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A balance between the anti-apoptotic activity of *Slug* and the apoptotic activity of *msx1* is required for the proper development of the neural crest

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Abstract

We have studied the pattern of programmed cell death in the neural crest and analyzed how it is controlled by the activity of the transcription factors *Slug* and *msx1*. Our results indicate that apoptosis is more prevalent in the neural folds than in the rest of the neural ectoderm. Through gain- and loss-of-function experiments with inducible forms of both *Slug* and *msx1* genes, we showed that *Slug* acts as an anti-apoptotic factor whereas *msx1* promotes cell death, either in the neural folds of the whole embryos, in isolated or induced neural crest and in animal cap assays. The protective effect of expressing *Slug* can be reversed by expressing the apoptotic factor *Bax*, while the apoptosis promoted by *msx1* can be abolished by expressing the *Xenopus* homologue of *Bcl2* (*XR11*). Furthermore, we show that *Slug* and *msx1* control the transcription of *XR11* and several caspases required for programmed cell death. In addition, expression of *Bax* or *Bcl2*, produced similar effects on the survival of the neural crest and on the development of its derivatives to those produced by altering the activity of *Slug* or *msx1*. Finally, we show that in the neural crest, the region of the neural folds where *Slug* is expressed, cells undergo less apoptosis, than in the region where the *msx1* gene is expressed, which correspond to cells adjacent to the neural crest. We show that the expression of *Slug* and *msx1* controls cell death in certain areas of the neural folds, and we discuss how this equilibrium is necessary to generate sharp boundaries in the neural crest territory, and to precisely control cell number among neural crest derivatives.

Keywords: Apoptosis; *Slug*; *msx1*; Bax; Bcl2; Caspases; Neural crest

Introduction

The neural crest is a specialized population of cells that develops at the border between the neural plate and the epidermis in all vertebrate embryos. The closure of the neural tube brings these cells together at the dorsal pole of the neural tube, from where they migrate to different regions of the embryo, and differentiate into many cell types (for reviews, see LaBonne and Bronner-Fraser, 1999; Mayor et al., 1999).

From studies in chick, amphibian and zebrafish embryos, some of the signals involved in the induction of the neural crest have been identified, e.g., BMPs, Wnts, FGF, Notch and retinoic acid (Deardorff et al., 2001; Endo et al., 2003; García-Castro et al., 2002; Glavic et al., 2004; LaBonne and Bronner-Fraser, 1998; Liem et al., 1995; Mayor et al., 1995; Saint-Jeannet et al., 1997; Selleck et al., 1998; Streit and Stern, 1999, 1997; Villanueva et al., 2002). In *Xenopus* and zebrafish, it appears that the initial induction of the neural crest depends on a gradient of BMP activity (reviewed in Aybar and Mayor, 2002; Chitnis, 1999). As such, the intermediate concentrations of BMPs found at the border between the neural plate and the epidermis are those required to specify the neural crest cells, i.e., where BMP4 activity is lower than that required to induce epidermis formation and higher than that which

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induces neural tissue (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Morgan and Sargent, 1997; Nguyen et al., 1998; Villanueva et al., 2002; Tríbulo et al., 2003; Wilson et al., 1997).

It is now accepted that the induction of the neural crest is a multistep process, whereby a precise sequence of signals is necessary to produce the exact number of crest cells at the right position. However, it is clear that the number of cells in a given organ or tissue is also determined by the rate of cell division and of cell death (Jacobson et al., 1997). Thus, once inductive signals have been transmitted to a population of cells, additional mechanisms are required to determine the exact size of the organ by controlling cell proliferation and death. Indeed, during animal development, numerous structures are formed that are later removed by apoptosis. Moreover, cells that have been incorrectly induced fail to receive the trophic signals necessary for their survival and consequently they activate their innate autodestructive program.

It has been shown that apoptosis plays an important role in neural crest patterning. This became apparent through analyzing the developing hindbrain, a structure that is subdivided into eight segments, known as rhombomeres (Lumsden, 1990). In the hindbrain, a premigratory neural crest population is produced in each rhombomere; however, in avian embryos, the odd-numbered rhombomeres (3 and 5) do not produce a significant number of migratory cells (Kontges and Lumsden, 1996; Lumsden et al., 1991). Rather, the large majority of neural crest cell from rhombomeres 3 and 5 undergo apoptosis (Ellies et al., 2000; Graham et al., 1993, 1994). For this reason, we became interested in analyzing the role that apoptosis plays on patterning the neural crest in *Xenopus* embryos, and how this programmed cell death might be controlled.

Members of the *Snail* family of transcription factors lie upstream of the genetic cascade responsible for neural crest specification. Indeed, in the chick embryo, inhibiting *Slug* prevents neural crest migration (Nieto et al., 1994), whereas its overexpression augments the production of neural crest cells (Del Barrio and Nieto, 2002). Similarly, in *Xenopus* embryos, inhibition of *Slug* with antisense RNA or expression of a dominant-negative form of *Slug* reduces the expression of neural crest markers and inhibits the migration of the crest from the neural tube (Aybar et al., 2003; Carl et al., 1999; LaBonne and Bronner-Fraser, 2000; Mayor et al., 2000). Furthermore, overexpression of *Slug* produces an enlargement of the neural crest territory (Aybar et al., 2003; LaBonne and Bronner-Fraser, 2000; Mayor et al., 2000). It is noteworthy that in *C. elegans*, CES-1, a member of the *Snail* family of transcription factors, acts as an anti-apoptotic factor, similar to *Bcl2* or *Bcl-X_l*, and promotes the survival of IL-3-dependent murine pro-B cells deprived of cytokine (Inukai et al., 1999; Metzstein and Horvitz, 1999). Moreover, it has recently been shown that *msx* genes play an important role on neural crest initial development, as dominant-negative constructs of *msx1* block the expression of several early neural crest markers (Tríbulo et al., 2003). Furthermore, the *msx* genes have been implicated in promoting programmed cell death (reviewed in Davidson, 1995), and BMP4, a factor that directly controls *msx* transcription, induces apoptosis in both the cephalic neural crest (Graham et al., 1994) and the chick limb (Ganan et al., 1996).

As a result of these relationships, we have undertaken a detailed spatial and temporal analysis of naturally occurring cell death during the neurula stages of *Xenopus* embryo development. Through the use of conditional *Slug* and *msx1* gain- and loss-of-function constructs, we demonstrate that *Slug* acts as an anti-apoptotic factor while *msx1* promotes apoptosis in isolated neural crest, in the neural folds of whole embryos, in neural crest induced in vitro, and in animal caps. This suggests that these two genes may exert opposing effects on apoptosis. In addition, we show that both factors lie upstream of the Bcl2 and Bax proteins, and that they control the transcription of several caspase genes that are important in regulating programmed cell death. We interfered with cell death by expressing *Bax* and *Bcl2* genes in the neural fold region and this consistently altered the expression of early neural crest markers as well as affecting the development of neural crest derivatives in a similar way to *Slug* and *msx1* expression. We also compared the patterns of TUNEL staining with the expression of *msx1* and the neural crest marker gene *Slug*. We found that particularly high levels of apoptosis were detected in the neural fold region, these being especially high at the border of the neural crest territory, where *msx1* is expressed, rather than in the neural crest territory itself, where *Slug* expression is found. Our results suggest that the balance of anti-apoptotic factors expressed by neural crest cells and apoptotic factors expressed at the border of the neural crest territory serves to correctly define the population of neural crest cells and to control the correct size of its derivatives.

Materials and methods

Embryonic manipulation and dexamethasone treatment

Embryos were obtained from adult *Xenopus laevis* by standard hormone-induced egg laying and artificial fertilization (Villanueva et al., 2002). The embryos were staged according to Nieuwkoop and Faber (1967) and, where necessary, the animal caps were dissected out from them using eyebrow knives as indicated in Aybar et al. (2003).

Plasmid constructs and in vitro mRNA synthesis

The inducible constructs *msx1-GR*, *HDmsx1-GR*, *Slug-GR*, and *ZnfSlug-GR* were synthesized as described in Tríbulo et al. (2003) and Aybar et al. (2003). CM-BMP4,

CM-BMP7, dnBMP1, and ΔBMPR constructs were kindly donated by Dr. K.W. Cho (Blitz et al., 2000; Hawley et al., 1995). The Bax and XR11 constructs were a gift from Dr. C. Finkielstein and Dr. J. Maller (Finkielstein et al., 2001). All cDNAs were linearized and transcribed using a GTP cap analog (New England Biolabs) and SP6, T3, or T7 RNA polymerases (as described by Harland and Weintraub, 1985). After DNAse treatment, RNA was extracted with phenol–chloroform, precipitated with ethanol, and resuspended in DEPC–water.

RNA microinjection, lineage tracing and dexamethasone induction

Dejellied *Xenopus* embryos were placed in 75% NAM containing 5% Ficoll, and one blastomere of a two-cell stage embryo was injected with differing amounts of capped mRNA and 1–3 μg/μl lysine fixable fluorescein dextran (40,000 MW; FLDX, Molecular Probes) as a lineage tracer. For overexpression of *XR11* and *Bax*, mRNA was injected into one animal blastomere of an 8- to 16-cell stage embryo. For animal cap assays, mRNA was injected into the animal side of the two blastomeres of two-cell stage embryos. Approximately 8–12 nl of diluted RNA was injected into each embryo. Ethanol dissolved dexamethasone (10 μM) was added to the culture medium at stage 15 and was maintained in the medium until the embryos were fixed.

Noggin treatment

Heparin acrylic beads (Sigma) were incubated overnight with 100 μg/ml of noggin protein (a kind gift from R. Harland). Treatment with noggin was achieved by bringing together two caps, conjugated with a noggin-soaked bead between them. PBS-soaked beads were used as controls.

Whole-mount TUNEL staining, sectioning and nuclei counting

TUNEL was performed on whole-mount embryos as described previously (Hensey and Gautier, 1998). Briefly, embryos or caps were fixed in MEMFA (100 mM MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO4, 4% formaldehyde) and stored in methanol at −20°C. They were rehydrated in PBT (0.1% Tween 20 in PBS), washed in PBS, and incubated in 150 U/ml terminal deoxynucleotidyltransferase (Roche) and 0.5 mM digoxigenin-dUTP (Roche). The reaction was terminated in PBS/1 mM EDTA for 2 h at 65°C, followed by extensive washes in PBS. The embryos were then washed twice with MAB, blocked in MAB/2% Roche blocking reagent, and incubated with an anti-digoxigenin antibody coupled to alkaline phosphatase at a dilution of 1:3000 (Roche). Embryos were washed in MAB and the antibody was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Embryos and animal caps were bleached in 5% hydrogen peroxide and sections were performed as described previously (Linker et al., 2000).

