the GFP gene as a reporter gene. These constructs were cloned in the Sleeping Beauty plasmid (14). Injected into \textit{X. laevis} at the two-cell stage, this plasmid remains present at high levels throughout early development, at least until late tail-bud stages, and enables expression of ectopic proteins under the control of specific promoters.2 This technique has the great advantage of being simple and harmless for the embryos. Thus, a large number of injected embryos can be obtained in a single experiment, making it possible to obtain statistically significant results with many different constructs.

We assayed various fragments of the \textit{X. tropicalis} slug promoter, from 200 bp to 4 kb in length, in which the GFP coding region was inserted just downstream from the CAP site. The GFP coding region inserted downstream from the 5'-UTR of the \textit{X. tropicalis} Slug cDNA was used as a negative control (Fig. 1A). Several series of injections were performed with each of these constructs, but no fluorescence was ever detected. We tested for GFP mRNA by semiquantitative RT-PCR of stage 19 NF injected embryos. The GFP transcript was detected in various quantities, depending on the construct injected (Fig. 1A). The constructs p200-GFP and p3900-GFP (including 200 and 3900 bp of the \textit{X. tropicalis} slug promoter, respectively) gave relatively high levels of expression. These results show that the \textit{X. tropicalis} slug promoter was able to drive GFP expression in \textit{X. laevis} embryos. Nevertheless, the efficiency of this promoter could not be compared with that of the endogenous promoter in this assay.

We investigated promoter efficiency by making new constructs with the two most efficient regions of the \textit{X. tropicalis} Slug promoter. These fragments were cloned upstream from a modified \textit{X. laevis} Slug cDNA containing an insertion (Fig. 1B). This enabled us to detect in injected embryos the products of both the endogenous and exogenous promoters with the same set of primers and thus to perform comparative RT-PCR. The results show that the exogenous and endogenous products were produced in similar amounts (Fig. 1B). As expected, the relative quantity of exogenous Slug decreased over time because the number of plasmid copies/cell decreased during the course of development. However, with this method, each cell should be considered to contain \textasciitilde 20 copies of the plasmid at stage 19 NF. Therefore, if the two promoters were similar in efficiency, the exogenous product would be produced in substantial excess over the endogenous product.

2 J. Vallin, E. Giaconello, I. S. Coulter, J. P. Thiery, and F. Broders, submitted for publication.
Our results indicate that the region of the X. tropicalis slug promoter isolated was inefficient in X. laevis embryos. There may be two reasons for this: either this region did not contain all the regulatory elements, or the regulatory process differed between X. tropicalis and X. laevis.

Cloning of Two X. laevis Slug Pseudoalleles—A PCR approach was used to clone the 5′-flanking region of the X. laevis slug gene. Comparison of the sequences of Slug promoters from X. tropicalis, humans (19), and mice3 revealed no similarity except a common 20-bp region, located at −3.2 kb in X. tropicalis, −1.5 kb in humans, and −1.2 kb in mice. We designed two primers in this common region and used them with two primers corresponding to the Slug coding region to perform nested PCR on X. laevis genomic DNA.

We obtained two types of amplicon containing 3 and 1.7 kb upstream from the ATG codon, respectively. Several subfragments were amplified, and a large number of clones were sequenced to obtain the complete sequences of these two genes. The genetic organization of these two clones was identical (Fig. 2A), and their predicted coding sequences were very similar (Table I), with 96% identity at the nucleotide level, compared with 67% identity between XSlug and the related zinc finger protein Xenopus Snail (22). This high level of similarity indicated that we had cloned two Slug pseudoalleles. The first (with 3 kb of 5′-flanking region) appeared to correspond to the published X. laevis slug gene and is referred to hereafter as slug α. In addition to a few differences in the coding region, the second clone is slightly divergent in the 5′- and 3′-UTRs (Table I). This clone is referred to hereafter as slug β.

