Transcription Factor AP-2 Is an Essential and Direct Regulator of Epidermal Development in Xenopus

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Expression of the Xenopus homolog of the mammalian transcription factor AP-2/\(H9251\) (XAP-2) is activated throughout the animal hemisphere shortly after the midblastula transition, and becomes restricted to prospective epidermis by the end of gastrulation, under the control of BMP signal modulation. Elevated expression in the future neural crest region begins at this time. Ectopic expression of XAP-2 can restore transcription of epidermal genes in neuralized ectoderm, both in ectodermal explants and in the intact embryo. Likewise, loss of XAP-2 function, accomplished by injection of antisense oligonucleotides or by overexpression of antimorphic XAP-2 derivatives, leads to loss of epidermal and gain of neural gene expression. These treatments also result in gastrulation failure. Thus, AP-2 is a critical regulator of ectodermal determination that is required for normal epidermal development and morphogenesis in the frog embryo. © 2002 Elsevier Science (USA)

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INTRODUCTION

The ancestral form of the transcription factor AP-2 presumably arose early in animal evolution, because recognizable homologs are now present in vertebrate and insect phyla (Bauer et al., 1998; Monge and Mitchell, 1998). Homologs have not been reported in yeast or plant genomes. In mammals, there are three unlinked AP-2 genes, AP-2/\(\alpha\), AP-2/\(\beta\) (the originally identified gene; Mitchell et al., 1987), and AP-2/\(\gamma\) (initially named AP2.2 in mouse; Chazaud et al., 1996). These share extensive protein sequence identity (70% α-β, 60% α-γ, and 57% β-γ for mouse), with higher conservation in the activation, DNA binding and protein dimerization domains (Wankhade et al., 2000; Williams and Tjian, 1991a,b). A range of potential regulatory target genes have been identified for AP-2/\(\alpha\), most—but not all—of which appear to respond in a positive manner to this factor (Hilger-Eversheim et al., 2000). Among these putative targets are several genes specifically expressed in epidermal cells, including some encoding epidermal keratins (Sinha et al., 2000). Promoter element mapping experiments conducted in our laboratory using transient DNA expression assays in Xenopus embryos lead to the conclusion that an AP-2 protein closely related to AP-2/\(\alpha\) was an essential factor in the ectoderm-specific expression of the XK81A1 gene, which encodes an embryonic type I keratin that is an early marker for ventral/epidermal specification in this species (Jonas et al., 1985; Snape et al., 1991; Winning et al., 1991). More recently, in situ hybridization of AP-2 probes to mutant and wild-type zebrafish embryos showed that this gene is ventrally expressed in early fish embryos, and regulated by the bone morphogenetic protein (BMP) signal modulation pathway (Nguyen et al., 1998). The earliest reported expression of AP-2 in mammalian embryos is in extraembryonic cell types, which have no direct equivalent in frog or fish embryos (Mitchell et al., 1991), and gene targeting experiments in mouse have not revealed developmental functions before neurulation for AP-2/\(\alpha\) or AP-2/\(\beta\) genes (Hilger-Eversheim et al., 2000). However, the studies in fish and frogs suggested that AP-2 could potentially mediate downstream effects of BMP signaling in gastrula stages. In Xenopus embryonic ectoderm, disruption of BMP signaling results in the transfiguring of cells otherwise destined to be epidermis into anterior neural plate tissue (Sasai, 2001). BMP antagonists secreted by cells in the organizer region are thought to function as neural inducers via this mechanism in amphib-
ians (Harland, 2000), while a somewhat different situation obtains in amniote embryos (Streat and Stern, 1999). The partitioning of ectoderm into its principal derivatives—epidermis, neural plate and neural crest—is an important aspect of early development, and the regulatory factors that mediate the attendant changes in structural gene activity are of considerable interest. We have examined the regulation and function of AP-2 in ectodermal differentiation in Xenopus, and find that AP-2 expression depends on BMP signaling, that this factor is essential for epidermis-specific gene expression, and that AP-2 function is necessary for gastrulation.

