Regulation of apoptosis in the *Xenopus* embryo by Bix3

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Summary

Members of the *Bix* family of homeobox-containing genes are expressed in the vegetal hemisphere of the *Xenopus* embryo at the early gastrula stage. Misexpression of at least some of the family members causes activation of mesoderm- and endoderm-specific genes and it is known that some of the proteins, including Bix2 and Bix3, interact with Smad proteins via a motif that is also present in the related protein Mixer. In this paper we study the function of *Bix3*. Misexpression of *Bix3*, similar to misexpression of other members of the *Bix* family, causes the activation of a range of mesendodermal genes, but the spectrum of genes induced by *Bix3* differs from that induced by *Bix1*. More significantly, we find that overexpression of *Bix3* also causes apoptosis, as does depletion of Bix3 by use of antisense morpholino oligonucleotides. The ability of Bix3 to causes apoptosis is not associated with its ability to activate transcription and nor with its possession of a Smad interaction motif. Rather, Bix3 lacks a C-terminal motif, which, in Bix1, acts in cis to inhibit apoptosis. Mutation of this sequence in Bix1 causes the protein to acquire apoptosis-inducing activity.

Key words: *Xenopus*, Cell adhesion, Gastrulation, Morphogenesis, Embryogenesis, Apoptosis

Introduction

Members of the *Bix* family of homeobox-containing genes are expressed in the vegetal hemisphere of the early *Xenopus* embryo (Tada et al., 1998). The four genes – *Bix1* (also known as Mix.4) (Mead et al., 1998), *Bix2* (also known as Milk) (Ecochard et al., 1998), *Bix3* and *Bix4* – are members of a larger family that includes *Mix.1* (Rosa, 1989), *Mix.2* (Vize, 1996) and *Mixer* (Henry and Melton, 1998) (also known as Mix.3) (Mead et al., 1998). The family members have in common their expression patterns (see references above) and the fact that at least some of them, including *Bix1*, *Bix2*, *Bix4* and *Mixer*, can cause isolated animal pole regions to activate genes normally expressed in the mesendoderm (Ecochard et al., 1998; Henry and Melton, 1998; Tada et al., 1998). All members of the family that have been tested function as activators of transcription (Mead et al., 1996; Henry and Melton, 1998; Tada et al., 1998).

Although members of the Bix/Mix family are similar in their amino acid sequences (particularly in their homeodomains), expression patterns and biological functions, they do differ in some respects. Most recently, for example, they have been found to differ in their abilities to interact with Smad proteins; the Smad-interacting motif present in Bix2 (Milk) and Mixer is also present in Bix3, but not in Bix1, Bix4, Mix.1 or Mix.2 (Germain et al., 2000; Randall et al., 2002). In the first part of this paper we add to these differences by showing that Bix3 induces a different spectrum of genes to that induced by Bix1. More significantly, however, we reveal a novel and unexpected property of members of the Bix family: the ability to regulate apoptosis. Apoptosis is observed in response to overexpression of Bix3, but not in response to Bix1; we show that it does not, however, correlate with the possession of a Smad-interaction motif. Interestingly, inhibition of Bix3 function by microinjection of an antisense morpholino oligonucleotide also causes apoptosis, suggesting that apoptosis might be regulated during normal development according to particular concentrations of Bix3.

In investigating the molecular basis of this phenomenon we found that Bix1 has cryptic apoptotic activity, which is revealed following deletion of approximately 25 C-terminal amino acids. This observation suggests that the C-terminal region of Bix1 represses the apoptotic activity of the rest of the protein, a property that presumably is not shared with the C-terminal region of Bix3. Consistent with this idea, a chimeric version of Bix1 in which the C-terminal domain is derived from Bix3 is able to induce apoptosis. The domain of Bix1 that represses apoptosis contains an essential threonine residue; if this is mutated to alanine, Bix1 again acquires the ability to cause apoptosis. We discuss the significance of these observations for the control of apoptosis and for the regulation of Bix protein activity.

Materials and methods

Embryos

Xenopus embryos were obtained by artificial fertilisation (Smith and Slack, 1983). They were maintained in 10% normal amphibian medium (NAM) (Slack, 1984) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975). Injections of RNA were performed in 75% NAM containing 4% Ficoll and embryos were transferred to 10% NAM before gastrulation. Cycloheximide treatment was performed by transferring embryos to 0.1 mg/ml cycloheximide in 10% NAM.

In situ hybridisation

In situ hybridisations were performed essentially as described (Harland, 1991), except that BM purple was used as a substrate. In an effort to improve probe penetration, some embryos were cut in half before performing the procedure.

Expression constructs

Capped RNA was transcribed as described (Smith, 1993). RNA was synthesised from the following plasmid constructs; full details are available on request: pBix1.64T, pBix1 (1-376).64T, pBix1 (1-315).64T, pBix1 (1-225).64T, pBix1 (1-225;315-401).64T, pBix1 (1-144;225-315).64T, pBix1/3.64T, pBix1 (1-397).64T, pBix1 SS378.64T, pBix1 T384A.64T, pBix1 EE393/4AA.64T, pBix1 Q132E.64T, pBix1 (1-376) Q132E.64T, pBix3.64T, pBix3 (1-360).64T, pBix3 (1-302).64T, pBix3 (1-214).64T, pBix3 PP313/3AA.64T, pBix1-HA.64T, pBix3-HA.64T, pBix1ΔUTR.64T, pBix3ΔUTR.64T, pHBcl2. All Bix constructs were HA-tagged.

DNA expression constructs were as follows: Bix1. pcDNA3, Bix1 (1-376). pcDNA3, Bix1 (1-315). pcDNA3, Bix1 (1-225). pcDNA3, Bix1 (1-397). pcDNA, Bix1 SS378. pcDNA3, Bix1 T384A. pcDNA3, Bix1 EE393/4AA. pcDNA3, Bix1 Q132E. pcDNA3, Bix1 (1-376) Q132E. pcDNA3, Bix3. pcDNA3, Bix3 (1-360). pcDNA3, Bix3 (1-302). pcDNA3, Bix3 (1-214). pcDNA3 and Bix3 PP313/3AA. pcDNA3. Details of these plasmids, all of which carry an HA tag, are available on request. The P3 reporter construct comprises 6 copies of the P3 site (TAATTGAATTA) (Wilson et al., 1993) cloned into a luciferase reporter containing the E4 minimal promoter (Latinkic and Smith, 1999).

Analysis of gene expression by real-time RT-PCR

RNA was extracted using the Roche TriPure reagent according to the manufacturer's protocol. Briefly, animal caps were homogenised in 800 μl TriPure reagent, extracted with 200 μl chloroform and incubated at room temperature for 15 minutes. Debris was removed by centrifugation and nucleic acids were precipitated by addition of isopropanol, following addition of 10 μg glycogen as carrier. DNA was removed by incubation for 30 minutes at 37°C in RNAase-free DNAase followed by Proteinase K treatment for a further 30 minutes. Samples were phenol/chloroform extracted and RNA was precipitated with 2 volumes of ethanol and 1/6 volume 5M LiCl at –20°C for 30 minutes.

Gene expression analyses were performed by real-time RT-PCR and a LightCycler System™ (Roche). Primer pairs for *cerberus*, *goosecoid*, *XHex* and *Sox17α* are described by Xanthos et al. (Xanthos et al., 2001). *plakoglobin* primers are described by Kofron et al. (Kofron et al., 1999) and ornithine decarboxylase primers by Heasman et al. (Heasman et al., 2000).

TUNEL assay

Whole-mount TUNEL staining was performed essentially as described (Hensey and Gautier, 1998). Briefly, embryos were fixed in MEMFA (100 mM MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 4% formaldehyde) and stored in methanol at –20°C. They were rehydrated in PBT (0.2% Tween 20 in PBS), washed in PBS, and incubated in 150 U/ml terminal deoxynucleotidyltransferase (Gibco BRL) and 0.5 μM digoxigenin-dUTP (Boehringer Mannheim). The reaction was terminated in PBS/1 mM EDTA for 2 to 4 hours at 65°C, followed by extensive washes in PBS. The embryos were then blocked in PBT/20% heat-inactivated lamb serum, followed by incubation in a 1:2000 dilution of anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim). Embryos were extensively washed in PBS and stained using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. The reaction product was visible within approximately 30 minutes. In some cases, embryos were bleached overnight in 70% methanol and 10% hydrogen peroxide and viewed following dehydration in methanol and mounting in 2:1 benzyl benzoate:benzyl alcohol.

tPARP cleavage assay

The poly(ADP) ribose polymerase (tPARP) cleavage assay was performed essentially as described by Hensey and Gautier (Hensey and Gautier, 1997). Briefly, embryos were homogenised in EB buffer [80 mM β-glycerophosphate (pH 7.3), 15 mM $MgCl_2$, 20 mM EGTA, 10 mM DTT plus protease inhibitors] and the homogenate was centrifuged three times at 4°C, to remove particulate matter. Five μl of each homogenate was mixed with 3 μl of ^{35}S-labelled PARP and incubated at 24°C for 15 minutes. Samples were denatured and run on a 12% PAGE gel at 130 V for one hour.