To count the number of apoptotic nuclei, high-magnification pictures from sections of the TUNEL-stained embryos were taken and the neural folds were divided in equal parts: the external, central, and internal regions. A grid was placed on each region and the number of stained nuclei was counted. Similar results were obtained by counting apoptotic nuclei in whole mount or in sectioned embryos, but here we have only presented the results obtained from the sections.

DNA fragmentation

Pieces of ectoderm, neural plate and neural fold were dissected from stage 15 embryos and the fragmentation of DNA was analyzed as in Kaito et al., 2001. Explants were homogenized in 10 mM Tris (pH 8.0) containing 0.1 mM EDTA, 50 μg/ml RNAse A and 0.5% sodium dodecylsulfate (SDS), and incubated for 1 h at 37°C. Proteinase K (100 ug/ml) was added to the homogenate and incubated for a further 2 h at 50°C. The mixture was then treated with phenol/chloroform (1:1) and the DNA precipitated with ethanol. Electrophoresis was performed on a 1.5% agarose gel and the DNA was stained with ethidium bromide.

Whole-mount in situ hybridization

For *Xenopus* embryos, antisense probes containing Digoxigenin-11-UTP (Roche Biochemicals) were prepared by in vitro transcription for *msx1* (Suzuki et al., 1997), *FoxD3* (Sasai et al., 2001), *Slug* (Mayor et al., 1995). Specimens were prepared, hybridized and stained according to Harland (1991) with the modifications described in Mancilla and Mayor (1996).

Cartilage staining

For cartilage staining, embryos were fixed in formaldehyde at stages 45–47, washed with PBS and stained overnight in 0.2% alcyan blue/20% acetic acid in ethanol. Embryos were washed extensively with ethanol and bleached with a 1% KOH solution. Finally, the embryos were washed with 20% glycerol/2% KOH and dehydrated through a glycerol series into 80% glycerol.

RNA isolation and RT-PCR analysis

Total RNA was isolated from embryonic tissue by the guanidine thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987), and cDNAs were synthesized using AMV reverse transcriptase (Roche Biochemicals) and an oligo(dT) primer. Primers for *H4* were as described in

Aybar et al., 2003, and the primers used to analyze the *Xenopus caspases* (*2*, *3*, *6*, *7*, *9*) and *XR11* expression were:

Caspase 2 (AB038168): 5′-CTGTGCCTATGCCTGTCTGA-3′ and 5′-CCTGGGCGTAAATGATATGG-3′
Caspase 3 (AW765027): 5′-AAGTCTGGAACATCGCAAGG-3′ and 5′-TAAATGAGCCCCTCATCACC-3′
Caspase 6 (AB038169): 5′-TGGACATCAAGGACTGTGGA-3′ and 5′-CTGAACATCAAACCCCAGGT-3′
Caspase 7 (AB038170): 5′-CGGGAAAGAATTGGAAGTCA-3′ and 5′-AGGGCCGTTCTGTAAACCTT-3′
Caspase 9 (AB038172): 5′-CCGATGGAGTTTCAAGCAAA-3′ and 5′-GACTGGGCAGAAGGATTCAG-3′
XR11(X82461): 5′-GTCGGCCTGTATGGAAAGAA-3′ and 5′-CATGATAGGCGACCCAGTG-3′.

PCR amplification using these primers was performed over 28 cycles, and the PCR products were analyzed on 1.5% agarose gels. As a control, PCR was performed with RNA that had not been subjected to reverse transcription to check for DNA contamination. Quantification of the PCR bands was performed using ImageJ software (NIH, USA) on 8-bit grayscale JPG files; the values were normalized to the levels of H4 from the same samples and they were expressed as relative intensities (sample/H4 × 10).

Results

Slug and msx1 control apoptosis

It has been proposed that the *msx* genes promote apoptosis while members of the *Snail* family of genes might act as anti-apoptotic factors, although it has not been tested yet if this is also correct for *Xenopus* ectoderm. To analyze whether these factors could control apoptosis in ectodermal cells, we proceeded to use the *Xenopus* animal cap assay. Apoptosis was analyzed by TUNEL staining. It should be noticed that as the animal caps are transparent and small, so both the superficial and inner apoptotic nuclei are visible.

A large number of apoptotic cells can be detected in animal caps cultured in vitro (Fig. 1A); however, this could be dramatically reduced by blocking BMP activity, either by treating the animal caps with a noggin-soaked bead, by expressing a dominant-negative BMP4 receptor construct or by a BMP4 dominant negative (Figs. 1B–D,K). It is significant that the inhibition of other members of TGFβ family (BMP1 and BMP7) with dominant-negative cleavage mutant BMP7 or dominant-negative BMP1 did not alter the pattern of apoptosis in animal caps (not shown).

Expressing *Slug* (Fig. 1E) or a dominant-negative *msx1* construct (Fig. 1F) produced a strong inhibition of apoptosis in the animal cap (Figs. 1L,M). The specificity of the *msx1* dominant negative was demonstrated by the reappearance of the apoptosis when it was coinjected with *msx1* mRNA (Figs. 1G,H,L). Interestingly, the effect of *Slug* as an anti-apoptotic factor was also reversed by the coinjection of *msx1* mRNA (Figs. 1I,J,M). Taken together, these results indicate that in animal caps, high levels of BMP4 and its downstream target *msx1* promote apoptosis, an effect that can be reversed by blocking BMP4 or *msx1* activity. In addition, the expression of *Slug* suppressed apoptosis in animal caps although this effect could be reversed by coinjecting *msx1*, suggesting that apoptosis is controlled by a balance of *msx1* and *Slug*.

Control of apoptosis by Slug and msx1 in neural crest cells

As *Slug* and *msx1* are expressed in the neural crest and that play important roles in the development of this tissue (Aybar et al., 2003; Del Barrio and Nieto, 2002; Tríbulo et al., 2003), we proceeded to analyze apoptosis in the neural crest and how was it controlled by *Slug* and *msx1*.

To analyze the characteristic normal developmental cell death, whole-mount TUNEL staining was used to detect in situ DNA fragmentation in *Xenopus* embryos. It has previously been shown that apoptosis can first be detected during gastrulation, and as development progresses, characteristic patterns of cell death have been observed, particularly at the neurula stage. Indeed, at these stages, high levels of cell death are found in neural tissue (Hensey and Gautier, 1997, 1998, Yeo and Gautier, 2003). The TUNEL staining that we observed reproduced the same patterns of apoptosis that were described previously.

At the neurula stage, we found more TUNEL-stained nuclei in the neural folds than in the neural plate or epidermis (Fig. 2A). It should be mentioned here that to see this pattern of TUNEL staining, the color reaction has to be precisely controlled as if it is allowed to develop for longer periods of time, excessive staining is observed in the epidermis.

An additional feature of apoptosis is the activation of endonucleases that cleave and fragment genomic DNA (Wyllie, 1980). To determine where this process could be detected in the early neurula, we dissected out pieces of epidermis, neural fold or neural plate, and analyzed the DNA fragmentation in this tissue. The DNA ladder characteristic of genomic DNA fragmentation was observed in the neural fold tissue but not in the epidermis or neural plate (Fig. 2B). This result confirmed our observation that more apoptosis occurs in the neural fold when compared to other tissues.

To analyze the role of *Slug* and *msx1* on apoptosis, we performed two kinds of experiments. First, neural crest tissue was dissected from a stage 14 neurula from control embryos or from embryos previously injected with different *Slug* and *msx1* constructs, cultured in vitro and processed for TUNEL staining (Fig. 2C). As expected, a high level of apoptotic nuclei was found in the control