Both X. laevis Slug Genes Are Expressed in NCCs—Before studying the regulatory capacities of the 5′-flanking regions of the two X. laevis Slug clones, we needed to determine the precise expression pattern of each gene. As previously in situ hybridization studies (7, 23) used part of the coding region as a probe, the domains expressed may be those of Slug α, Slug β, or both Slug mRNAs.

To discriminate between the two pseudoalleles, we first used an RT-PCR approach. Amplification was performed with primers derived from a divergent region of the 5′-UTR, which were therefore specific for a particular Slug gene. Semiquantitative PCR using embryos at various stages of development showed that both pseudoalleles were expressed at very similar levels and in exactly the same temporal pattern (Fig. 3A). Moreover, in situ hybridization with neurula embryos and short specific probes derived from the 5′-UTR showed that the two X. laevis Slug genes were expressed in the same location, at the border of the neural plate, as expected (Fig. 3B).

The 5′-Flanking Regions of Both X. laevis Slug Genes Drive Specific Expression in NCCs—We assessed the efficiency of the X. laevis promoters obtained using constructs consisting of the GFP coding region under the control of the full-length slug α or slug β flanking region (α3000-GFP and β1700-GFP, respectively). Both promoters drove strong GFP expression in NCCs at the edge of the neural plate throughout the neurulation process and later both in premigratory and migrating cells (Fig. 4). Expression was detected at stage 14 NF at the border of the neural plate. The highest level of expression was observed at stage 19 NF, with the strong expression in the cephalic NCCs and a lower level of expression in the rest of the neural crest territory. At stage 25 NF, GFP was strongly expressed in the cephalic premigratory NCCs. The migrating cells colonizing the branchial arches gave a positive, but weaker GFP signal. The cells from the mandibular pathway displayed the strongest expression among cells colonizing the branchial arches. This expression pattern closely resembled that of endogenous Slug according to in situ hybridization data (23). This suggests that the isolated flanking regions of both slug α and slug β contain the key regulatory elements of the promoter.

3 P. Savagner, unpublished data.
Comparison of the Three Xenopus Slug Promoters—The three Xenopus Slug genes presented similar expression patterns, but their 5' flanking regions were different. More detailed analysis revealed two conserved regions (Fig. 2A and Table I). Region A was −250 bp long and was located just upstream from the CAP site. Region B was −300 bp long, and its position was variable: around −3 kb in X. tropicalis slug, around −2.7 kb in X. laevis slug α, and around −1.5 kb in slug β. The sequences between these two regions were divergent. X. tropicalis presented a 315-bp fragment of GT repeats, located around −800 bp. These repeats were absent from the X. laevis Slug promoters, but slug α and slug β displayed 11 and 4 repeats, respectively, of a 79-nucleotide motif. Although this motif has been identified in regulatory as well as transcribed regions of the X. laevis genome (24–26), it has never been shown to have a regulatory function.

Additionally, comparison of the sequences of the genes from X. tropicalis and X. laevis revealed a striking difference at the transcription initiation site. Although the surrounding regions are almost identical in the three promoters, both X. laevis genes have a 9-bp insertion just downstream from the identified CAP site of the X. tropicalis promoter (Fig. 2B). This insertion creates a second putative CAP site, with a score even higher than that for the first site. This may account for the inefficiency of the X. tropicalis promoter in X. laevis. Alternatively, divergent regions, such as the repeats present in X. laevis but not in X. tropicalis, may be involved in Slug regulation.