In vitro translation

pBix1.64T and pBix3.64T (both of which include the 5′ untranslated regions of the cDNAs) were transcribed and translated in the Promega TNT-coupled Reticulocyte Lysate System. Briefly, 1 μg DNA was incubated with lysate, buffer, SP6 RNA polymerase, amino acids, [^{35}S]-methionine and RNAase inhibitor in the presence or absence of 10 μM antisense Bix3 morpholino oligonucleotide (see Fig. 5). Reactions were incubated at 30°C for 90 minutes. A 1 μl aliquot from each reaction was run on a 12% polyacrylamide gel and developed overnight.

Detection of HA-tagged Bix proteins by immunoprecipitation, western blots

Different amounts of a Bix3 antisense morpholino oligonucleotide were injected into *Xenopus* embryos at the one-cell stage followed by RNA (100 pg) encoding HA-tagged versions of Bix1 or Bix3 at the two-cell stage. Embryos were allowed to develop to early gastrula stage 10.5 and were then frozen at –80°C. Batches of 10 embryos were homogenised in 500 μl of solubilisation buffer (10% glycerol, 0.15 M NaCl, 50 mM Tris pH 7.5, 1% Triton X100, 0.2% SDS plus protease inhibitors) and then centrifuged at 13,000 rpm. Supernatants were transferred to a fresh tube, 10 μl anti-HA affinity resin (WB) was added to each extract, and samples were incubated overnight at 4°C. The beads were pelleted by centrifugation and washed four times in 1 ml solubilisation buffer. SDS polyacrylamide gel loading buffer (20 μl) was added, the samples were boiled for 2 minutes, and after centrifugation they were applied to a 12% polyacrylamide gel.

Western blots were performed as described (Tada et al., 1997), using monoclonal antibody 12CA5 to detect the HA epitope.

Transient transfections in cell culture

COS cells were transiently transfected by lipofection with variable amounts of effector DNA, 100 ng of firefly luciferase reporter construct, 100 ng reference plasmid pRL-TK (Promega), and appropriate amounts of pcDNA3 (InVitrogen) to a total of 800-1000 ng. Each experiment was performed in duplicate. Cells were harvested approximately 48 hours after transfection and Renilla and firefly luciferase activities were determined according to the manufacturer's instructions (Dual luciferase kit, Promega). For western blots, cells were transiently transfected with 1 μg effector DNA and harvested as described (Sambrook et al., 1989). Western blots to detect the HA epitope were performed as described above.

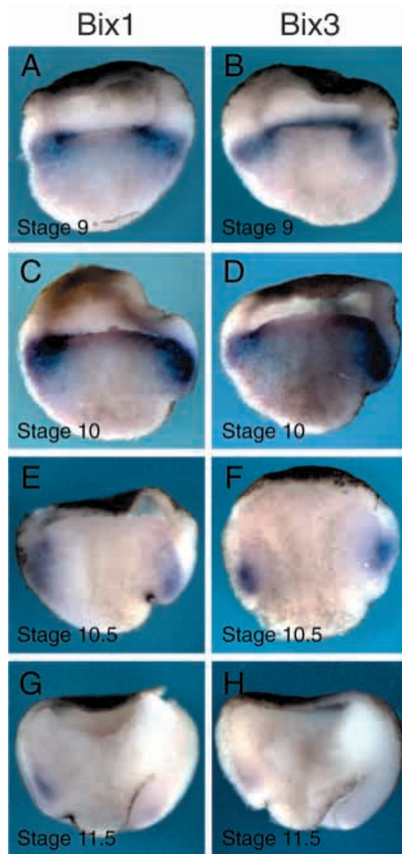

Fig. 1. The expression patterns of *Bix1* and *Bix3*. In situ hybridisation was performed on bisected embryos at the indicated stages using probes specific for *Bix1* and *Bix3*. In C-H, dorsal is to the right.

Results

Bix1 and *Bix3* have similar expression patterns

This paper concerns the ability of *Bix3* to activate gene expression and to induce apoptosis, in which respects, as we show below, it differs from some other members of the *Bix* family. In the first series of experiments we therefore used in situ hybridisation to compare the expression pattern of *Bix3* with that of *Bix1*. Analysis of intact and of bisected embryos shows that the two genes have similar expression patterns, and that they also resemble the published expression patterns of other members of the *Bix* and *Mix* families (Fig. 1 and data not shown) (Rosa, 1989; Vize, 1996; Ecochard et al., 1998; Henry and Melton, 1998; Mead et al., 1998; Tada et al., 1998; Casey et al., 1999; Germain et al., 2000). Thus, expression of both genes is first detectable at late blastula stage 9, in the marginal zone (Fig. 1A,B) and in prospective endoderm (where transcripts are more clearly visible in intact embryos). Transcripts persist until the early gastrula stage (Fig. 1C,D) but begin to decline soon thereafter, especially in the vegetal hemisphere (Fig. 1E,F). By mid-gastrula stage 11.5, low levels of expression are detectable in the ventral marginal zone of the embryo, but transcripts are absent from the dorsal marginal zone (Fig. 1G,H).

Misexpression of *Bix1* and *Bix3* in *Xenopus* embryos causes distinct phenotypes

Previous work has shown that injection of *Bix1* or *Bix4* RNA into the animal pole of *Xenopus* embryos at the 2-cell stage causes those embryos to develop abnormally. They appear normal at late blastula stage 9, but gastrulation and subsequent development are then impaired, because of both ventralisation of dorsal mesoderm and conversion of ventral mesoderm to endoderm (Fig. 2E-G) (Tada et al., 1998; Casey et al., 1999). We had expected a similar phenotype to be observed with the closely related *Bix3*, but surprisingly, abnormalities proved to be visible from the late blastula stage, with some animal pole cells becoming arrested in the cell cycle and strongly pigmented (Fig. 2I). A little later, during gastrulation, these cells appear white, they lose the ability to adhere to their neighbours, and they dissociate from the remaining mesendodermal mass (Fig. 2J,K). This phenotype was observed with doses of

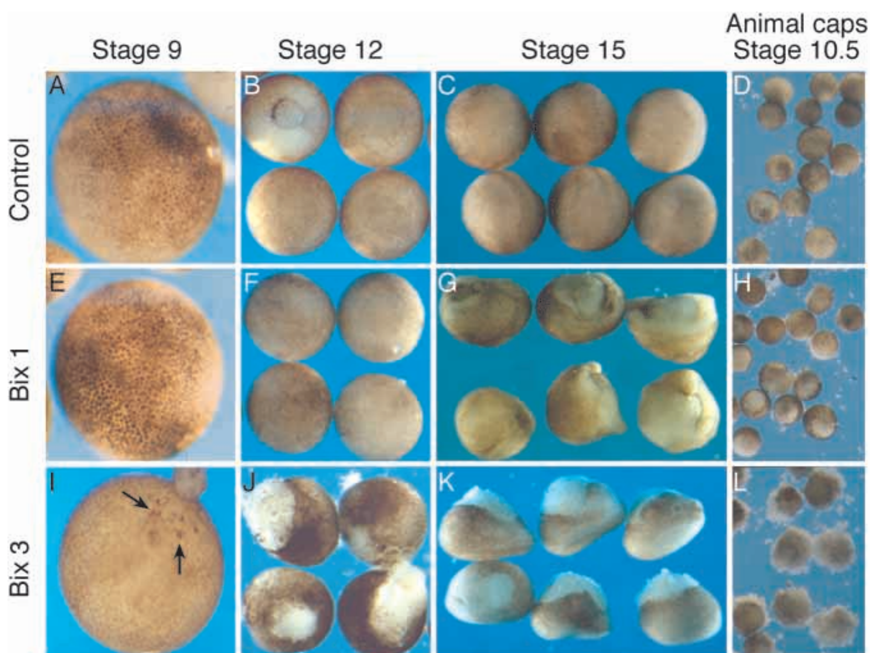

Fig. 2. Effects of *Bix1* and *Bix3* on *Xenopus* embryos and animal pole regions. *Bix1* (E-H) or *Bix3* (I-L) RNA (400 pg) was injected into the animal pole regions of *Xenopus* embryos at the one-cell stage. Other embryos (A-D) were left uninjected. Embryos were fixed at the indicated stages and photographed. (A,E,I) Animal view of stage 9 embryos. (B,F,J) Animal view of stage 12 embryos (except the embryo in the upper-left corner of B, which is viewed from the vegetal pole). (C,G,K) Stage 15 embryos. (D,H,L) Animal caps dissected at stage 8.5 and allowed to develop to the equivalent of early gastrula stage 10.5. Note that animal caps derived from embryos injected with *Bix3*, but not *Bix1*, RNA are in the early phases of disaggregation. Arrows in I indicate darkly pigmented cells.