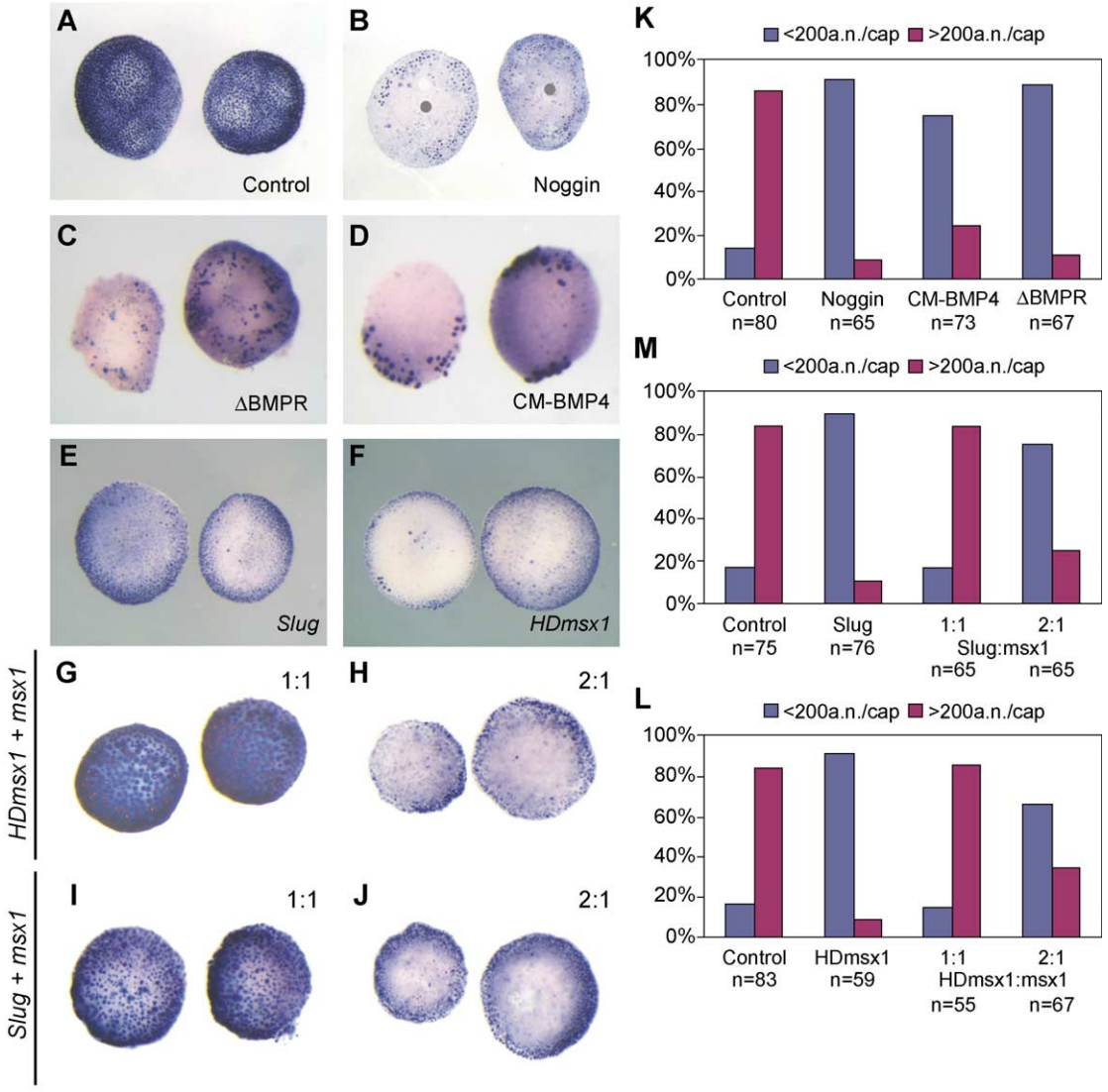
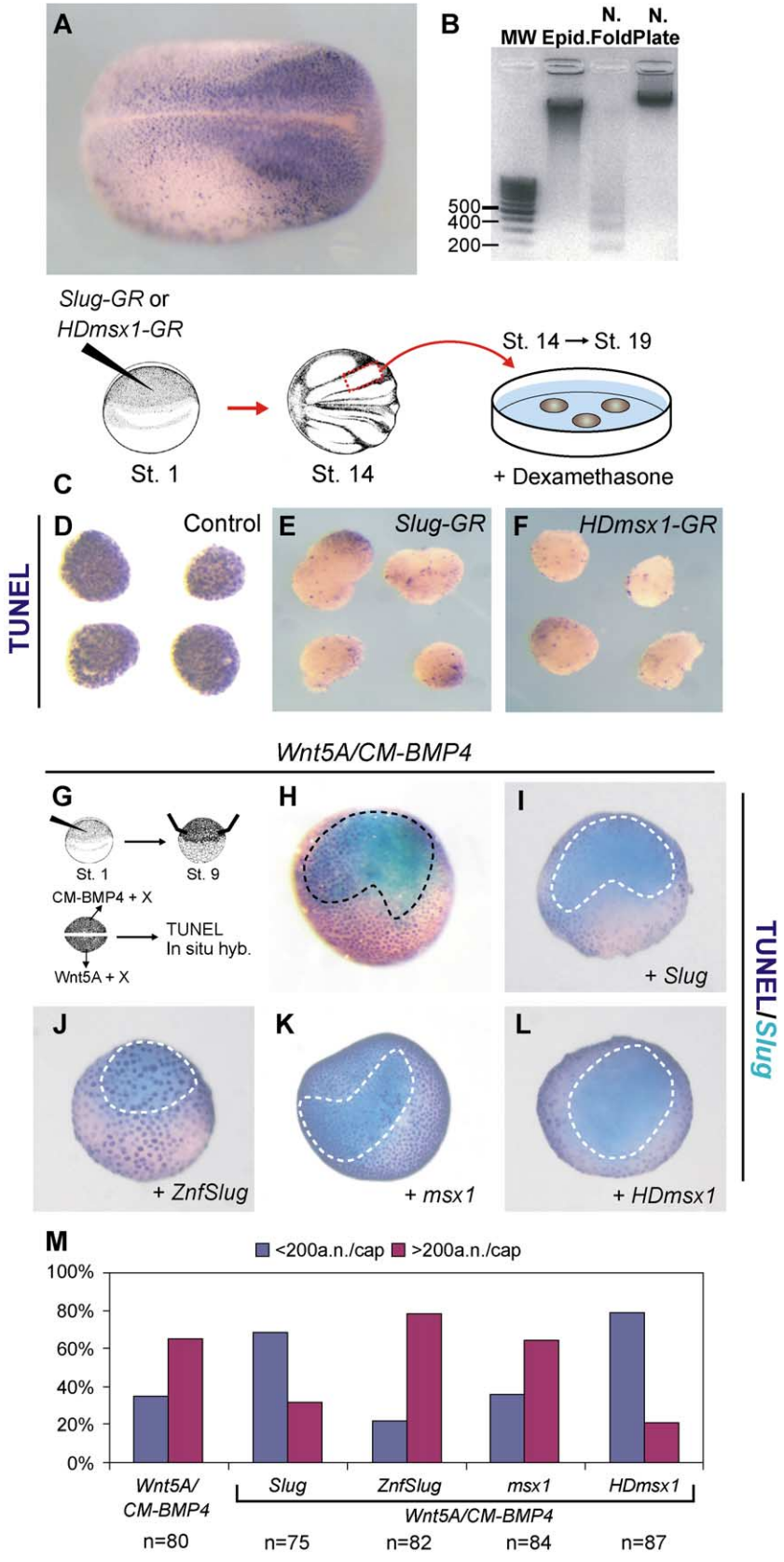


Fig. 1. Apoptosis in animal caps. TUNEL staining of animal caps isolated from blastula stage embryos and cultured to the equivalent of stage 19. (A) Control animal cap. (B) Animal cap treated with noggin-soaked bead. (C–F) Injected animal caps. Animal caps were dissected from embryos injected with 1 ng of (C) ΔBMPR mRNA, (D) CM-BMP4 mRNA, (E) *Slug* mRNA, (F) *msx1* dominant-negative mRNA. (G–H) Embryos were injected with 500 pg of *HDmsx1* and 500 pg of *msx1* (G) or 1 ng of *HDmsx1* and 500 pg of *msx1* (H). (I–J) Embryos were injected with 500 pg of *Slug* and 500 pg of *msx1* (I) or with 1 ng of *Slug* and 500 pg of *msx1* (I). (K–M) Summary of the analysis of apoptosis in the animal caps. a. n.: apoptotic nuclei. Blue bars: less than 200 stained nuclei per animal cap; red bars: more than 200 stained nuclei per animal cap. *n*: total number of animal caps analyzed.

neural fold (Fig. 2D). However, when the inducible *Slug* construct was expressed in these cells and activated at stage 14, a dramatic reduction of TUNEL staining was observed (Fig. 2E). A similar inhibition of apoptosis was observed with the *msx1* dominant negative when was expressed and activated at stage 14 (Fig. 2F). These results indicate that in the neural crest, *Slug* can work as an antiapoptotic factor, and that *msx1* is likely to work as a proapoptotic factor, as its dominant negative blocks apoptosis in the neural crest cells. A second experiment was performed to analyze the role of these factors on neural crest apoptosis. Many signals have been found to be able to induce neural crest cells in animal caps cultured in vitro. Thus, a combination of anti-BMPs and Wnts signals can induce neural crest in *Xenopus* animal cap (LaBonne and Bronner-Fraser, 1998; Tríbulo et al., 2003). We injected one-cell-stage embryos with 200 pg of *CM-BMP4* mRNA and several *Slug* or *msx1* constructs; at the blastula stage, the animal caps were dissected and conjugated with an animal cap taken from an embryo injected with 500 pg of *Wnt5A* mRNA. After culturing the conjugate in vitro until the equivalent of a stage 19 embryo, a double staining for TUNEL and *Slug* in situ hybridization was performed (Fig. 2G). We found that *Slug* was induced in most, not in all, animal caps; therefore, we proceeded to analyze TUNEL staining only on those animal caps that had a strong *Slug* induction. Animal caps induced as neural crest show high levels of TUNEL

staining but interestingly these levels are reduced in the region where neural crest marker is expressed (dotted area in Fig. 2H). As expected, when *Slug* is expressed in these animal caps, a drastic inhibition of apoptosis is observed (Figs. 2I,M). These levels of apoptosis are again increased when a dominant negative of *Slug* is expressed (*ZnfSlug*)



and in this case the apoptotic nuclei can be observed in the region of *Slug* expression (Figs. 2J,M). When *msx1* is expressed, high levels of apoptosis are observed in the cap but they are lower in the region of *Slug* expression (Figs. 2K,M); however, when a dominant negative of *msx1* is used, even lower levels of TUNEL staining are observed in the animal cap (Figs. 2L,M). Taken together, these results indicate that in the neural crest cultured in vitro, the *Slug* gene works as an antiapoptotic factor and the *msx1* promotes apoptosis.

Control of apoptosis by Slug and msx1 in neural crest cells of whole embryos

We analyzed the role that *Slug* and *msx1* play on neural crest apoptosis in the whole embryo. One blastomere of two-cell-stage embryos was injected with inducible forms of *Slug*, *msx1* or their dominant-negative constructs (Aybar et al., 2003; Tríbulo et al., 2003). To overcome the early effects of *Slug* and *msx1* in mesoderm and neural crest induction (Mayor et al., 1999; Yamamoto et al., 2000; Yamamoto et al., 2001), the inducible fusion constructs were not activated by dexamethasone treatment until stage 15, and, subsequently, cell death was analyzed by TUNEL. Due to the variation in TUNEL staining observed between different embryos, we always analyzed both the injected and uninjected side of the same embryo carefully. The normal patterning of apoptosis (Fig. 3A) was inhibited in the injected side by the expression of *Slug* (Fig. 3D), while the injection of the *Slug* dominant negative only produced a moderate increase in the proportion of apoptotic nuclei (not shown). Similarly, apoptosis was strongly inhibited on the injected side after expressing a dominant-negative construct of *msx1* (Fig. 3C). As *msx1* is a downstream target of BMP4, we also analyzed the effect of expressing a dominant-negative form of the BMP4 receptor and found that it strongly inhibited apoptosis on the injected side (Fig. 3B). As it has been described that inhibition of *msx1* can suppress *Slug* expression when it is activated at the late gastrula stage (Tríbulo et al., 2003), we analyzed if a similar relation was taking place between these two factors at the mid neurula stage. Embryos injected at the one-cell stage with the inducible constructs of the dominant negative of *msx1* or *Slug*, were induced at stage 15 and the expression of *Slug* or *msx1* was analyzed, respectively. No effect in the expression of *Slug* was observed when *msx1* dominant negative was expressed (Fig. 3E), and no effect on *msx1* was observed when *Slug* was expressed (Fig. 3F); therefore, when any of these factors are activated after neural crest specification (stage 12; Mancilla and Mayor, 1996), no mutual control of transcription take place. In conclusion, these results indicate that *Slug* can indeed act as an anti-apoptotic factor while *msx1* promotes apoptosis in the neural crest of *Xenopus* embryos.