The Extra CAP Site in X. laevis Is Necessary for Efficient Transcription—We investigated whether the extra CAP site is necessary for efficient transcription in X. laevis by producing a construct derived from β1700-GFP. Seven nucleotides were deleted at the 3'-end of the promoter, eliminating the second putative CAP site (construct β1700ΔCAP-GFP) (Fig. 5A). This construct did not drive detectable GFP expression when injected into X. laevis embryos (data not shown). GFP mRNA was detected in these embryos by RT-PCR, but at a level of about one-fifth that found in embryos injected with β1700-GFP (Fig. 5A). Thus, the second CAP site present in the X. laevis genes is

---

**TABLE I**

| CAP sites | X. laevis (X.l.) | X. tropicalis (X.t.) |
|-----------|------------------|----------------------|
| X. laevis | aagaagtcttagGctgcacttg | -----------gtgatgg |
| X. tropicalis | aagaagtcttagGctgcacttg | -----------gtgatgg |

**Fig. 2. Comparison of the three Xenopus Slug genes.** A, all three genes displayed the same organization, with three exons (dark-gray boxes) and two introns (i1 and i2; white boxes) of similar length. Asterisks indicate the positions of the stop codons in the coding regions. The 5' flanking regions are divergent except for two conserved regions, each ~300 bp long (regions A and B). Region A is located just upstream from the CAP site, whereas the position of region B is variable. The repeat boxes indicate repeats of a 79-nucleotide motif in X. laevis (X.l.) sequences. The GT box indicates a region of GT repeats in the X. tropicalis (X.t.) sequence. B, comparison of the sequences of X. tropicalis and X. laevis promoters around the transcription start site (indicated by arrows). Asterisks indicate differences between the two species. We predicted CAP sites and determined score as described by Bucher (21). In the X. laevis promoter, a 9-bp insertion creates an extra CAP site, with a better predicted score than the initial CAP site and that of X. tropicalis.

---

**a** Xr, X. tropicalis; Xl, X. laevis; aa, amino acids.
necessary for efficient transcription in this species.

The β1700ΔCAP-GFP construct was very similar to the X. tropicalis constructs used in terms of the CAP site (Fig. 5B). Interestingly, the level of GFP mRNA detected with this β1700ΔCAP-GFP construct was similar to that obtained with the most efficient X. tropicalis constructs (compare Figs. 1A and 5A, the same unit is used in both cases). This suggests that the inefficiency of the X. tropicalis promoter in X. laevis was mainly due to the absence of the second CAP site in this promoter.

We also investigated the putative role of the first intron in Slug regulation. We injected a construct containing the β1700 promoter, the first exon, the first intron, and the start of the second exon in frame with the GFP cDNA (construct β1700int1-GFP) (Fig. 5A). Similar results were obtained with this construct and with β1700-GFP, suggesting that the first intron of the slug gene contains no major regulatory elements.

**Combination of the Two Common Regions Is Necessary and Sufficient for Targeted Expression**—We investigated the regulatory properties of the various regions of Slug promoters using several constructs containing different portions of the slug α promoter (Fig. 6). The amount of GFP and its distribution in injected embryos were analyzed both by fluorescence monitoring throughout development and by semiquantitative RT-PCR using 5′-UTR sequences as short specific probes. The two genes were expressed at the same site in the presumptive cephalic NCCs (arrowheads) as previously described for Slug mRNA.
levels obtained with \(a70\)TATA-GFP. This indicates the existence of a negative regulatory element in the 5' fourth of region A. With 8 × injections, this \(a270\)A-GFP construct gave a similar nonspecific expression pattern compared with \(a200\)A-GFP, but with lower mRNA levels. The \(a1000\)-GFP and \(a2200\)-GFP constructs (with 1 and 2.2 kb of promoter, respectively, and containing region A, but lacking region B) had similar effects compared with \(a270\)A-GFP for both 1 × and 8 × injections. This suggests that the intermediate divergent regions of the promoters are not very important for transcription regulation. If the region B was present, as in the full-length \(a3000\) promoter, the specific Slug pattern was observed in 1 × injected embryos, as described above. This suggested the presence of another positive regulatory element in region B. No GFP expression was ever observed with constructs containing region B and the TATA box, but lacking the rest of region A (see, for example, \(a500\)B-GFP and \(a2300\)B-GFP), even for 8 × injections. These results suggest that both conserved regions A and B were necessary for specific expression in NCCs. In addition, the intermediate region did not seem to possess regulatory elements. As the relative positions of regions A and B differ in the three promoters, we wondered whether the intermediate region might play an essential role as a spacer. We tested this hypothesis by injecting the \(a700\)BA-GFP construct, which contains a very short intermediate region. This construct gave a similar expression pattern compared with the full-length promoter, indicating that the two conserved regions alone are sufficient to drive specific expression in NCCs.