Bix3 RNA as low as 50 pg, and maximal effects were noted with 400 pg. Similar results have been observed with *Bix2* (data not shown).

Direct observation of injected embryos indicates that the defects in cell adhesion caused by *Bix3* develop rapidly. For example, the interval between the appearance of a few white cells and the disaggregation of more than half the animal hemisphere can be as little as one hour (data not shown). Injection of *Bix3* RNA into individual blastomeres at the 32-cell stage suggests that the effects of the protein are cell autonomous because the area of cell disaggregation is reduced (data not shown).

Defects in cell adhesion caused by *Bix3* can also be observed in isolated animal pole regions. When *Bix3*-expressing animal caps are dissected from *Xenopus* embryos at the late blastula stage, they fail to adhere and disaggregate into single cells, whereas control caps, or those expressing *Bix1*, form compact spheres (Fig. 2D,H,L).

Bix1 and *Bix3* activate different genes to different extents

The results described above suggest that *Bix1* and *Bix3* exert different biological effects. In support of this observation we find that although *Bix3*, similar to *Bix1*, activates the expression of endoderm-specific genes, the spectra of genes activated by *Bix1* and *Bix3* differ (Fig. 3). In assessing these experiments we note that the levels of our reference gene, *plakoglobin*, are reduced by *Bix3* (Fig. 3). This is probably because of loss of cells before the samples are harvested (see Fig. 2); a similar effect is seen using another reference gene, *ornithine decarboxylase* (data not shown). When corrections are made for this loss (see inset, Fig. 3), *Bix3* proves to be a particularly powerful inducer of *Sox17α* and *goosecoid*, but it cannot induce expression of *Xhex* or *cerberus*. By contrast, *Bix1* induces *XHex* strongly and *cerberus* to some extent. We reach the same conclusion following experiments in which animal caps are harvested before the onset of apoptosis and in which levels of the reference gene are similar in *Bix1*- and *Bix3*-injected animal caps (data not shown). *Bix1* and *Bix3* therefore differ in their inducing activities as well as in their effects on cell adhesion.

Bix3 misexpression induces apoptosis

A decrease in cell adhesion, as documented in Fig. 2, is frequently accompanied by apoptosis, in a phenomenon known as anoikis (Frisch and Francis, 1994). We first investigated whether overexpression of *Bix3* causes apoptosis by subjecting *Xenopus* embryos to TUNEL staining. Control embryos showed little or no TUNEL staining during gastrula stages (Fig. 4A-C) (Hensey and Gautier, 1998), whereas embryos exposed to cycloheximide, which arrests cell division and triggers apoptosis, contained many apoptotic cells, which were visible at mid-gastrula stage 11 (Fig. 4D-F) (Hensey and Gautier, 1998). Embryos injected with RNA encoding Bix3 also contained apoptotic cells. A few TUNEL-staining cells were visible at stage 11, but highest levels were observed at stages 12 and 12.5 (Fig. 4G-I). We suspect that the apoptotic cells revealed by TUNEL staining are those that are undergoing disaggregation.

To confirm that overexpression of *Bix3* causes apoptosis, embryo extracts were assayed for caspase activity by testing their ability to cleave the well-characterised caspase substrate tPARP (Cohen, 1997; Hensey and Gautier, 1998; Stancheva et al., 2001). Extracts of control embryos, or of embryos injected with RNA encoding Bix1, did not cause cleavage of tPARP (Fig. 4J). Extracts of cycloheximide-treated embryos, however, do cause tPARP cleavage, with caspase activity first detectable at early gastrula stage 10 (see also Hensey and Gautier, 1997). Extracts of *Bix3*-injected embryos also caused tPARP cleavage, with caspase activity first detected at stage 11 (Fig. 4J). This observation confirms that overexpression of *Bix3* causes apoptosis.

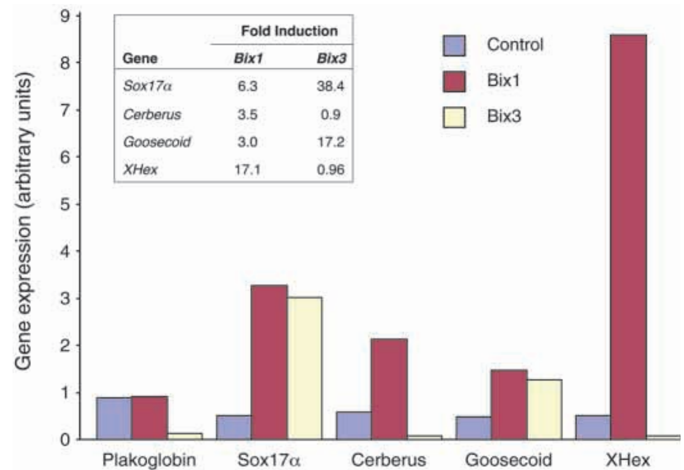

Fig. 3. Gene activation by *Bix1* and *Bix3*. *Xenopus* embryos were injected with RNA (200 pg) encoding Bix1 or Bix3 or were left uninjected. Animal pole regions were dissected at late blastula stage 9 and cultured to the equivalent of early gastrula stage 10.5, when they were assayed for expression of the indicated genes by real-time PCR. *Bix3* causes a decrease in expression of the control transcript *plakoglobin* but activates *Sox17α* and *goosecoid* to high levels. It does not induce expression of *cerberus* or *XHex*. *Bix1* is a powerful inducer of *Xhex* and also activates expression of *cerberus*. Inset shows data normalised to levels of *plakoglobin* expression.

We note that TUNEL-positive cells only appear after the decrease in embryonic cell adhesion illustrated in Fig. 2, and even the more sensitive tPARP assay only detects caspase activity at mid-gastrula stage 11 (Fig. 4J). One interpretation of these observations is that the primary effect of *Bix3* is to reduce cell adhesion during gastrulation and that this leads to apoptosis through anoikis. Interestingly, however, the anti-apoptotic protein human Bcl2 can delay the onset of caspase activity (Fig. 4K), and in five out of seven experiments it also delayed the onset of cell disaggregation (Fig. 4L-M), suggesting that loss of adhesion is a consequence of apoptosis and that the later appearance of TUNEL-positive cells and caspase activity are because of lower sensitivities of these techniques.

Loss of Bix3 function by antisense morpholino oligonucleotides also causes apoptosis

Our results show that overexpression of *Bix3* causes apoptosis: what is the effect of loss of function of this gene? To investigate this question we designed an antisense morpholino oligonucleotide directed against the *Bix3* 5′ untranslated region. The *Bix* family is highly conserved (Tada et al., 1998),