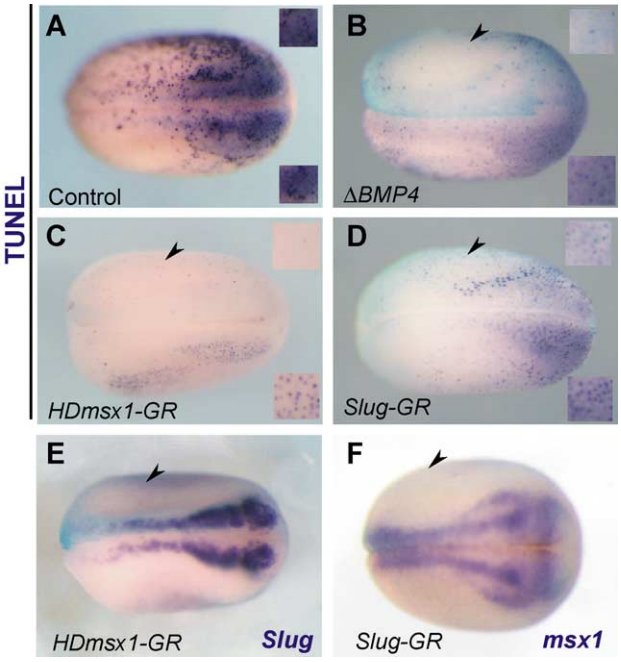


Fig. 3. *Slug* and *BMP4/msx1* control apoptosis in embryos. One blastomere of a two-cell stage embryo was injected with 700 pg of ΔBMPR mRNA (B), 700 pg of *HDmsx1-GR* mRNA (C, E), or 700 pg of *Slug-GR* mRNA (D, F). Expression was induced at stage 15. TUNEL (A–D) or in situ hybridization (E ,F) was performed at stage 18. Anterior is to the right. Inset corresponds to a higher magnification of the injected (upper inset) and uninjected (lower inset) neural folds. Arrowhead: injected side. (A) Control embryo showing the normal distribution of apoptotic nuclei. (B) ΔBMPR injected embryo, 78% of embryos exhibited less apoptosis on the injected side, $n = 57$. (C) *msx1* dominant-negative injected embryo, where apoptosis was inhibited in 68%, $n = 73$. (D) *Slug* injected embryos, where 68% apoptosis was inhibited, $n = 66$. (E) *msx1* dominant-negative injected embryo, showing the normal expression of *Slug* in the injected side. (F) *Slug-GR* injected embryo, with normal *msx1* expression in the injected side.

Fig. 2. Apoptosis in the neural crest is controlled by *Slug* and *msx1*. (A) Apoptosis was detected by TUNEL staining in neural fold stage whole-mount embryos. (B) Apoptosis was determined by analyzing DNA fragmentation in embryo explants. Genomic DNA was extracted from epidermal (Epid.), neural fold (N. Fold) and neural plate (N. Plate) tissue dissected from stage 15 embryos, and it was separated by electrophoresis in 1.5% agarose gel. (C) Embryos were injected at the one-cell stage with 700 pg of *Slug-GR* or 700 pg of *HDmsx1-GR* mRNA, at the mid neurula stage (14) the neural fold were dissected an cultures in vitro in the presence of dexamethasone, until the equivalent of stage 19, when apoptosis was analyzed by TUNEL. (D) TUNEL staining of control neural crest. (E) TUNEL staining of neural crest expressing *Slug-GR*. (F) TUNEL staining of neural crest expressing *HDmsx1-GR*. (G) Embryos were injected at the one-cell stage with 200 pg of CM-BMP4 mRNA together with several *Slug* or *msx1* constructs (X), or with 500 pg of Wnt5A mRNA, at the blastula stage conjugates between animal caps expressing CM-BMP4 and Wnts5A were performed, cultured until the equivalent of stage 19, when the expression of *Slug* and TUNEL was analyzed. Dotted line: *Slug* expression. (H) Control conjugate injected with CM-BMP4 and Wnt5A. Note the induction of the neural crest marker *Slug*. (I) Conjugate expressing *Slug*. (J) Conjugate expressing the *Slug* dominant negative, *ZnfSlug*. (K) Conjugate expressing *msx1*. (L) Conjugate with overexpression of *msx1* dominant negative, *HDmsx1*. (M) Summary of the analysis of apoptosis in the animal caps. a. n.: apoptotic nuclei. Blue bars: less than 200 stained nuclei per animal cap; red bars: more than 200 stained nuclei per animal cap. *n*: total number of animal caps analyzed.

An interaction of Slug and msx1 with the Bcl2/Bax proteins is required to control apoptosis

Bcl2 and *Bax* proteins form part of the core apoptotic machinery, which is conserved across species as diverse as *C. elegans* and mammals. At the functional level, *Bcl2* inhibits apoptosis while *Bax* promotes it, although the final decision of a cell to execute the program of cell death depends on the balance between all the proteins of the apoptotic machinery. Because *Slug* and *msx1* are involved in controlling apoptosis, we decided to analyze the interaction between all these factors in isolated animal caps and in whole embryos. We injected mRNA encoding *Bax* at the one-cell stage, animal caps were dissected, cultured in vitro, and TUNEL staining was analyzed. No significant difference in the number of apoptotic cells was observed between the control animal caps (Fig. 4A) and the animal caps injected with *Bax* mRNA (Figs. 4B,G). However, apoptosis was dramatically inhibited in animal caps by the expression of the *Xenopus* homologue of *Bcl2*, *XR11* (Cruz-Reyes and Tata, 1995; Figs. 4C,G). The inhibition of apoptosis produced by expressing *Slug* (Figs. 1E and 4G) was reversed by coinjection of *Bax* (Fig. 4D; *Slug* + *Bax* in Fig. 4G), suggesting that the *Bax* protein lies downstream of *Slug* in the apoptotic cascade. Similarly, the inhibition of apoptosis by the dominant-negative *msx1* construct (Figs. 1F and 4G), was also reversed by coexpressing the *Bax* protein, indicating that *Bax* activity is also downstream of the apoptotic cascade activated by *msx1* (Fig. 4E; *HDmsx1* + *Bax* in Fig. 4G). Finally, when *msx1* was co-expressed with *XR11*, less apoptosis was detected in the animal cap, suggesting that *XR11* is downstream of *msx1* in the apoptotic cascade (Fig. 4F; *msx1* + *XR11* in Fig. 4G).

To confirm these results in whole embryos, similar injections of mRNA were performed in one blastomere of a two-cell-stage embryo, and TUNEL staining was analyzed at neurula stages. Although similar results were obtained in whole embryos and animal caps, it should be noted here that the high levels of apoptosis observed in normal embryos made it more difficult to detect an increase in apoptosis promoted by proapoptotic factors. When mRNA encoding for *Bax* was injected into one side of an embryo, the normal pattern of apoptosis was only moderately affected by the expression of *Bax* (compare Fig. 4H with 4I). In contrast, injection of the *Xenopus* homologue of *Bcl2*, *XR11* (Cruz-Reyes and Tata, 1995), strongly inhibited apoptosis (Fig. 4J). We then performed a series of rescue experiments. Coinjection of *Bax* mRNA with that of *Slug* reversed the inhibition of apoptosis produced by injecting *Slug* mRNA alone (compare Fig. 4K with 4N). Similarly, the inhibition of cell death provoked by expressing the *msx1* dominant-negative construct (Fig. 4L) was also reversed by coinjecting *Bax* mRNA (Fig. 4O). On the other hand, coinjection of *msx1* and *XR11* reversed the inhibitory effect on apoptosis produced by expressing *XR11* alone (compare Fig. 4P with 4J and 4M). Taken together, our results show that the transcription factors *Slug* and *msx1* activate the Bcl2/Bax proteins to control apoptosis.