Region B Contains a Functional Lef/β-Catenin-binding Site Necessary for Its Positive Regulatory Action—In silico analysis

**FIG. 5.** The second CAP site is necessary for *X. laevis* Slug promoter function, whereas the first intron is not. A, the second CAP site was deleted from the slug β promoter (β1700-ΔCAP construct) (left panel). This construct was injected into embryos, and GFP expression was quantified by RT-PCR analysis and compared with that driven by the full-length promoter (right panel). No fluorescence was detected with this construct in living embryos, and the mRNA level at stage 19 NF was about one-fifth that obtained with the full-length promoter. A construct containing the full-length promoter and the first intron gave results similar to those obtained with the promoter alone. Int1, intron 1; n, number of embryos analyzed. B, shown is the comparison of the sequences surrounding the transcription initiation site in the β1700-ΔCAP construct and in the *X. laevis* and *X. tropicalis* Slug promoters. The transcription start sites are indicated by an uppercase G. Note that the *X. laevis* construct lacking the second CAP site was very similar to the *X. tropicalis* promoter in this region. a.u., arbitrary units.

**FIG. 6.** Combination of the two conserved regions is necessary and sufficient to drive specific expression in NCCs. Various constructs (left panel) were injected into one blastomere of a two-cell stage embryo at two different concentrations. In 1 × injections, \(2 \times 10^{-17}\) mol of plasmid was injected in 15 nl. This corresponded to 100 pg of the \(a3000\)-GFP construct. In 8 × injections, eight times as many plasmids were injected in the same volume. GFP expression was analyzed by fluorescence monitoring in living embryos and by semiquantitative RT-PCR with stage 19 NF total RNA. n, number of embryos analyzed; fluo, localization and intensity of the observed fluorescence (major phenotype); nc, neural crest; ep, presumptive epidermis. The bars indicate the amount of GFP mRNA relative to histone H4 mRNA. The images show the GFP expression pattern obtained with the \(a70\)TATA-GFP (TATA); \(a200\)GA-GFP (A\(^1\)); \(a270\)A-GFP (A), and \(a700\)BA-GFP (BA) constructs.
of Xenopus Slug promoter sequences predicted a number of putative binding sites for transcription factors. These sites included a consensus binding site for Lef-1 in the middle of region B (centered on -2839 in the slug α promoter). The Lef/Tcf transcription factors form complexes with β-catenin, acting as downstream targets of the Wnt signaling pathway, which has been shown to be directly involved in NCC determination (10). We investigated the possible role of this site in Slug regulation by performing electrophoretic mobility shift assays to study the in vitro interaction of Lef with the slug promoter. Purified Lef-1 protein specifically bound a 22-bp fragment from region B containing the putative site (Fig. 7A). This binding was inhibited by a nonradioactive competitor containing the Lef-1 consensus binding site (27). Furthermore, the addition of β-catenin led to the detection of a ternary DNA-Lef-β-catenin complex that was supershifted upon addition of an anti-β-catenin antibody (Ab; upper arrow). The β lane is a negative control in which the probe was incubated with GST/β-catenin alone. The GST lane is a negative control in which Lef-1 was incubated with GST alone before adding the probe. The arrowhead indicates free probe. C, GFP expression in stage 19 NF embryos injected with the indicated constructs was assessed by RT-PCR. The bars indicate the means ± S.D. of three independent experiments. The α3000ΔLef construct, which contained a promoter lacking the Lef-binding site, gave levels of GFP much lower than those obtained with the full-length α3000 promoter, but similar to those obtained with the negative α70TATA control. a.u., arbitrary units.