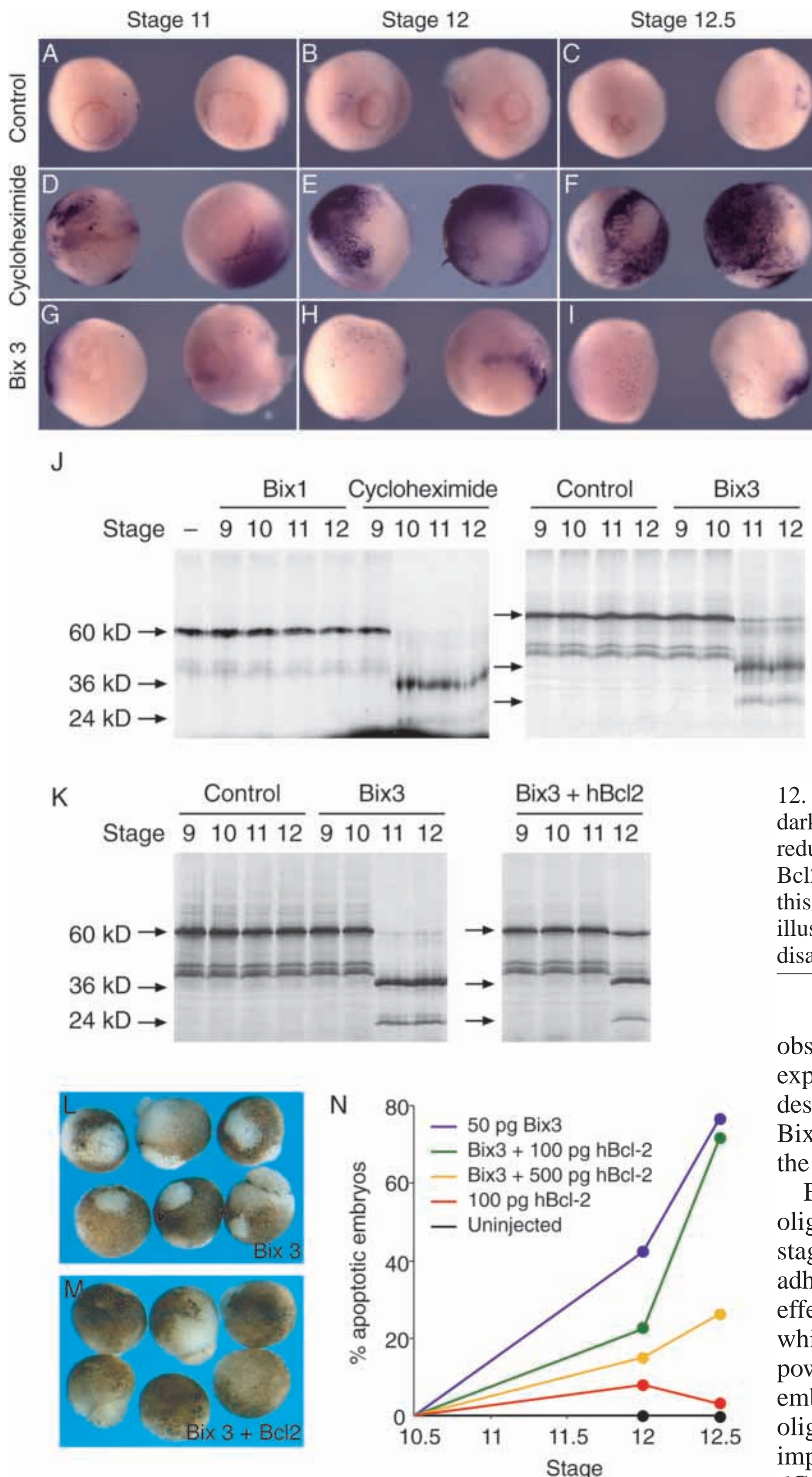

Fig. 4. Apoptosis in *Xenopus* embryos injected with RNA encoding Bix3. (A-F) One group of embryos was left uninjected whereas another (G-I) was injected with 400 pg of *Bix3* RNA at the one-cell stage. At stage 8.5, half of the uninjected embryos were transferred to 0.1 mg/ml cycloheximide (D-F), whereas the other half was allowed to develop normally (A-C). Embryos were collected at the indicated stages and used for TUNEL staining. Positive cells are dark blue. Note that loosely adherent cells in *Bix3*-injected embryos, as seen in Fig. 2, are lost during the TUNEL procedure. (J) tPARP assay shows that Bix1 does not activate caspase activity whereas induction of apoptosis by Bix3 was first detectable by stage 11. Cycloheximide induces caspase activity from early gastrula stage 10. Intact tPARP has a relative molecular mass of 60 kD (upper arrows in both gels), and this is cleaved into fragments of 36 kD and 24 kD (lower arrows). (K) Human Bcl2 delays the onset of Bix3-induced apoptosis. Caspase assays were performed on control embryos at the indicated stages, embryos injected with RNA encoding Bix3, or embryos injected with *Bix3* RNA together with RNA encoding human Bcl2 (500 pg). Note that human Bcl2 delays the onset of caspase activity. (L-N) Partial rescue of the cell adhesion and apoptosis defect by human Bcl2. *Bix3* RNA alone (50 pg; L), or in combination with 500 pg of human Bcl2 RNA (M), was injected in the animal pole of one-cell stage embryos. Embryos were left to develop and photographed at mid-gastrula stage 11.5-12. Note that human Bcl2 does not prevent the appearance of darkly pigmented cells in the animal hemisphere but does reduce or delay the incidence of cell disaggregation. Human Bcl2 delayed disaggregation in five out of seven experiments of this sort. (N) Quantitation of an experiment of the type illustrated in (L) and (M). Embryos undergoing cell disaggregation were scored at the indicated stages.

and it proved difficult to design an oligonucleotide specific for Bix3. In particular, it seemed probable that our Bix3 oligonucleotide might also inhibit translation of Bix1 (Fig. 5A), and indeed the antisense Bix3 oligonucleotide blocked translation of Bix1 as well as Bix3 both in an in vitro coupled transcription/translation system (Fig. 5B) and after injection of RNA encoding HA-tagged forms of Bix1 and Bix3 into *Xenopus* embryos (Fig. 5C). No effect was observed on translation of Bix2 or Bix4 (Fig. 5B). Although these observations complicate the interpretation of our experiments, we believe the apoptotic phenotype described below is because of depletion of Bix3 and not Bix1, because cell death can be 'rescued' by injection of the former and not the latter (see Fig. 7).

Embryos injected with the Bix3 antisense morpholino oligonucleotide appeared normal until the mid-gastrula stage, but cells of the neural plate then became less adhesive and holes appeared in the neural plate. No such effect was observed with a control oligonucleotide in which four base pairs were mutated (Fig. 6A-C; higher-power views in Fig. 6D,E). TUNEL staining of intact embryos suggested that the Bix3 morpholino oligonucleotide was causing apoptosis (Fig. 6F), and this impression was confirmed in bisected embryos (Fig. 6G,H), where apoptotic cells were visible in the archenteron roof and in dorsal mesoderm, both of which are derived from *Bix3*-expressing cells, and also in the endodermal mass. Apoptosis was also visible in cells of the neural plate (Fig. 6H). Comparison of Keller's fate map (Keller, 1975) with the expression domain of *Bix3* (Fig. 1) suggests that the progeny of *Bix3*-expressing cells may give rise to a limited portion of the neural plate, but it is probable that a substantial proportion of the apoptosis observed in the neural plate is because of non-cell autonomous effects of the Bix 3 antisense morpholino oligonucleotide. It is unlikely to

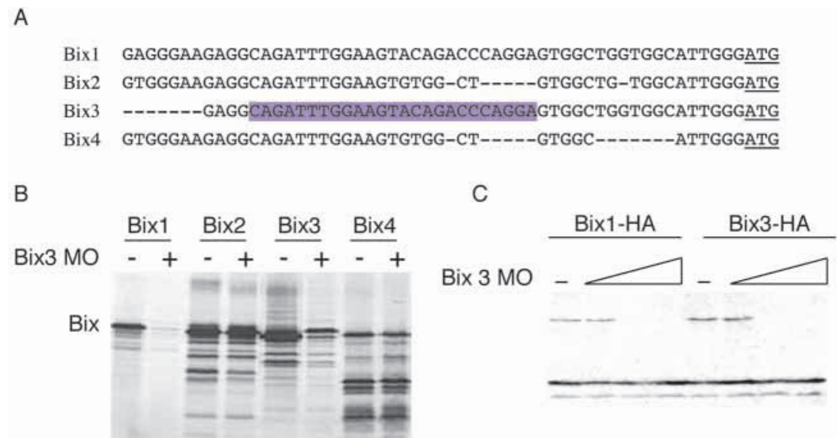

Fig. 5. Specificity of an antisense *Bix3* morpholino oligonucleotide. (A) The 5′ untranslated regions of *Bix1-4*. The highlighted region of *Bix3* indicates the sequence recognised by the antisense oligonucleotide: 5′ TCCTGGGTCTGTACTTCCAAATCTG 3′. An oligonucleotide with sequence 5′ TCCTCGGTCAGT-ACTACCAATTCTG 3′ was used as a control (underlined nucleotides indicate differences from the Bix3 sequence). (B) The antisense Bix3 morpholino oligonucleotide inhibits in vitro translation of both Bix1 and Bix3. See Materials and Methods for experimental details. Note that translation of the most abundant *Bix3* gene product is inhibited by the antisense oligonucleotide, but that a higher molecular weight form persists. Such a form is not detected in the western blot experiment described in C. (C) The two higher concentrations of an antisense *Bix3* morpholino oligonucleotide inhibit translation of HA-tagged forms of both Bix1 and Bix3 following RNA injection into *Xenopus* embryos. Quantities of Bix3 antisense morpholino oligonucleotide (10, 20 and 50 ng) were injected.

be because of non-specific effects of the morpholino oligonucleotide, because no apoptosis is observed following injection of a Bix3 antisense morpholino oligonucleotide into control animal pole cells, and nor is it observed in animal pole cells that have been induced to form neural tissue by injection of noggin (Lamb et al., 1993). In contrast, animal caps induced by activin to form mesendodermal tissue, and therefore to express *Bix3*, do undergo apoptosis in response to the Bix3 antisense morpholino oligonucleotide (Fig. 6I-O).