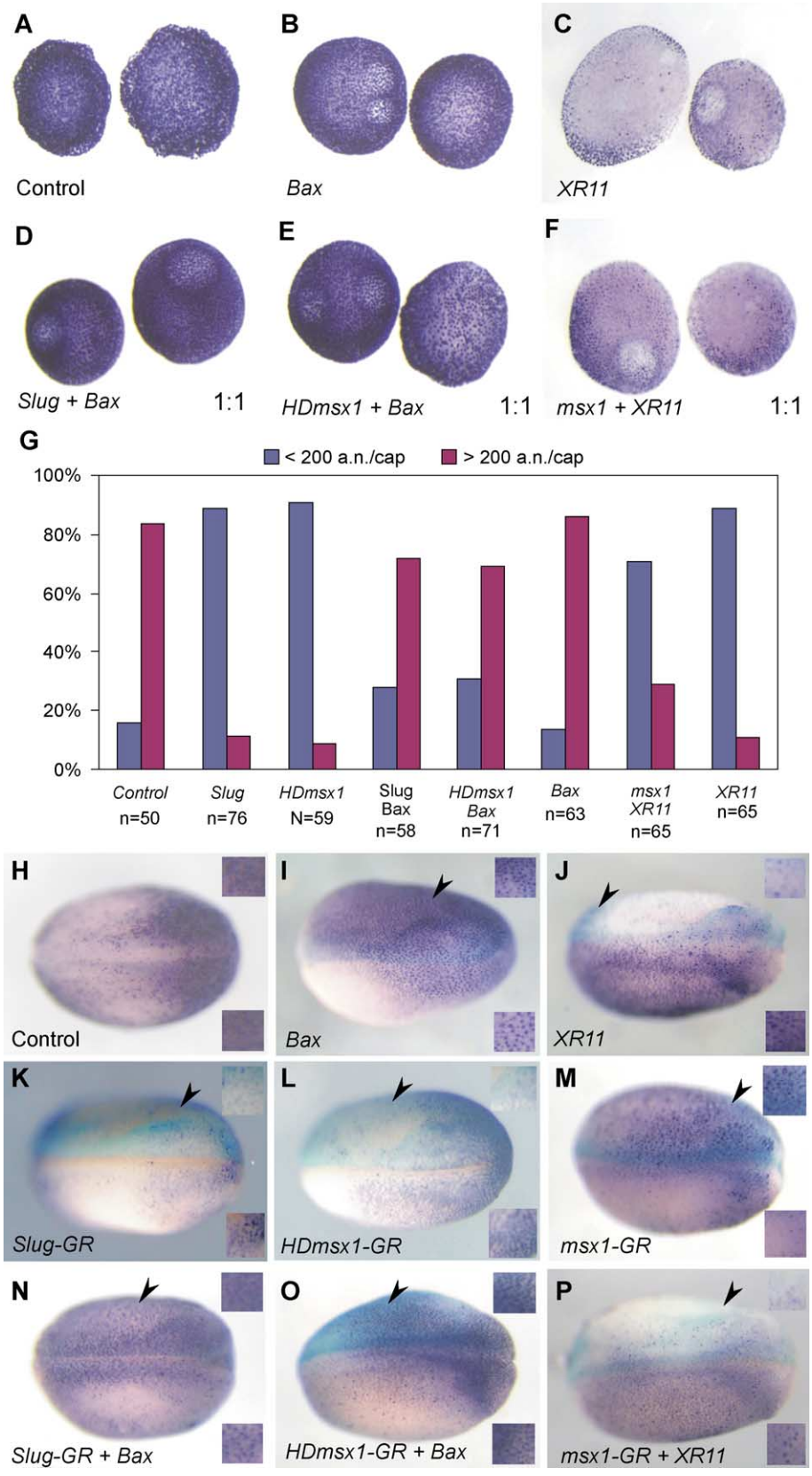
Slug and msx1 control programmed cell death at the transcriptional level in Xenopus embryos

A group of cysteine proteases, now called caspases, have been recognized as the proteins principally responsible for executing programmed cell death. It is now accepted that apoptosis is mediated by the sequential and coordinated activation of two different groups of cellular caspases. The first group, called the 'initiator caspases', is comprised of caspases 2, 8, 9, and 10, which are able to activate caspases 3, 6, 7, termed the 'effector group'. Although the mechanisms that underlie the initiation of apoptosis have been well established in the recent years, there is little evidence regarding the transcriptional control of caspases in different cellular processes.

We have shown that *Slug* and *msx1* can regulate apoptosis in the neural crest and that this control involves the participation of Bcl2/Bax family members. Thus, we investigated whether *Slug* and *msx1* might regulate the transcription of the different members of the caspase family and the *XR11* gene. The *msx1* dominant-negative or *Slug* mRNAs were expressed in animal caps, and after culturing until the equivalent of stage 17, the expression of two initiator *caspases 2* and *9*, of the effector *caspases 3*, *6*, and *7*, and of an anti-apoptotic *Bcl2* family member, *XR11*, was analyzed by RT-PCR. The expression of *Slug* reduced the expression of all the caspases analyzed while the injection of the dominant-negative *msx1* mRNA only decreased the expression of *caspases 2*, *3*, *7* and *9*, but not caspase *6* (Figs. 5A,B). In contrast, *XR11* expression could only be increased by injecting *Slug* mRNA (Figs. 5A,B). Our results

Fig. 4. *XR11* and *Bax* rescue animal caps and embryos from the effects of *Slug* and *msx1*. (A–G) Analysis of TUNEL staining in animal caps. (A) Control animal cap. (B–F) One-cell stage embryos were injected with 1 ng of *Bax* mRNA (B), 1 ng of *XR11* mRNA (C), 1 ng of *Slug* and 1 ng of *Bax* mRNA (D), 1 ng of *HDmsx1* and 1 ng of *Bax* mRNA (E) or 1 ng of *msx1* and 1 ng of *XR11* mRNA (F). Animal caps were dissected at stage 9, and apoptosis was analyzed when the caps reached stage 19. (G) Summary of animal cap assays. a. n.: apoptotic nuclei. Blue bars: less than 200 stained nuclei per animal cap; red bars: more than 200 stained nuclei per animal cap. *n*: total number of animal caps analyzed. (H) TUNEL staining in control embryos at stage 18. (I–P) Embryos were injected in one blastomere at the two-cell stage with 1 ng of Bax (I), XR11 (J), *Slug-GR* (K), *HDmsx1-GR* (L), *msx1-GR* (M), or with a combination of 700 pg of *Slug-GR* and 1 ng of *Bax* (N), 700 pg of *HDmsx1-GR* and 1 ng of *Bax* (O), 700 pg of *msx1-GR* and 1 ng of *XR11* (P). Expression was induced at stage 15 and apoptosis was analyzed in the embryos at stage 18. Anterior is to the right. The injected side is labeled with fluorescein and is indicated by an arrowhead. *Bax* was able to overcome the antiapoptotic effect of *Slug* (N; *n* = 67), in 59% of embryos apoptosis was seen in the injected side, and of *HDmsx1* where apoptosis occurred in 54% of embryos (O; *n* = 55). In contrast, *XR11* was able to reduce the apoptotic effect of *msx1* (P; *n* = 70, 57%). Note the increase in apoptosis in the embryo injected with *Bax* (I) and the reduction in apoptosis in the embryo injected with *XR11* (J).

support the idea that *Slug* and *msx1* control programmed cell death by the transcriptional regulation of some components of the apoptotic pathway. These results also indicate that *Slug* and *msx1* differentially control the transcription of the members of apoptosis pathway or its effectors.



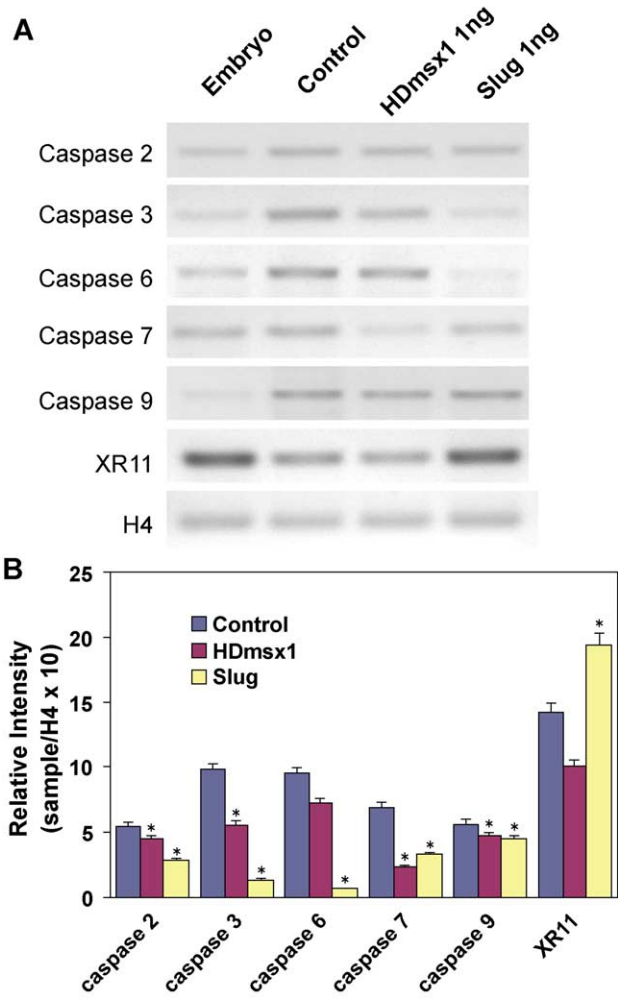


Fig. 5. *Slug* and *msx1* control the transcription of caspases and *XR11*. One-cell stage embryos were injected with 1 ng of *Slug* mRNA or 1 ng of *HDmsx1* mRNA, whereas control embryos were not injected. Animal caps were dissected at stage 9 and the expression of the caspases 2, 3, 6, 7 and 9 of XR11 and of histone H4 was analyzed by RT-PCR. (A) Embryo and animal cap samples are shown. (B) Quantification of the gel is shown in B where the results are expressed as Relative Intensity (Sample/H4 × 10). Note that when *Slug* was injected, the expression of all the caspases analyzed decreased and the expression of XR11 increased. *HDmsx1* injection produced a decrease in the expression of the caspases 2, 3, 7 and 9. Student's *t* test was used to analyze the differences between each of the groups with respect to the corresponding control group. Differences were considered statistically significant at $P < 0.001$ (*).

Apoptosis in the neural crest is controlled in a cell autonomous manner

To analyze whether extracellular signals influenced apoptosis in the neural crest, or rather that it was activated by a cell autonomous program, cephalic neural crest was dissected from a stage 14 neurula embryo and grafted into the epidermal region of another embryo (Fig. 6A). The donor neurula had initially been injected at the one-cell stage with fluorescein as a lineage marker. After receiving the graft, the host embryo was cultured until stage 18 when TUNEL and in situ hybridization for *Slug* and *msx1* was combined with the visualization of the fluorescein. High levels of apoptosis were observed in fluorescein-labeled tissue along with *Slug* and *msx1* expression (Figs. 6B–D). As control, we grafted a piece of epidermis dissected from a stage 14 embryo into the epidermal region of another embryo (Fig. 6E). No apoptosis, *Slug* or *msx1* expression was observed in the graft (Figs. 6F–H). These results suggest that the signals present in the cephalic neural crest territory are sufficient to maintain a high level of apoptosis, and that the apoptosis in the neural crest is apparently not influenced by external signals. However, we cannot rule out the possibility that other signals are present in the graft site.