DISCUSSION

The X Slug Promoter Is an Efficient Tool for Targeting NCCs—We report in this study the cloning of X Slug 5'-flanking regions, which contain the regulatory elements required for specific expression in NCCs. This promoter is a powerful tool for investigating the molecular mechanisms controlling the behavior and fate of these cells. Only a few studies have investigated these mechanisms, mainly through difficult grafting experiments (28–31). In such experiments, donor tissues injected with an exogenous mRNA must be transplanted to the precise position of the presumptive neural crest. These manipulations are delicate and time-consuming and result in only a small number of analyzable embryos. In addition, the position of the graft may differ slightly between embryos, rendering data interpretation difficult. Overall, such experiments require a considerable amount of work if statistically significant results are to be obtained.

In contrast, the X Slug promoter, combined with an efficient vector like Sleeping Beauty, facilitates the targeting of exogenous proteins to NCCs. Given the pattern of expression driven by this promoter, the role of various molecules can be investigated in at least three important phases of NCC evolution: 1) the quiescent state, corresponding to the entire neurulation process in Xenopus; 2) the epithelium-mesenchyme transition and delamination from the neural tube; and 3) migration along various specific pathways.

Xenopus Slug Gene Evolution—We report here the complete sequencing and comparison of the two X laevis pseudoalleles and the X. tropicalis homolog of the same gene. This kind of comparison has been reported only once before, for the short α-globin gene (32). Such comparisons are very informative, both about the isolated gene itself and about the relative evolution of these two species.

As far as Slug is concerned, this comparison identified regions that may be important in the action of the protein and for its regulation. As expected, the three predicted amino acid sequences of the Slug proteins show a high level of conservation, with >97% of residues identical. The five zinc fingers are totally identical in the three genes, confirming their crucial importance. In contrast, the 29-amino acid Slug-specific box described by Sefton et al. (33) displays two nonequivalent substitutions in these very closely related species, calling into question the role of this region in Slug function. At the nucleotide level, comparison of the divergent 5'-flanking sequences led to the identification of two conserved regions that seem to contain the key regulatory elements of the promoter. These regions are highly conserved, displaying ~90% identity; and domains thought to be of particular importance, such as the TATA box and the Lef-binding site, are totally identical in the two species.

These findings indicate that there has been strong selection
pressure and emphasize the importance of a functional Slug protein produced at precisely the right time and place for NCC determination. The related gene snail is also precociously expressed in NCCs, suggesting that Slug and Snail are both important in neural crest formation in *Xenopus*, whereas only one of these two factors is maintained in this area in other vertebrate species (33).

The analysis of divergent regions provides some information about the similarity and relative evolution of the *X. laevis* and *X. tropicalis* genomes. These two species are thought to be closely related, but very little is known about similarities between them in terms of nucleic acid sequence. One study compared rDNA and concluded that there was a sister group relationship between *X. laevis* and *X. tropicalis*, which seem to be more closely related to each other than to other morphologically similar pipid frogs (34). One possible mechanism of genomic evolution is the insertion of DNA fragments into non-essential regions. Two examples are found in the divergent regions of *Xenopus* Slug genes. There is a 79-nucleotide motif in the intermediate region of the *X. laevis* Slug promoters, but not in the equivalent region of the *X. tropicalis* promoter. Eleven repeats of this motif are present in *slug α*, whereas only four are present in *slug β*. This suggests that an ancestral motif was inserted after separation of the two species but before *X. laevis* genomic duplication. In the first intron, 16 repeats of a 24-nucleotide motif are present in *slug α* only, accounting for the difference in the length of this intron between this gene and the other two genes. In this case, the ancestral motif must have been inserted after genomic duplication. Further analysis of the divergent regions should make it possible to date more precisely this genomic duplication in the evolution of *Xenopus* frogs.