Use of the tPARP cleavage assay confirmed that depletion of Bix3 causes premature apoptosis. In control embryos, and embryos injected with a control morpholino oligonucleotide, caspase activity was first detected at stage 17. In embryos injected with the *Bix3* morpholino oligonucleotide caspase activity was present by stage 14 (Fig. 6P).

Although the Bix3 antisense morpholino oligonucleotide caused apoptosis in the *Xenopus* embryo, it had little effect on regional specification. Patterns of expression of *Sox3*, *Otx2* and *Krox20* were normal in morpholino-injected embryos, although expression levels were considerably lower (data not shown).

To confirm that the observed phenotype is a specific response to the depletion of Bix3 protein, embryos were injected with Bix3 antisense morpholino oligonucleotide together with RNA encoding Bix1 or Bix3 (Fig. 7). The *Bix* constructs lacked the 5′ untranslated region against which the morpholino oligonucleotide was directed. Coinjection of *Bix3* RNA 'rescued' apoptosis caused by the Bix3 morpholino oligonucleotide in a dose-dependent manner. Thus 100 pg *Bix3* RNA had little effect (Fig. 7D), whereas 200 pg RNA elicited good rescue (Fig. 7E). Embryos coinjected with 400 pg Bix3 *RNA* did undergo apoptosis (Fig. 7F), perhaps because Bix3 levels were now too high. By contrast, no concentration of RNA encoding Bix1 was able to rescue the apoptotic effects of the Bix3 morpholino (Fig. 7G-I). These results suggest that the phenotype described in Fig. 6 is specifically because of the absence of Bix3 protein.

Smad-binding and transcriptional activation are not required for Bix3-induced apoptosis

The results described above indicate that *Bix3* plays a role in cell survival during early *Xenopus* development and in this respect it resembles *Bix2* (data not shown) but differs from *Bix1* and *Bix4* (Fig. 2 and data not shown). In an effort to map the domain of *Bix3* responsible for the induction of apoptosis, RNA encoding C-terminal truncations of Bix3 was injected into the animal pole regions of *Xenopus* embryos at the one-cell stage. All truncated *Bix3* constructs proved to cause apoptosis (Fig. 8A), indicating that apoptotic activity is not localised to amino acids 214-389. We note that Bix3(1-302) and Bix3(1-214) lack the Smad interaction motif (SIM) (Germain et al., 2000; Randall et al., 2002), suggesting that interaction with Smads is not necessary for *Bix3*-induced apoptosis. To reinforce this conclusion, a mutant version of Bix3 was constructed in which two conserved prolines of the SIM (amino acids 313 and 314) are replaced by alanines, thus creating Bix3 PP313/4AA. RNA encoding this protein also caused apoptosis, confirming that interaction with Smad proteins is not necessary for Bix3-induced cell death (Fig. 8A).

Mixer and Milk (Bix2) function as activators of transcription (Henry and Melton, 1998; Germain et al., 2000). To ask if the same is true of Bix3, and to determine which region of the protein might be responsible for transcription activation, the constructs depicted in Fig. 8A were transfected into COS cells along with a reporter in which six P3 sites are positioned upstream of a minimal promoter (Wilson et al., 1993; Latinkic and Smith, 1999). Bix proteins bind to P3 sites with high affinity (M.T. and J.C.S., unpublished) (Mead et al., 1998). All transfected constructs generated protein in approximately equal amounts, as determined by western blotting to detect the HA tag (data not shown).

Fig. 8B shows that Bix3 is an activator of transcription and that this activity is distributed throughout the C-terminal region. Mutation of the SIM (Bix3 PP313/4AA) does not affect transcriptional activation by Bix3, indicating that interaction with Smad proteins is not required for this aspect of Bix3 function. We note, however, that although transcriptional activation is greatly reduced in (for example) the construct Bix3(1-214), this is not associated with a decrease in the ability of the protein to induce apoptosis. There is, therefore, no strong correlation between transcriptional activation and the ability to cause apoptosis.

There was no evidence of apoptosis in cultured cells transfected with any of the Bix3 constructs described above