The influence of apoptosis on neural crest development

We have shown here that *Slug* acts as an anti-apoptotic factor in the neural crest whereas *msx1* promotes apoptosis. However, it is not clear what is the biological function that underlies this pattern of apoptosis. To identify a biological role for this program of cell death, we analyzed the effect of directly expressing proteins involved in the apoptotic machinery, such as *Bax* and *XR11* (*Bcl2*), and comparing its effect with the expression of *Slug* and *msx1*. Injecting *XR11* mRNA in one half of the embryo provoked an expansion of the territory in which the early neural crest marker *FoxD3* was expressed (Fig. 7A). More interestingly, the sharp boundary of *FoxD3* expression found on the control side was lost, and on the injected side the cells expressing this marker were somewhat dispersed (Fig. 7B). When the pro-apoptotic factor *Bax* was expressed, a dramatic reduction in expression of the neural crest marker *FoxD3* was observed on the injected side of the embryo (Figs. 7C,D). Very similar results are obtained when *Slug* or its dominant negative are expressed in neural crest cells (Aybar et al., 2003). Then, we analyzed what happened with some of the neural crest derivatives after these treatments. Accordingly, in the half of the embryo where the anti-apoptotic factors *Slug* or *XR11* were expressed, an expansion of the cephalic cartilages, such as the Meckel, ceratohyal and ceratobranchial cartilages was subsequently observed (Figs. 7E–K). In contrast, a dramatic reduction in the same cartilages, or even a complete loss in the case of the ceratobranchial cartilage, was found after expressing the pro apoptotic factors *msx1* or *Bax* (Figs. 7L–R). No effect on melanocyte development was observed under any of these treatments. In conclusion, inhibiting apoptosis in the neural fold region leads to both an enlargement of the territory in which early neural crest markers are expressed and to an enlargement in the size of neural crest derivatives. In contrast, inducing apoptosis produces a reduction in the quantity of neural crest cells and derivatives.

Analysis of apoptosis in the neural folds

We have shown in this work that *Slug* and *msx1* have opposite effect on controlling apoptosis in ectodermal and

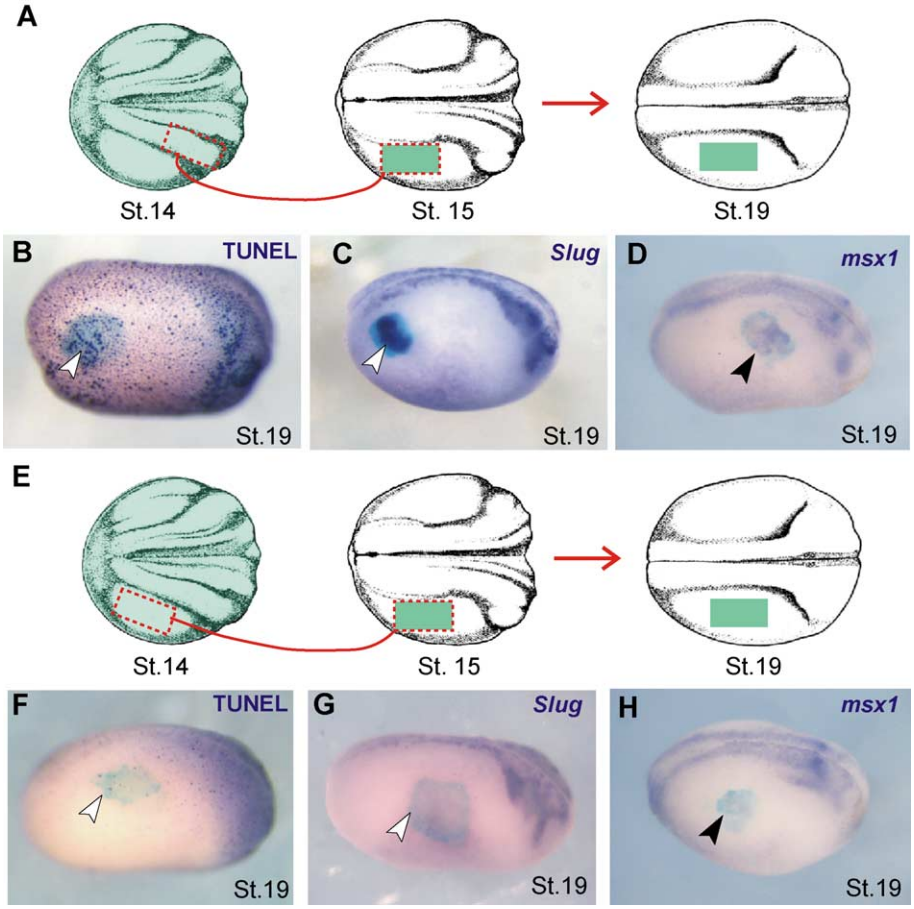


Fig. 6. Cell autonomous control of apoptosis. A piece of anterior neural crest taken from a stage 14 embryo previously injected with FLDx was transplanted into the ventral region of a normal stage 15 embryo (A). The host embryo was cultured to stage 19 at which point apoptosis was assessed by TUNEL (B) and *Slug* (C) or *msx1* (D) expression by in situ hybridization. In the host embryos and the grafted neural crest tissue, the normal pattern of apoptosis and *Slug* and *msx1* expression was observed (arrowheads). (E) Control experiment where a piece of epidermis was transplanted into the ventral region of a normal stage 15 embryo. (F) TUNEL staining, no label is found in the graft. (G) *Slug* and (H) *msx1* staining, no expression is found in the graft.

neural crest cells. However, it has been reported that both genes are expressed in the neural folds. To understand how apoptosis was controlled in the neural fold/crest region by these factors, we performed a careful analysis of the expression of these two genes at the mid neurula stage (stage 14–15), and compared it with the pattern of apoptosis in the neural folds. To confirm that the neural folds was a region of more intense apoptosis, we compared the distribution of TUNEL staining with that of the neural crest marker *Slug* in the same embryo. Although the *Slug* and TUNEL staining did not coincide exactly, it was clear that the increase density of apoptotic nuclei occurred in the neural folds (Figs. 8A,B). Then, we compared the staining for apoptotic nuclei with that *msx1* and *Slug* in situ hybridization in sections of embryos. In a section of the cephalic neural crest region (Fig. 8C), it is clear that apoptotic nuclei can be found not only in the superficial tissues but also in the deep layer of the ectoderm. We noticed that most of the apoptotic nuclei did not exactly coincide with the neural crest territory but rather were in the adjacent tissue (i, e in Fig. 8C). Indeed, the regions with the highest levels of apoptosis corresponded to those at the border of the neural crest territory in which the cells expressed *msx1* (i, e in Figs. 8F,H), surrounding the *Slug* expressing cells (nc in Fig. 8H). Thus, the expression of *msx1* and *Slug* is not found in the same region of the neural fold in a mid neurula stage embryo; instead, *Slug* is expressed in the center of the fold while *msx1* is expressed at the border, as it can be seen in a double in situ hybridization analyzed in whole mount and in sections (Figs. 8G,H). A clear correlation between the expression of these genes and the apoptotic pattern can be seen by analyzing TUNEL-stained whole-mount embryos at higher magnifications (Fig. 8A), where less staining could be observed in the epidermal or neural side of the neural fold, when compared to the center of the neural fold where the prospective neural crest cells lie (Fig. 8D).

To quantify this phenomenon, we counted the number of positive TUNEL nuclei in each territory, dividing the neural fold into three equal regions (Fig. 8D): the external neural fold (epidermal side of the fold, “e”); the central neural fold (prospective neural crest cells, “c”); and the