The most striking difference observed in our comparison of Slug genes concerns the transcription initiation site. A 9-bp insertion in *X. laevis* has created an extra CAP site that seems to be the functional site in this species, as its removal renders the promoter ineffective *in vivo*. Consistent with this result, the *X. tropicalis* promoter was unable to drive physiological expression when injected into *X. laevis* embryos, although it possesses all the other important regulatory regions. This demonstrates that closely related species may present different mechanisms, even in a regulatory process that is quite similar overall between the two species. Thus, care should be taken when using *X. tropicalis* material (especially DNA material) in *X. laevis* or vice versa.

**Mechanism of XSlug Activation**—The three *Xenopus* Slug promoters cloned displayed two conserved regions, which are critical for the specific regulation of this gene: the combination of these two regions is necessary and sufficient to drive expression in NCCs. These conserved sequences are not present in the 5′-flanking regions of the human (19) and mouse*β* Slug genes, but this is consistent with the observed differences in the Slug expression pattern between *Xenopus* and mouse (33). The only sequence conserved between these four species is a 20-bp motif at the 5′-end of region B in Xenopus, suggesting that this motif may be involved in regulation.

The first conserved region in *Xenopus* Slug promoters (region A) is located just upstream from the CAP site and contains basal transcription regulation elements and at least two specific responsive elements. In the middle of this region, between the TATA box and −200, there is a positive element that can be activated in all non-neural ectodermal cells, but not in neural tissue, as shown by injection of the α200A*-GFP construct. This specificity suggests interaction with factors specific to the presumptive epidermis. The 5′-fourth of region A contains a negative element, which is also efficient in non-neural ectodermal cells. Indeed, injected at physiological levels, the α270A*-GFP construct gave no expression. Injected at higher doses, this construct promoted expression at the same location as α200A*-GFP. This indicates that its negative regulation is probably mediated by one or several nuclear factors: in overexpression experiments, some of the injected plasmid would titrate these factors, and “free” plasmid would be responsible for the observed expression. These factors may be specific to non-neural ectodermal cells or ubiquitous.

The second conserved region (region B) contains a positive regulatory element that, in combination with those of region A, is responsible for the specific activation of XSlug genes in NCCs. The distance between these two regions is not critical, as it differs greatly between the three genes, and the elimination of almost all of the intermediate region does not affect expression. Region B contains a functional Leφ/β-catenin-binding site that seems to be required for Slug activation in NCCs. This is consistent with the finding that Wnt signaling is required for the activation of this marker (10) and indicates that Slug is a direct target of this pathway. As Wnt is thought to be one of the initial signals in NCC determination, Slug induction would be one of the first steps of this determination. Nevertheless, slug is not a master gene in NCC formation, as its overexpression in ectodermal explants is insufficient to elicit neural crest gene expression (10). Thus, other specific genes, such as *snail*, may be regulated by the same determination signals.

**Mechanism of Xenopus NCC Determination**—Our study confirms that the Wnt signaling pathway plays a direct role in *Xenopus* NCC induction. It also indicates that other signals are necessary, as the Wnt-responsive element of the XSlug promoter is insufficient to promote expression, as shown by injection of the α2300B-GFP construct. This is consistent with previous experiments showing that Wnt alone does not induce NCC markers in ectodermal explants (10). Based on previous data on *Xenopus* NCC induction, BMP4 is an attractive candidate for the second signaling molecule. Interestingly, some promoters have been shown to be directly regulated by interaction between the Wnt and BMP pathways. For example, in *Drosophila melanogaster*, Wingless and Decapentaplegic (Wnt and BMP orthologs, respectively) act in synergy to stimulate transcription of the *Ultrabithorax* gene (35). Similar cooperation between these two signaling pathways was recently demonstrated in *Xenopus* during formation of the Spemann organizer (36, 37). However, the mechanism is likely to be different in NCCs, as Slug expression requires activation of the Wnt pathway, but inhibition of the BMP pathway (10). It is not clear whether total or partial inhibition is required, and there are therefore two possible hypotheses: 1) NCC induction results from combination of the Wnt signal with low levels of BMP4, or 2) the total absence of BMP4 is required for NCC induction in response to the Wnt signal. Further studies of the Slug promoter, particularly of the regulatory elements in region A, should help us to understand the sophisticated mechanism underlying NCC induction.