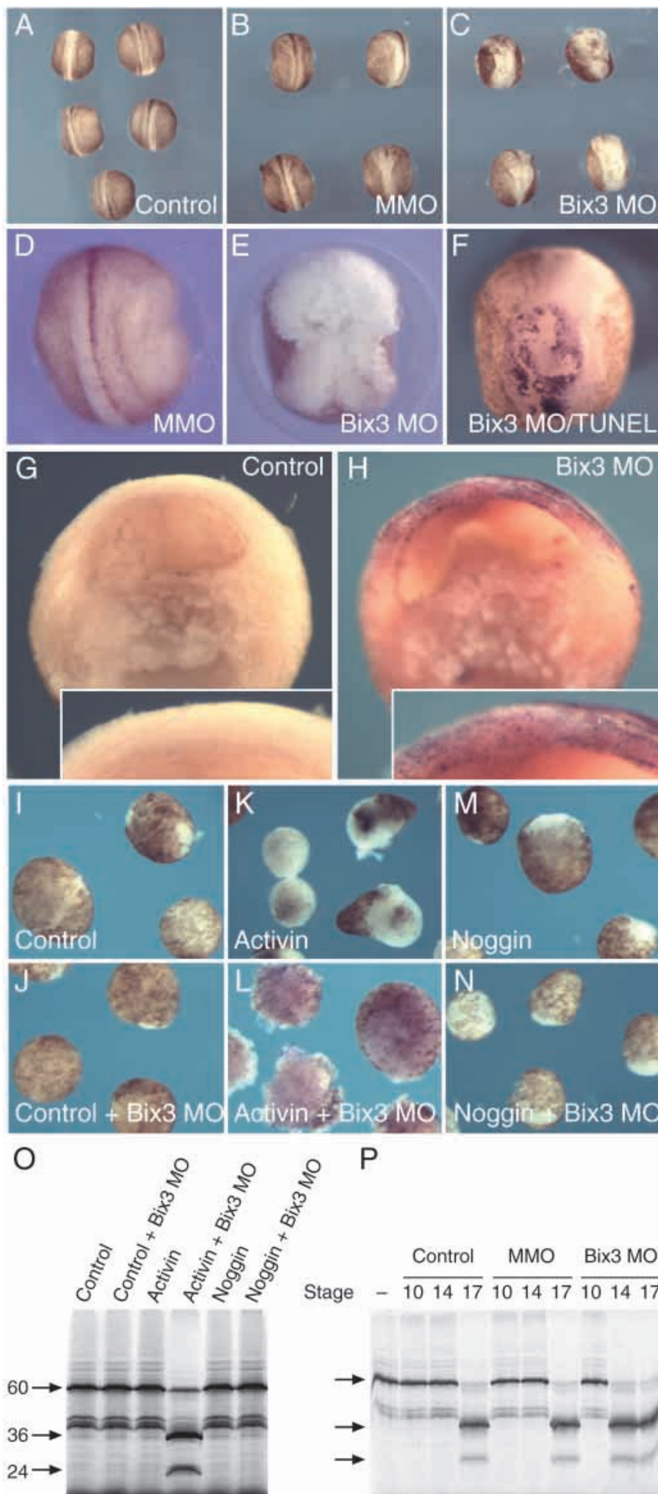

Fig. 6. An antisense Bix3 morpholino oligonucleotide causes apoptosis in the early *Xenopus* embryo without affecting regional specification. (A) Group of control embryos at stage 20. (B) Group of embryos injected at the one-cell stage with 50 ng of the control morpholino oligonucleotide described in Fig. 5. (C) Group of embryos injected with 50 ng of the Bix3 antisense morpholino oligonucleotide shown in Fig. 5. Notice the onset of apoptosis in dorsal axial structures. (D) Higher-power view of an embryo injected with the mutated morpholino oligonucleotide. (E) Higher-power view of an embryo injected with a Bix3 antisense morpholino oligonucleotide. (F) High-power view of an embryo injected with a Bix3 antisense morpholino oligonucleotide and stained using the TUNEL technique. Notice apoptotic cells in the neural plate. (G) Transversely bisected late gastrula control embryo stained using the TUNEL technique. Very few apoptotic cells are visible. Inset shows higher-power view of the archenteron roof. (H) Transversely bisected late gastrula embryo previously injected with a Bix3 antisense morpholino oligonucleotide. Note apoptotic cells in the endodermal mass and in dorsal axial structures. Inset shows higher-power view of the archenteron roof, with apoptotic cells in all three germ layers. (I-N) Control animal caps (I) or animal caps derived from embryos injected with RNA encoding activin (6 pg) or noggin (250 pg) together with 50 ng of antisense Bix3 morpholino oligonucleotide (J-N, as indicated) were fixed at the equivalent of stage 11 and stained using the TUNEL technique. Only animal caps treated with activin and injected with Bix3 morpholino undergo apoptosis. (O) Samples equivalent to those illustrated in I-N were subjected to a tPARP assay. Only animal caps treated with activin and injected with Bix3 morpholino undergo apoptosis. (P) A tPARP assay confirms that a Bix3 antisense morpholino oligonucleotide causes premature apoptosis. In control embryos, and in embryos injected with the control morpholino oligonucleotide, caspase activity is first detected at stage 17. In embryos injected with the Bix3 oligonucleotide caspase activity is present at stage 14.

(data not shown), suggesting that Bix3 apoptotic activity is context-dependent.

Bix1 has cryptic apoptotic activity that is revealed after deleting C-terminal amino acids

In the course of experiments designed to ask why Bix1 differs from Bix3 in its ability to cause apoptosis, we prepared a series of constructs encoding C-terminally truncated forms of Bix1. Consistent with previous work (Tada et al., 1998), injection of full-length *Bix1* RNA did not induce apoptosis (Fig. 9A). To our surprise, however, all C-terminally truncated versions of Bix1, including Bix1 (1-376) which lacks just 25 amino acids, did cause apoptosis (Fig. 9A). These observations suggest that Bix1 has cryptic apoptotic activity which is usually masked by its 25 most C-terminal amino acids.

Further deletions confirmed this conclusion and allowed us to map the apoptotic domain of Bix1 to a region between amino acids 144 and 225. Thus, an internal deletion lacking amino acids 226-314 does not cause significant apoptosis, presumably because the most C-terminal amino acids are retained (Fig. 9A). But a construct lacking amino acids 316-401 (and therefore the ability to prevent apoptosis) as well as amino acids 145-224 does not cause significant apoptosis either, suggesting that this activity resides in the 80 amino acids C-terminal to the homeodomain (Fig. 9A). This conclusion is consistent with the data described in Fig. 8A, which map the apoptotic activity of Bix3 to the N-terminal half of the protein.

The apoptotic activity of truncated Bix1 requires DNA binding but not the ability to activate transcription

We also assessed the ability of Bix1 to activate transcription, using the methods employed for Bix3 (see Fig. 8). Bix1 proved to be a powerful activator (Fig. 9A), and, as observed with Bix3, activity was distributed throughout the C-terminal region

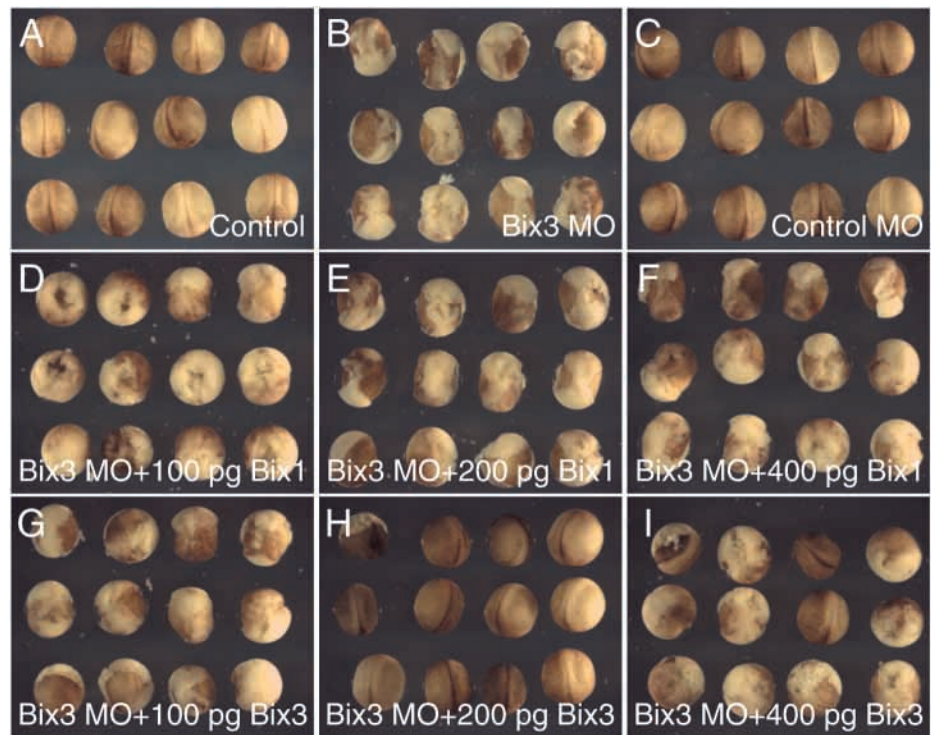

Fig. 7. Rescue of apoptosis by injection of 200 pg *Bix3* RNA but not *Bix1*. (A) Uninjected embryos at stage 19. (B) Embryos injected at the one-cell stage with a Bix3 antisense morpholino oligonucleotide begin to undergo apoptosis. (C) Embryos injected at the one-cell stage with a control morpholino oligonucleotide appear normal. (D-F). Embryos injected at the one-cell stage with Bix3 antisense morpholino oligonucleotide together with the indicated amounts of *Bix1* RNA which lacks the 5′ untranslated region against which the oligonucleotide is directed. Rescue is not observed at any concentration of RNA. (G-I) Embryos injected at the one-cell stage with Bix3 antisense morpholino oligonucleotide together with the indicated amounts of *Bix3* RNA which lacks the 5′ untranslated region against which the oligonucleotide is directed. Apoptosis is 'rescued' by 200 pg RNA (H) but not by 100 pg (G) or 400 pg (I).

(although deletion of amino acids 376-401 and of amino acids 225-315 caused a particularly marked decrease in activation). These results were confirmed in experiments in which Bix1 constructs were fused to the GAL4 DNA-binding domain and co-transfected into cultured cells together with a reporter containing GAL4-binding sites (data not shown). This analysis revealed strong transcriptional activity between amino acids 225 and 255 and between amino acids 376 and 401 of Bix1.

Deletion of amino acids 377-401 caused a slight decrease in transcriptional activation activity and conferred on Bix1 the ability to induce apoptosis (Fig. 9A). There is no correlation, however, between the loss of transcriptional activity and the loss of ability to repress apoptosis because Bix1 (1-376) does retain significant transcriptional activity (Fig. 9A) and, as discussed below, Bix1 T384A has full transcription activation function but also causes cell disaggregation (Fig. 10C).

The induction of apoptosis by Bix3 is not correlated with the ability to activate transcription (Fig. 8), and the same is true for truncated versions of Bix1; the transcription activation activity of Bix1 (1-225) is significantly reduced, yet the ability to induce apoptosis is unaffected (Fig. 9A). DNA binding, however, does seem to be required for apoptosis; the construct Bix1 (1-376) Q132E, which bears a mutation in helix 3 of its homeodomain, cannot bind DNA (data not