MATERIALS AND METHODS

XAP-2 Plasmid Constructions

For expression of full-length XAP-2, an open reading frame (ORF) from the original cDNA clone (Winning et al., 1991) was subcloned by PCR as a CiaI-Stul fragment into pC7S, a derivative of pCS2+ (Turner and Weintraub, 1994) containing dual SP6 and T7 promoters (Feledy et al., 1999), with an optimal translation initiation context (ATCGATAACATG; Koiz, 1987). A deletion derivative of this plasmid, ΔAD, was generated by PCR, using the same vector, with two pairs of primers (Forward: TCT CAC ACC CCC AAT CTA CCA CAT CAA CTT, Reverse: AAG TTG ATG TGG TAG GGG GGT GTG AGA) that result in the removal of amino acids 40–102, corresponding to the mammalian AP-2 activation domain (Williams and Tjian, 1993a), and a construct encoding the Drosophila engrailed repressor domain fused to the N-terminus of ΔAD (ENr-ΔAD) was prepared by ligating a PCR-generated fragment, with XhoI (5’) and Xbal (3’) restriction sites (Forward: AAA CTC GAG CAT GGG GGA ATG GCA GGA TCG. Reverse: GCC TCT AGA GCT TTC GGT GTT TCT CAT CTT), into ENG-NpCS2 (a gift from D. Kessler). A fusion of XAP-2 to the C-terminus of the ligand binding domain of human glucocorticoid receptor (GRXAP-2) was generated by combining the XAP-2 ORF (5’ end: filled-in BamHI, 3’ end: XhoI) with amino acid residues 512–778 (Stul-Xhol digestion) of the human glucocorticoid receptor (Kolm and Sive, 1995) cloned in pCS2+. For the silent point-mutated XAP-2 used as a control in antisense experiments (XAP-2*), substitutions were made by PCR, changing 281-ACAGCACCCAGGT-293 to TCAACATCCTGGA. This alters five nucleotides but does not change the amino acid sequence. These modifications resulted in an RNA that was efficiently translated in vitro, but no longer significantly cleaved in vitro or in vivo by combined activities of ASO281 and Runx2 (data not shown). All PCR-generated constructs were verified by DNA sequence analysis.

Embryo Manipulation and in Situ Hybridization

Embryos were obtained from adult Xenopus laevis by hormone-induced egg laying and artificial fertilization using standard methods, and staged according to Nieuwkoop and Faber (1967). Full-length capped transcripts encoding chordin, XAP-2, XAP-2*, Enr-ΔAD, GRXAP-2, type I keratin XK81 and type II keratin XK76 were generated using an SP6 mMessage Machine kit (Ambion Inc, Austin, TX). All synthetic mRNAs were checked for size by denaturing agarose gel electrophoresis, and tested and determined to be approximately equally efficient templates for protein synthesis as shown by in vitro translation using a rabbit reticulocyte kit from Promega (Madison, WI; data not shown). Following microinjection, embryos were cultured in 3% Ficoll-400/1X MMR until sibling embryos reached stage 7. For ectodermal explants, approximately 2/3 of the pigmented ectoderm was dissected from stage 7–8 embryos and cultured in 0.3X MMR until sibling embryos reached the desired stage. Embryos were dissociated with calcium, magnesium-free medium as described previously (Sargent et al., 1986). For experiments with GRXAP-2, fertilized eggs injected with RNAs encoding chordin (500 pg) and GRXAP-2 (1 ng) were treated with 10 μg/ml cycloheximide and 10 μm dexamethasone as described by Gammill and Sieve (1997).

Whole-mount in situ hybridization was carried out according to Harland (1991), with some modifications (Luo et al., 2001). Antisense probes labeled with digoxigenin or fluorescein were synthesized using an in vitro transcription kit (Roche, Diagnostics, Indianapolis, IN). For double in situ, digoxigenin-labeled XAP-2 probe was stained with BM-purple (Roche Diagnostics, Indianapolis, IN), and fluorescein-labeled chordin probe with Magenta-Phos (5-bromo-6-chloro-3-indolyl-phosphate 4-toluidine salt; Biosynth International Inc., Naperville, IL).