**Acknowledgments**—We thank Robert Grainger and Lyle Zimmermann for the *X. tropicalis* genomic library and Enrique Amaya for the GFP cDNA. We thank Pierre Savagner for communication of the mouse *slug* promoter sequence. We thank Andre Mariadassou and David du Pasquier for helpful discussions and technical advice.

**REFERENCES**

1. Le Douarin, N. M., and Kalcheil, C. (1999) *The Neural Crest*, Cambridge University Press, Cambridge, United Kingdom
2. Christiansen, J. H., Coles, E. G., and Wilkinson, D. G. (2000) *Curr. Opin. Cell Biol.* 12, 719–724
3. Nieto, M. A., Sargent, M. G., Wilkinson, D. G., and Cooke, J. (1994) *Science* 264, 835–839
4. Nieto, M. A., Bennett, M. F., Sargent, M. G., and Wilkinson, D. G. (1992) *Development* 116, 227–237
5. Smith, D. E., Franco del Amo, F., and Gridley, T. (1992) *Development* 116,
Characterization of Xenopus Slug Promoters

1033–1039
6. Thiese, C., Thiese, B., and Postlethwait, J. H. (1995) Dev. Biol. 172, 86–99
7. Mayor, R., Morgan, R., and Sargent, M. G. (1995) Development 121, 767–777
8. Essex, L. J., Mayor, R., and Sargent, M. G. (1993) Dev. Dyn. 198, 108–122
9. Mancilla, A., and Mayor, R. (1996) Dev. Biol. 172, 86–99
10. LaBonne, C., and Bronner-Fraser, M. (1998) Development 125, 2403–2414
11. Mayor, R., Guerrero, N., and Martinez, C. (1997) Dev. Biol. 189, 1–12
12. Marchant, L., Linker, C., Ruiz, P., Guerrero, N., and Mayor, R. (1998) Dev. Biol. 198, 319–329
13. Saint-Jeannet, J. P., He, X., Varmus, H. E., and Dawid, I. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13713–13718
14. Ivics, Z., Hackett, P. B., Plasterk, R. H., and Izsvak, Z. (1997) Cell 91, 501–510
15. Sive, L. J., Mayor, R., and Sargent, M. G. (1993) Dev. Dyn. 198, 108–122
16. Mancilla, A., and Mayor, R. (1996) Dev. Biol. 172, 86–99
17. Mayor, R., Guerrero, N., and Martinez, C. (1997) Dev. Biol. 189, 1–12
18. LaBonne, C., and Bronner-Fraser, M. (1998) Development 125, 2403–2414
19. Mayor, R., Guerrero, N., and Martinez, C. (1997) Dev. Biol. 189, 1–12
20. Marchant, L., Linker, C., Ruiz, P., Guerrero, N., and Mayor, R. (1998) Dev. Biol. 198, 319–329
21. Saint-Jeannet, J. P., He, X., Varmus, H. E., and Dawid, I. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13713–13718
22. Vallin, J., Girault, J. M., Thiery, J. P., and Broders, F. (1998) Mechan. Dev. 75, 171–174
23. Harland, R. M. (1991) Methods Cell Biol. 36, 685–695
24. Stedmann, K., Boecker, J., Kosan, C., Erment, A., Kunz, J., and Koch, M. C. (1999) Mutat. Res. 406, 63–69
25. Jiang, R., Norton, C. R., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Grindley, T. (1996) Biochim. Biophys. Acta 1443, 251–254
26. Bucher, P. (1990) J. Mol. Biol. 212, 563–578
27. Sargent, M. G., and Bennett, M. F. (1990) Development 109, 967–973
28. Linker, C., Bronner-Fraser, M., and Mayor, R. (2000) Dev. Biol. 234, 215–225
29. Mayor, R., Guerrero, N., and Martinez, C. (1997) Dev. Biol. 189, 1–12