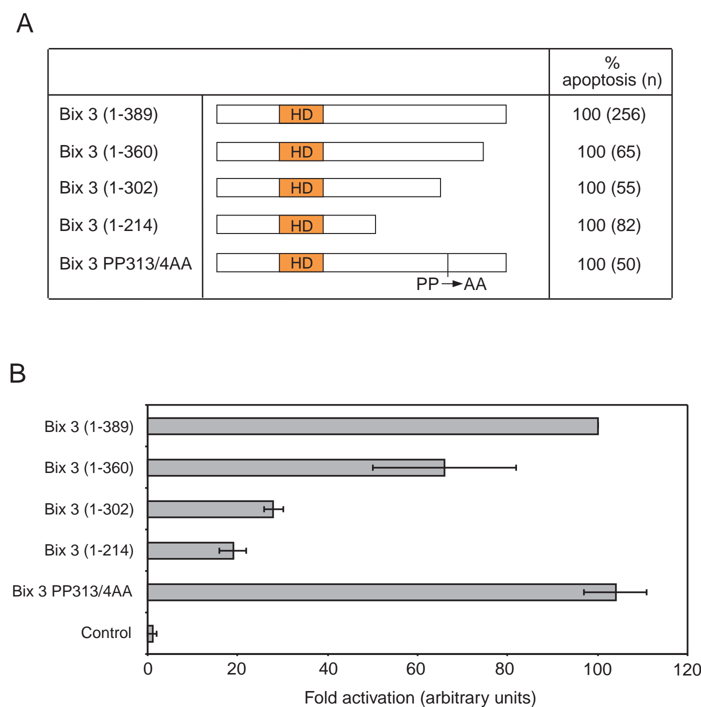

Fig. 8. The ability of Bix3 to activate transcription is not correlated with its ability to induce apoptosis. (A) Embryos at the one-cell stage were injected with RNA encoding full-length Bix3 [Bix3(1-389)], truncations of Bix3 (middle three constructs), or a mutation of Bix3 in which two prolines at positions 313 and 314 are replaced by alanines (Bix3 PP313/4AA), thereby mutating the Smad interaction motif. All five constructs induce apoptosis with equal efficiency. (B) Transient transfection assays in cultured cells. The Bix3 constructs described in A were co-transfected into COS cells with a reporter construct containing 6 copies of the P3 site, which is known to bind Bix proteins (Mead et al., 1998), and a reference plasmid. The average of three experiments are shown. In each the luciferase elicited by full-length Bix3 was defined as 100% and activity elicited by the other constructs was normalised relative to this. All transfected constructs used generate protein in approximately equivalent amounts (not shown). Note that the transcription activation activity of Bix3 (1-214) is substantially reduced, but its ability to induce apoptosis resembles that of the full-length protein.

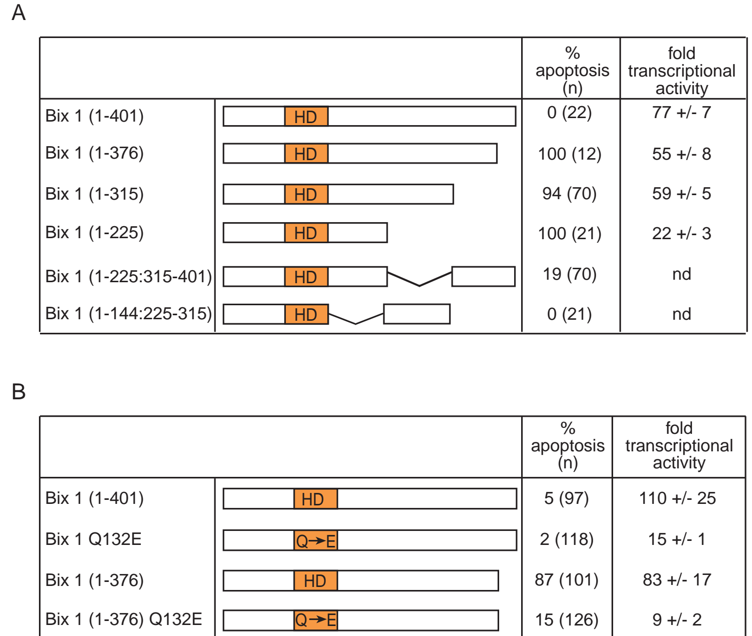

Fig. 9. Truncated versions of Bix1 cause apoptosis; the ability to cause apoptosis is not correlated with the ability of the protein to activate transcription but does require DNA binding. (A) RNA encoding truncated and deleted versions of Bix1 was injected into *Xenopus* embryos and the same constructs were introduced into COS cells to assess their transcriptional activity as described in Fig. 8. All transfected constructs generated protein in approximately equivalent amounts (not shown). Deletion of 25 C-terminal amino acids of Bix1 causes the protein to acquire apoptosis-inducing activity. Further deletions suggest that the apoptosis-inducing domain resides between amino acids 144 and 225 and that optimal apoptotic activity can be obtained in a construct [Bix1 (1-225)] in which 78% of transcriptional activity is lost. (B) Mutation of Q132 to E reduces transcriptional activation, substantially decreases the ability of the protein to cause apoptosis abolishes DNA binding (not shown). The ability of truncated Bix1 to induce apoptosis requires DNA binding. Bix1 (1-376) induces apoptosis, is a powerful activator of transcription and binds DNA (not shown).

shown) and it is a poor activator of transcription. It is also a very weak inducer of apoptosis (Fig. 9B).

The ability of Bix1 to prevent apoptosis is associated with threonine 384

Our results suggest that there is a C-terminal domain of Bix1 that is capable of suppressing *Bix*-induced apoptosis and that this domain is absent in Bix3. To confirm this conclusion, we made a chimeric version of Bix1 in which the 45 most C-terminal amino acids are derived from Bix3. As predicted, this chimeric molecule is capable of causing apoptosis (Fig. 10A).

The ability of Bix1 to repress cell disaggregation is contained within the 25 most C-terminal amino acids (Fig. 9A). The amino acid sequences of Bix1 and Bix3 are very similar within this region, but there are three positions of interest (Fig. 10B). First, Bix1 lacks a pair of serines between amino acids 377 and 378; second, Bix1 contains a threonine at position 384 whereas Bix3 contains an alanine; and finally (and perhaps of less significance), amino acids 396-399 of Bix1 comprise the residues IIRT whereas Bix3 consists of LLKS. To determine which of these groups of amino acids might be involved in repressing cell disaggregation, we designed a series of mutated versions of Bix1 (Fig. 10C). RNAs encoding Bix1 (1-397), which lacks the four C-terminal amino acids of Bix1, or Bix1 SS378, which contains two additional serines at position 378, behave in a similar manner to wild-type Bix1 when injected into *Xenopus* embryos at the one-cell stage. However, mutation of threonine 384 into an alanine, creating Bix1 T384A, caused apoptosis, as did mutation to alanine of two conserved glutamate residues at

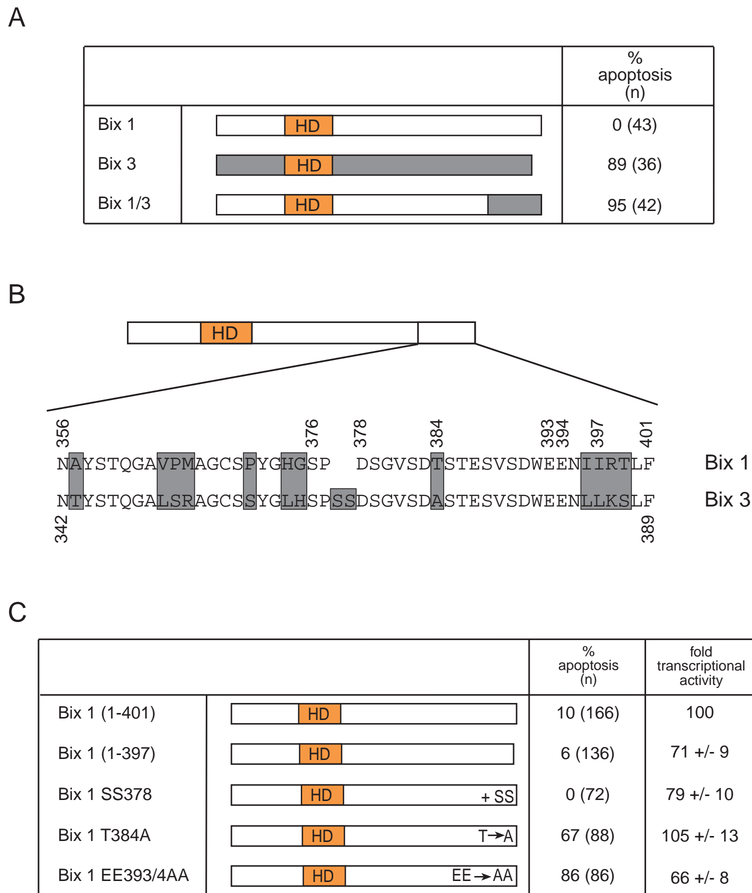

Fig. 10. A C-terminal domain of Bix1 acts in cis to prevent induction of apoptosis; the N terminus of Bix3 does not possess this activity. Threonine 384 is necessary for the anti-apoptotic function of the Bix1 C-terminal region. (A) A chimeric form of Bix1, in which the 45 C-terminal amino acids are derived from Bix3, induces apoptosis. (B) Comparison of the 45 C terminal amino acids of Bix1 and Bix3. (C) Mutation of threonine 384 of Bix1 to alanine causes the protein to acquire apoptosis-inducing activity, as does mutation of the two glutamic acids of the DWEEN motif.

positions 393 and 394 of Bix1, creating Bix1 EE393/4AA (Fig. 10C).