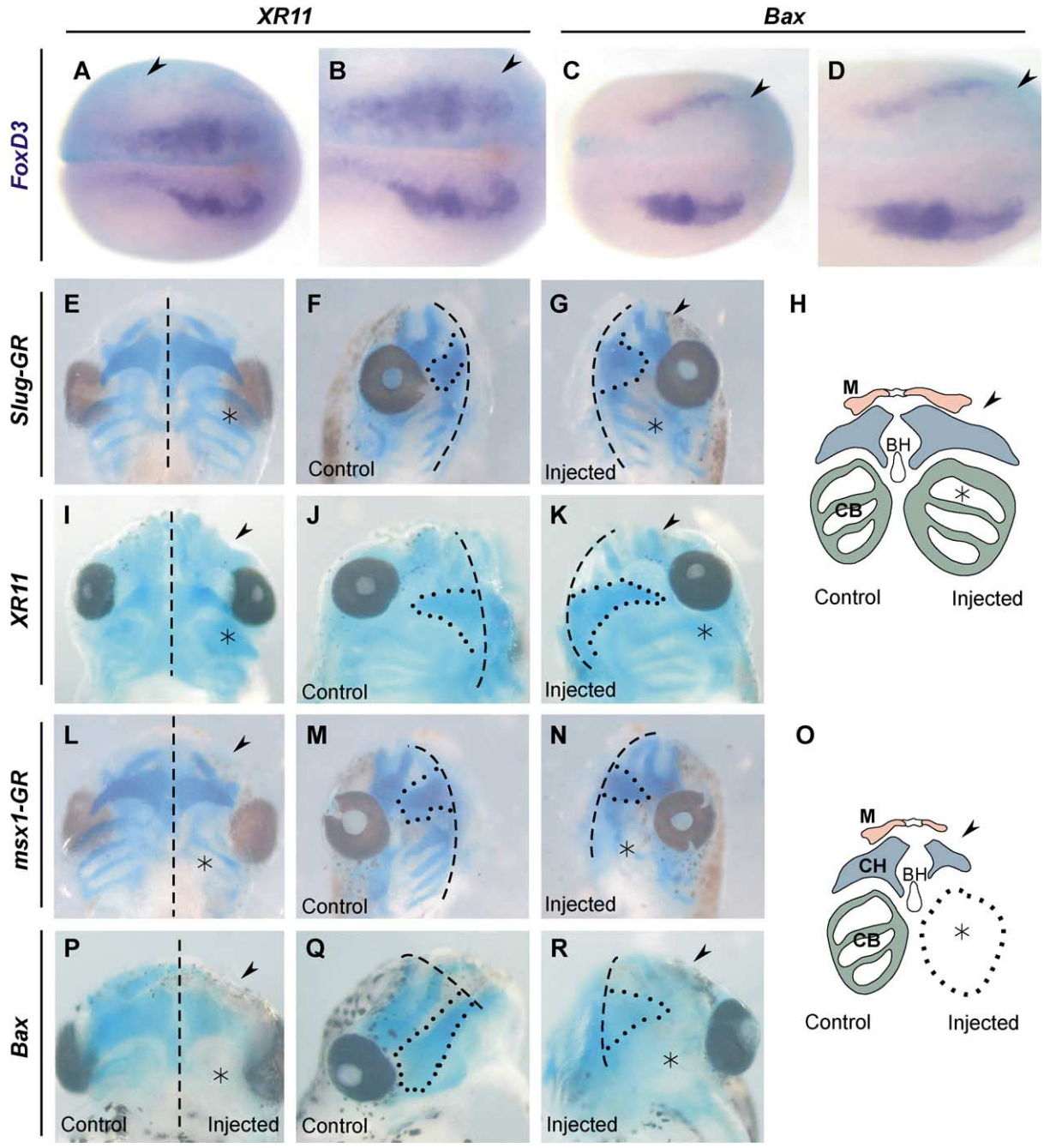


Fig. 7. Apoptosis regulate cell number in the neural crest. Embryos were injected in one blastomere at the two-cell stage with *XR11* mRNA (A, B, I–K), *Bax* mRNA (C, D, P–R), *Slug-GR* mRNA (E–G), *msx1-GR* mRNA (L–N). Expression was induced at stage 15. The expression of *FoxD3* was analyzed at stage 17 (A–D). Anterior is to the right. Cartilage was visualized after alcyan-blue staining and was analyzed at stage 45 (E–O). E, I, L, P: Ventral view. F, J, M, Q: View of the control side of tadpoles. G, K, N, R: View of the injected side of the tadpoles, the arrowheads indicate the injected side. Note the expansion of the *FoxD3* expression domain in the side injected with *XR11* (A, B) and the reduction of *FoxD3* expression in the embryos injected with *Bax* (C, D). At stage 45, embryos injected with *Slug* or *XR11* show an expansion of the cranial cartilage, while the embryos injected with *msx1* or *Bax* show a dramatic reduction of the same cartilage. The dotted line delineates the shape of ceratohyal cartilage and the asterisk indicates the ceratobranchial cartilage. (H, K) Schematic representation of the effects of *Slug*, *XR11*, *msx1* and *Bax* overexpression on head cartilage of a *Xenopus* tadpole. CH, ceratohyal cartilage; CB, ceratobranchial cartilage; BH, basihyal cartilage; M, Meckel cartilage.

internal neural fold (neural plate side of the fold, "n"). The nuclei stained by TUNEL were counted in whole mount embryos as well in sections, (see Materials and methods) and the neural crest population contained the lowest number of apoptotic nuclei (Fig. 8E). In summary (Fig. 8I), we found high levels of apoptosis in the neural fold region where the dying cells were concentrated among *msx1*-expressing cells that surround the neural crest. Apoptosis is much less frequent among the neural crest cells that express *Slug*.

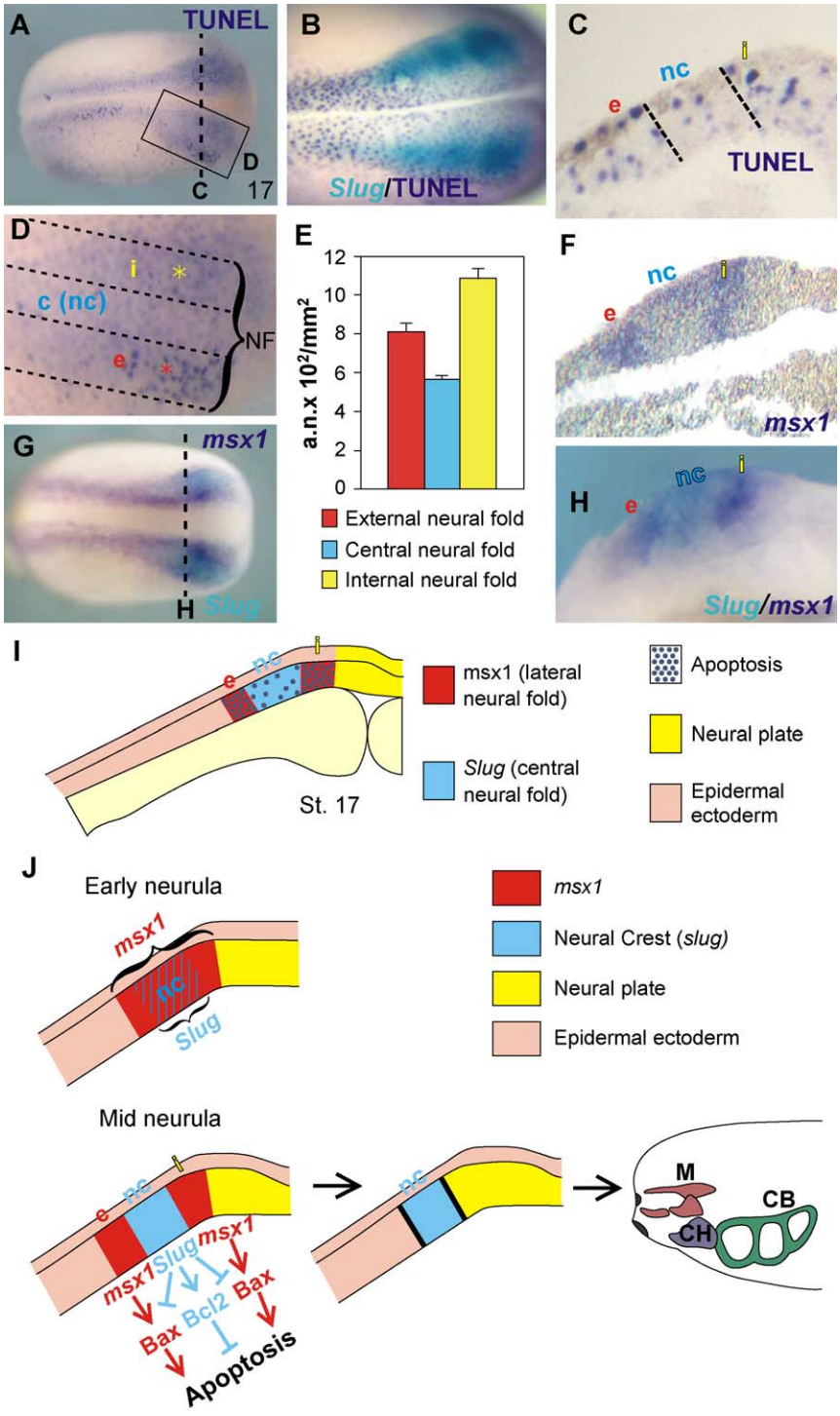


Fig. 8. Pattern of apoptosis in the neural fold. (A) Dorsal view of the pattern of apoptosis in a stage 17 embryo stained by TUNEL. (B) Double staining of apoptosis visualized by TUNEL and expression of the *Slug* gene visualized by in situ hybridization. (C) Transverse histological section of a TUNEL stained stage 17 embryo taken from the position indicated in A. (D) Higher magnification of the whole-mount TUNEL staining shown in A. Asterisks indicate regions with a high number of apoptotic nuclei. (E) TUNEL-stained nuclei were counted in different regions of the neural fold and the results presented graphically as apoptotic nuclei per square micrometer. (F) Expression of *msx1* in a transverse section of a stage 17 embryo. (G) Double in situ hybridization of a 17 stage embryo stained for *msx1* (purple) and *Slug* (green). (H) Transverse section of an embryo double stained *msx1* and *Slug*, as indicated in G. (I) Diagram of a transverse section summarizing the regions where apoptosis occurs and the expression patterns of *Slug* and *msx1*. (J) Model of control of apoptosis in the neural folds. Early neurula: expression of *msx1* and *Slug* in the neural fold region of an early neurula embryo. Mid neurula: Apoptosis in the neural folds is controlled by *msx1* and *Slug*. Left column: gene expression in the neural folds; central column: neural crest territory; right column: neural crest derivatives. See text for more details. e, external neural fold; nc, neural crest; i, internal neural fold. CH, ceratohyal cartilage; CB, ceratobranchial cartilage; M, Meckel cartilage.

Discussion

In *C. elegans* and *Drosophila*, apoptosis is largely restricted to early life and ends at birth or metamorphosis. In contrast, apoptosis in vertebrates is sustained at a high level in many tissues. Nevertheless, the apoptotic machinery in vertebrates is substantially homologous to that of invertebrates, although it is more elaborated and has incorporated a greater degree of redundancy. Indeed, in vertebrates, a bewildering array of proteins exist: caspases, Bcl2 and IAP family proteins, and survival signaling pathways multiplicity, consistent with the more sophisticated levels of control of apoptosis needed in vertebrate tissues (Meier et al., 2000; Strasser et al., 2000; Walter et al., 2003). Cell death is fundamental in some developmental processes and serves many functions, such as to sculpt or remove structures, control cell number, or to eliminate abnormal, misplaced or nonfunctional cells (Ellis et al., 1991; Sanders and Wride, 1995). Cell death has been reported as early as the blastocyst stage where it is believed to be important for cavitation (Coucouvanis and Martin, 1995) and it has also been detected in the gastrulating chick and mouse embryo (Sanders et al., 1997), as well as in urodele amphibians (Imoh, 1986). Two of the classical areas where cell death has been studied are the developing limb bud and the nervous system (Burek and Oppenheim, 1996; Hinchliffe, 1981). The role of programmed cell death in the formation of the digits in the vertebrate limb and in the morphogenetic reorganization of organs such as heart and kidney has been clearly demonstrated (Coles et al., 1993; Pexieder, 1975).

Neuronal death plays a major part in patterning the developing nervous system and it is thought to be regulated by many different mechanisms (Burek and Oppenheim, 1996; Oppenheim, 1991). In the chick, cell death is detected during the folding and closing of the neural tube, and later in development it has also been shown to have a clear role in sculpting the cephalic neural crest (Graham et al., 1993; Jeffs et al., 1992). An apoptotic program has also been found in *Xenopus* and this program of cell death is activated at the onset of gastrulation and remains active in many tissues, for example, in the Rohon–Beard neurons and during metamorphosis (Anderson et al., 1997; Coen et al., 2001; Hensey and Gautier, 1997, 1998; Sible et al., 1997; Stack and Newport, 1997).

In this study, through TUNEL staining and by analyzing DNA fragmentation, we show that a large number of apoptotic cells accumulate in the neural fold as opposed to other regions of the ectoderm.