30. Linker, C., Bronner-Fraser, M., and Mayor, R. (2000)Dev. Biol. 234, 215–225
31. Mayor, R., Guerrero, N., and Martinez, C. (1997) Dev. Biol. 189, 1–12
32. LaBonne, C., and Bronner-Fraser, M. (2000) Dev. Biol. 224, 215–225
33. Mayor, R., Guerrero, N., and Martinez, C. (1997) Dev. Biol. 189, 1–12
34. Marchant, L., Linker, C., Ruiz, P., Guerrero, N., and Mayor, R. (1998) Dev. Biol. 198, 319–329
35. Saint-Jeannet, J. P., He, X., Varmus, H. E., and Dawid, I. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13713–13718
36. Vallin, J., Girault, J. M., Thiery, J. P., and Broders, F. (1998) Mechan. Dev. 75, 171–174
37. Harland, R. M. (1991) Methods Cell Biol. 36, 685–695
38. Stedmann, K., Boecker, J., Kosan, C., Erment, A., Kunz, J., and Koch, M. C. (1999) Mutat. Res. 406, 63–69
39. Jiang, R., Norton, C. R., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Grindley, T. (1996) Biochim. Biophys. Acta 1443, 251–254
40. Sargent, M. G., and Bennett, M. F. (1990) Development 109, 967–973
41. Linker, C., Bronner-Fraser, M., and Mayor, R. (2000) Dev. Biol. 234, 215–225
42. Ho, L., Mervola, M., and Guisas, L. J. (1994) Mech. Dev. 47, 56–64
43. Kloc, M., Spohr, G., and Etkin, L. D. (1993) Science 262, 1712–1714
44. Tsujimura, A., Yasojima, K., Kuboki, Y., Suzu, A., Ueno, N., Shiokawa, K., and Hashimoto-Gotoh, T. (1995) Biochem. Biophys. Res. Commun. 214, 422–439
45. Giese, K., Amsterdham, A., and Grosschedl, R. (1991) Genes Dev. 5, 2567–2578
46. Borchers, A., Epperlein, H. H., and Wedlich, D. (2000) Dev. Genes Evol. 210, 217–222
47. Carl, T. F., Dufton, C., Hanken, J., and Klymkowsky, M. W. (1999) Dev. Biol. 213, 101–115
48. LaBonne, C., and Bronner-Fraser, M. (2000) Dev. Biol. 221, 195–205
49. Mayor, R., Guerrero, N., Young, R. M., Gomez-Skarmeta, J. L., and Cuellar, C. (2000) Mech. Dev. 77, 47–56
50. Stalder, J., Wirthmuller, U., Beck, J., Gruber, A., Meyerhof, W., Knochel, W., and Weber, R. (1988) J. Mol. Evol. 28, 64–71
51. Sefton, M., Sanchez, S., and Nieto, M. A. (1998) Development 125, 3111–3121
52. de Sa, R. O., and Hillis, D. M. (1998) Mol. Biol. Evol. 7, 365–376
53. Waltzer, L., and Bienz, M. (1999) EMBO J. 18, 1630–1641
54. Labbe, E., Letamendia, A., and Attisano, L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8358–8363
55. Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H., and Cho, K. W. (2000) Nature 403, 781–785
Cloning and Characterization of Three *Xenopus* Slug Promoters Reveal Direct Regulation by Lef/ β-Catenin Signaling

Jérôme Vallin, Raphaël Thuret, Emiliana Giacomello, Marisa M. Faraldo, Jean P. Thiery and Florence Broders

*J. Biol. Chem.* 2001, 276:30350-30358.
doi: 10.1074/jbc.M103167200 originally published online June 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103167200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 34 references, 12 of which can be accessed free at
http://www.jbc.org/content/276/32/30350.full.html#ref-list-1