These results first suggest that the ability of the C terminus of Bix1 to repress apoptosis resides in part in threonine 384. In this respect, it is interesting that Bix4 (which, similar to Bix1, does not cause apoptosis) also contains a threonine at this position, whereas Bix2 (which does cause apoptosis) contains an alanine. Mix.1 and Mix.2 also contain a threonine residue at this position, and to our knowledge these proteins have not been reported to cause cell disaggregation or apoptosis. Mixer contains a glycine residue at the equivalent position; it has not been reported to cause apoptosis, although Henry and Melton (Henry and Melton, 1998) comment on the dumbbell shape of animal caps derived from embryos injected with RNA encoding Mixer, and the fact that one end is yolky in appearance. The significance of threonine 384 is discussed below.

The pair of glutamate residues at positions 393 and 394 is part of the DWEEN motif, which is conserved in all Bix family members as well as in Mix.1 and Mix.2 (but not in Mixer). It is probable that this motif represents an essential structural element of the Bix family, and that mutation impairs its function.

Discussion

This paper describes an unusual phenotype, but one that is highly specific. Overexpression of the Bix family member *Bix3* in the *Xenopus* embryo causes dramatic cell disaggregation and apoptosis. Apoptosis is not observed with the closely related protein Bix1, although the apoptotic activity is present in latent form because C-terminally truncated versions of Bix1 do cause apoptosis. Similar to other members of the Bix family, Bix3 is an activator of transcription, but there is no correlation between the ability to activate transcription and the ability to cause apoptosis. Experiments with point mutants of Bix1 suggest, however, that DNA binding is required for induction of apoptosis.

The function of Bix3 during normal development was studied by microinjection of an antisense morpholino oligonucleotide. The results indicate that loss of Bix3 function in the *Xenopus* embryo also causes apoptosis, and ‘rescue’ experiments with different concentrations of *Bix3* RNA suggest that cell survival requires Bix3 levels to be maintained within a certain range. These results, and others, are discussed below.

Different effects of Bix family members: Bix3 causes apoptosis

Members of the Bix family of homeodomain-containing proteins have similar expression patterns (Fig. 1) but different biological actions. One example described in this paper concerns the inductive activities of the two genes, which differ markedly (Fig. 3), but more significant are their effects on apoptosis (Figs 2, 4). Overexpression of *Bix3* causes dramatic cell death in the *Xenopus* embryo, whereas *Bix1* has no apparent effect on cell survival whatsoever. That the cell death caused by Bix3 is indeed because of apoptosis is confirmed by TUNEL staining (Fig. 4A-I), by a tPARP cleavage assay (Fig. 4J), and by the fact that death can frequently be rescued, at least in part, by co-expression of human Bcl2 (Fig. 4K-N). We do not know whether the apoptotic effects of Bix3 are related to its inducing activity, which differs from that of Bix1; one way to address this question might be to compare the inducing activities of Bix1 and Bix1(T384A) or those of chimeric proteins in which the C-terminal regions of Bix1 and Bix3 are swapped.

Depletion of Bix3 causes apoptosis

The overexpression experiments described in this paper are complemented by experiments using antisense morpholino oligonucleotides, which indicate that depletion of Bix3 protein also causes apoptosis (Figs 6, 7). TUNEL staining revealed that apoptosis occurred in dorsal tissue and in the endodermal mass, both of which are derived from *Bix3*-expressing cells, and also in the neural plate, at least some of which may derive from *Bix3*-expressing cells (see Keller, 1975). Bix3 depletion had no effect on patterning of the early embryo; the spatial expression patterns of three neural markers were normal, although their levels were low. These experiments, together with those described above, indicate that one function of Bix3 during normal development is to protect cells from apoptosis, but that too much Bix3 is not a good thing, because this also causes cells to die.

This dose-dependent effect of *Bix3* is reminiscent of the dose-dependent effects of *Fgf8* in regulating apoptosis in the developing forebrain (Storm et al., 2003). Complete absence of *Fgf8*, or higher-than-normal levels of transcription, both increase apoptosis, whereas a reduction from normal levels has the opposite effect. This phenomenon is not completely understood, but the authors suggest a mechanism in which cell survival requires FGF8 (which is why apoptosis is high in the absence of signalling) but that FGF8 also induces, in a concentration-dependent manner, inhibitors of cell survival (which is why high concentrations of FGF8 also increase cell death). The regulation of apoptosis by Bix3 may involve a similar mechanism, and we are searching for targets of this transcription factor that may function in such a scheme.

The molecular basis of induction of apoptosis

The observation that Bix1 differs from Bix3 in its ability to cause apoptosis offers an opportunity to study the molecular basis of the phenomenon, and our results suggest that induction of apoptosis requires DNA binding but not transcriptional activation (Fig. 8, Fig. 9B). Use of a series of deletion constructs, chimeras and point mutations (Figs 9, 10) suggests that Bix1 contains a cryptic apoptotic domain whose activity is suppressed by a short sequence at the C terminus of the protein. The corresponding region of Bix3 lacks this activity, thus rendering the protein apoptotic, and we have identified a crucial threonine residue in Bix1 that is required for suppressor activity; mutation of this amino acid to an alanine turns Bix1 into an inducer of apoptosis. We are now asking whether this residue is phosphorylated and whether it interacts with components of the cell death (or anti-cell death) apparatus.

Regulation of apoptosis during early *Xenopus* development

Little is known about the regulation of apoptosis during *Xenopus* embryogenesis, although three potential pathways have recently been identified. The first involves the methylation status of the embryo, where Stancheva and colleagues have

shown that loss of a maintenance methylase causes the onset of apoptosis (Stancheva et al., 2001). They suggest that hypomethylation alters the state of embryonic blastomeres such that apoptosis is triggered by cell differentiation. A second pathway involves signalling by Xfz8, a member of the Frizzled family of Wnt receptors, which triggers apoptosis through a pathway involving activation of c-Jun N-terminal kinases (JNKs) (Lisovsky et al., 2002).

The third pathway, which may be the most relevant to the present data, involves signalling by members of the BMP family, although, as with the other pathways, the link to apoptosis is rather poorly understood. It is possible that this pathway is linked to the Frizzled pathway described above. The first piece of evidence concerns TAK1 (TGF-β activated kinase), a MAP kinase kinase kinase (MAPKKK) whose activity is stimulated by TGF-β1 or BMP-4 (Yamaguchi et al., 1995). Overexpression of *xTAK1* in the *Xenopus* embryo causes dramatic apoptosis which can be rescued by human *BCL2* RNA, thereby revealing the ventralising activity of *xTAK1* (Shibuya et al., 1998). It seems probable that TAK1 participates in a kinase cascade culminating in the activation of JNK family members that, as described above, are involved in the regulation of apoptosis (Xia et al., 1995; Moriguchi et al., 1996; Davis, 2000).

Recent evidence suggests that TAK1 might be activated not only by TGF-β family members, but also by non-canonical Wnt signalling (Ishitani et al., 2003). However, further evidence implicating BMP signalling in the regulation of cell death comes from the observation that depletion of maternal Smad8 in the *Xenopus* embryo also causes apoptosis (Miyanaga et al., 2002). Smad8, similar to Smads 1 and 5, is thought to mediate BMP signalling, yet other means of interfering with BMP function have not been reported to cause apoptosis in *Xenopus* (see Miyanaga et al., 2002). Interestingly, the effect of depleting the embryo of maternal Smad8 can be rescued with Smad8 but not by Smad1, suggesting that different Smads of the 1/5/8 family have different functions (Miyanaga et al., 2002).

Why should depletion of Smad8 give the same phenotype as overexpression of TAK1? It is possible, of course, that the two pathways are completely independent, but another idea is that cell survival in the embryo requires a balance of signalling through the two pathways, and that disrupting, or enhancing, one of them changes the extent of signal through the other. We are investigating this possibility.

Bix3 may be involved in this pathway as a gene that is regulated by BMP signalling. *Bix1* is known to be activated by BMP-4 (Tada et al., 1998), and the same may be true of *Bix3*. If so, *Bix3* may not be the only apoptotic transcription factor, for overexpression of *Xenopus Fli*, a member of the ETS family, also causes apoptosis and its apoptotic effects can be reduced by a dominant-negative BMP receptor (Goltzené et al., 2000). The interactions between different BMP signalling pathways and genes such as *Bix3* and *XFli* require further study.

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