The *ces1* gene, a transcription factor belonging to the *Snail* family of zinc finger proteins, has been shown to have anti-apoptotic activity (Inukai et al., 1999; Metzstein and Horvitz, 1999). Here we show that *Slug*, another member of this family, also acts as an anti-apototic factor both in whole embryos as well in isolated neural crest and animal caps, as might be predicted from other animal models. We should mention than the *ces1* gene from *C. elegans* is probably not the true homologue of *Xenopus Slug*, as it is more likely that it corresponds to the *scratch* gene, another member of the *Snail* family (Manzanares et al., 2001). However, as the function of some members of the *Snail* genes have been exchanged during evolution (Nieto, 2002; Sefton et al., 1998), it does not seem unlikely that *Slug* might act as the functional equivalent of *ces1* in amphibian embryos. Our animal cap assay shows that *Slug* has anti-apoptotic activity in the ectoderm that will probably differentiate into epidermis. Furthermore, based on the normal expression pattern of *Slug* in the neural crest and in our experiments using isolated or induced neural crest, we propose that one of the normal activities of *Slug* in these cephalic crest cells is to inhibit apoptosis.

Msx genes have been implicated in promoting apoptosis in tissues such as the developing limb and the cephalic neural crest (Davidson, 1995; Graham et al., 1993; 1994; Gomes and Kessler, 2001; Krabbenhoft and Fallon, 1992; Marazzi et al., 1997; Song et al., 1992; Woloshin et al., 1995). We show here that *msx1* promotes apoptosis in whole embryos as well as in isolated neural crest and animal caps. Thus, inhibiting *msx1* activity by expressing its dominant-negative form reduced the amount of apoptosis in the embryo, in neural crest and in animal caps. In general, it was easier for us to detect a reduction in the amount of apoptosis than an increase. Thus, although our results suggest that expressing *msx1* promote apoptosis, our conclusions are reinforced by the inhibitory effect on cell death of the *msx1* dominant negative.

The pattern of *msx1* expression is complex during the development of *Xenopus* embryos and it seems to play multiple roles. Thus, at the blastula stage, it has been implicated in the ventralization of the mesoderm (Maeda et al., 1997), while its expression in the ventral ectoderm has been related to epidermal differentiation (Suzuki et al., 1997). It has also recently been shown that *msx1* is expressed in the neural folds, including the neural crest territory at the early neural stage, where it plays an important role in specifying the neural crest (Tríbulo et al., 2003). Here, we show that at the mid neurula stage the dynamic expression of *msx1* is excluded from the neural crest cells, it remains only at the border of the neural folds. A similar change in expression has been described for the Notch ligands *Delta1* and *Serrate*, where an initial but transient expression of these genes in the neural crest region is followed by an up-regulation of their expression in the cells that surround the neural crest (Glavic et al., 2004). Interestingly, by using inducible activators and inhibitors of Notch signaling, it has been shown that effect of Notch signaling on neural crest development depends on the time at which the signal is received (Coffman et al., 1993; Glavic et al., 2004). Similarly, it appears that *msx1* promotes neural crest development at the early neurula stage (Tríbulo et al., 2003), but provokes apoptosis in crest cells at the mid neurula stage. Other transcription factors that have different

effects on neural crest development depending on the time of their activation have also been described, such as the *Dlx* (Woda et al., 2003), *Snail* and *Slug* genes (Aybar et al., 2003; LaBonne and Bronner-Fraser, 2000).

We found that *msx1* is expressed in the cells that undergo programmed cell death and that surround the *Slug*-expressing cells within the neural crest territory. The expression of these genes is interesting as it correlates with the apoptotic activity of these factors. However, many other genes are also known to be expressed in these territories. While a recent report has shown that the *Notch* ligands *Delta1* and *Serrate* are expressed in the cells surrounding the neural crest (Glavic et al., 2004), the neural crest population in *Xenopus* expresses: *Snail* (Aybar et al., 2003; Essex et al., 1993; Linker et al., 2000; Mayor et al., 1993), *Slug* (Mayor et al., 1995), *zic5* (Nakata et al., 2000), *FoxD3* (Dirksen and Jamrich, 1995; Sasai et al., 2001), *twist* (Hopwood et al., 1989), *Sox9* (Spokony et al., 2002) and *Sox10* (Aoki et al., 2003; Honoré et al., 2003).

The specific influence of *Bcl2* or *Bax* on apoptosis when expressed in *Xenopus* embryos is similar to the effects of expressing the *Slug* or *msx1* genes at the mid neurula stage. However, in both cases, the expression of these apoptotic factors was not restricted to the neural folds but they were expressed in half of the embryo. As a consequence, some of the effects observed could be due to effects on tissues other than the neural crest. Although we cannot rule out this alternative, the fact that at the border of the neural crest territory a higher number of apoptotic cells were detected suggests that this is the tissue that is most sensitive to the inhibition of apoptosis. In addition, by looking at the general morphology of the embryos, as well as specific molecular markers, our manipulations appear to specifically affect neural crest development.

Considering our results together, we propose a model for the regulation of apoptosis during neural crest development (Fig. 8J). By the early neurula stages, the neural crest has already been specified (Aybar and Mayor, 2002; Mancilla and Mayor, 1996), and the crest cells already express the *Slug* gene, while *msx1* is expressed in a wider domain that includes the neural crest territory (Fig. 8J; Tríbulo et al., 2003). At the mid neurula stage, the expression of *msx1* is down-regulated in the neural crest cells (Fig. 8J). At this stage, *Slug* exerts an anti-apoptotic influence on the crest region and *msx1* promotes cell death in the adjacent cells, both *Slug* and *msx1* lying upstream of Bcl2 and Bax (Fig. 8J). We speculate that the balance between these antagonistic activities is required to generate sharp boundaries in the neural crest region, to control the precise number of crest cells, or to avoid any ectopic development of the prospective crest cells. As a result of this control, a sharp limit for the neural crest territory is generated, which, in turn, allows the neural crest derivatives to develop properly (Fig. 8J). Indeed, when *Slug* is overexpressed or apoptosis is reversed by expressing *Bcl2* (*XR11*), the sharp borders of the prospective neural crest are lost and the neural crest domain is enlarged, as is that of its derivatives. Finally, if *msx1* activity is augmented, or its apoptotic activity is mimicked by expressing *Bax*, a decrease in the size of the neural crest territory and its derivatives is observed.

Our model explains how the medio-lateral patterning of the neural crest territory is controlled by apoptosis, but it does not specify any influence in its anterior–posterior patterning. In the chick, it has been clearly shown that a specific anterior–posterior pattern of apoptosis exists in the hindbrain region. Apoptosis in chick premigratory neural crest is observed specifically in rhombomeres 3 and 5 (Ellies et al., 2000, 2002; Farlie et al., 1999; Graham et al., 1993, 1996; Golding et al., 2002; Kulesa and Fraser, 2000; Trainor et al., 2002). While we were unable to detect a similar pattern of apoptosis in the *Xenopus* hindbrain, we did observe a pattern of *Slug* and *msx1* expression that may reflect such a phenomenon. We detected expression of the proapoptotic gene *msx1* in rhombomere 3, a region from which the anti-apototic factor *Slug* was excluded (not shown). Based on the expression of these genes, we might predict that more cell death should occur in rhombomere 3 of *Xenopus* embryos, in a similar manner to that described for chick hindbrain. It is possible that our inability to detect such a pattern of apoptosis in the *Xenopus* hindbrain could simply be because this pattern does not exist, as has been previously proposed for amphibian and fish embryos (Ellies et al., 1997; 2002; Del Pino and Medina, 1998; Hensey and Gautier, 1998). Alternatively, the shorter hindbrain in *Xenopus*, much shorter than the chick hindbrain, may make it difficult to detect this apoptosis given the resolution of the techniques used, below that required to find such a pattern in a small territory. In fact, the rhombomeres in *Xenopus* are only 2 or 3 cell diameters wide, and since apoptosis never occurs in all the nuclei within a territory at the same time, it would be practically impossible to detect a pattern in such a small field. Based on the expression pattern of *Slug* and *msx1* that we describe here, and given that *Slug* expression in chick is absent from the rhombomeres in which more prominent apoptosis occurs (Del Barrio and Nieto, 2002), we favor this latter explanation.

In this report, we also provide evidence regarding the molecular mechanisms through which *Slug* and *msx1* might influence apoptosis. By carrying out rescue experiments, we showed that *Slug* and *msx* lie upstream of the apoptotic factors *Bax* and *Bcl2* (*XR11*). Coinjecting *Bax* reversed the effects of *Slug* on apoptosis, indicating that *Slug* is upstream of *Bax* in the apoptotic cascade. The expression of *msx1* did not provoke apoptosis when co-expressed with *XR11*, indicating that *msx1* is upstream of *XR11* in controlling apoptosis. Moreover, we showed that *Slug* controls the transcription of *XR11*, being a positive regulator of this anti-apoptotic factor. In addition, *Slug* and *msx1* control the levels of transcription of several caspases

directly involved in the apoptotic machinery. *Slug* represses the transcription of *caspases 2*, *3*, *6*, *7* and *9*, which are required to trigger cell death and also is able to increase the expression of *XR11*, while the expression of dominant negative of *msx1* promotes the expression of *caspases 2*, *3*, *7* and *9*.These results indicate that *Slug* and *msx1* differentially control the transcription of components of the apoptosis pathway.

It is possible that *msx1* and *Slug* mutual repress one another. However, expressing *Slug* in whole embryos does not have any important effect on *msx1* expression. In addition, the expression of *Slug* in animal caps does not affect the expression of any other neural crest or neural plate marker (Aybar et al., 2003; LaBonne and Bronner-Fraser, 1998). Conversely, the expression of *msx1* in whole embryos or animal caps does not inhibit *Slug* expression (this work and Tríbulo et al., 2003). Furthermore, the fact that *Slug* or *msx1* expression does not alter the overall expression of marker genes, but rather specifically affects the transcription of genes of the apoptotic machinery, argues against the possibility that the changes in apoptosis observed in animal caps are a consequence of changes in the fate of the ectodermal tissue. Instead, they support the notion that *Slug* and *msx1* work directly as apoptotic factors, controlling the expression and/or activity of different elements of the apoptotic pathway.

In conclusion, *msx1* and *Slug* have complementary patterns of expression and opposing activities, not only in controlling apoptosis but also on the transcriptional regulation of *XR11* and several caspases.

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