Northern and Southern Blots

RNAs were analyzed using denaturing methylmercury hydroxide RNA gels as described previously (Sargent et al., 1986). Northern blots were washed in 0.2X SSPE at 65°C. Probes for XK81 (Jonas et al., 1985), XK76 (Jamrich et al., 1987), Dlx5 (Feledy et al., 1999), Dlx6 (Luo et al., 2001), Mesx (Su et al., 1991), Otx2 (Panouse et al., 1995), Zic-r1 (Mizuseki et al., 1998), Zic3 (Nakata et al., 1997), and Efi (Krieg et al., 1989) were labeled with 32P-dCTP by primer extension (Life Technologies, Inc., Gaithersburg, MD).

Antisense Oligonucleotide Injections

Two different approaches to antisense oligonucleotide inhibition were attempted. Initial attempts used morpholino oligonucleotides (GeneTools, Corvallis, OR). Embryos injected with these oligonucleotides showed none of the characteristic molecular or phenotypic defects we observed using dominant negative AP-2 mRNA injections. A second approach used antisense oligonucleotides where many of the bridging phosphodiester linkages were modified with diethylthlenediamine (Dagle et al., 2000). The target site in the AP-2 mRNA was selected in a two step process. The optimal folding of the AP-2 mRNA sequence was determined using the mFold algorithm of M. Zuker (http://bioinfo.math.rpi.edu/~mfold/rna/form1.cgi; Zuker et al., 1999; Mathews et al., 1999). Four different sites were selected for testing, including one site that spanned the translational start site, one all loop, one including a loop and an adjacent stem, and one all stem site. Unmodified oligonucleotides complementary to the selected sites were injected into fertilized eggs that has been previously injected with in vitro synthesized AP-2 mRNA. In this assay, the efficiency of oligonucleotide mediated cleavage of the AP-2 mRNA ranged from nearly complete (with target sites predicted to have all loop or loop and an adjacent stem) to moderate (all stem target site) to nearly none (the AUG region of the mRNA). We note that comparing these data, and target site selection screening of other mRNAs we have analyzed have not yet found a strong correlation between predicted structure and efficacy of target site. One of the better sites, starting at position 281 was used to design the following antisense oligo: G +
Early expression of AP-2 in Xenopus

Of the three mammalian AP-2 genes, AP-2α is the most homologous to XAP-2 (92% identity vs. 67% for β and 58% for γ). Using low-stringency Southern and Northern blot analysis with murine AP-2 probes we determined that while there is some evidence for sequences corresponding to β and γ AP-2 isoforms in the Xenopus genome, only AP-2α transcripts could be detected through tailbud stages (data not shown). Consequently analysis of AP-2 function in Xenopus development, at least at early stages, may be limited to a single gene (i.e. XAP-2).

The XAP-2 gene has been previously shown by Northern blot analysis to be expressed in the early embryonic ectoderm (Winning et al., 1991), and by whole-mount in situ hybridization studies to be active in early ventral ectoderm, and dependent upon bmp2b/swirl gene function in zebrafish (Nguyen et al., 1998). Fig. 1 shows whole-mount in situ hybridizations of Xenopus AP-2 (XAP-2) probes to early and late gastrula stage frog embryos. XAP-2 RNA is readily detectable by this method at the beginning of gastrulation and is present throughout the ectoderm. By late gastrula (Fig. 1B) XAP-2 RNA has been cleared from prospective neural plate, and is just beginning to accumulate in areas corresponding to future neural crest cells.

The spatiotemporal expression pattern of XAP-2 suggested that as in zebrafish, this gene is a target of BMP signaling in the Xenopus embryo. To test this, ectoderm was explanted from embryos injected with RNA encoding the BMP antagonist chordin (Sasai et al., 1994). XAP-2 expression in ectoderm isolated from such embryos was strongly inhibited, supporting the conclusion that at the earliest stages, this gene depends on BMP signaling in Xenopus (Fig. 2A). Several ventrally expressed genes have been shown to similarly depend on BMP in this organism. Some, such as Msx1 (Suzuki et al., 1997) and XOM/XVENT2 (Trindade et al., 1999) can be activated by BMP in the absence of protein synthesis, and have thus been considered to represent an “immediate early” response to BMP in this context. Other genes, including Dlx3 (Federy et al., 1999) and Dlx5 (Luo et al., 2001) also require BMP signals for expression, but will not respond to BMP treatment in the presence of cycloheximide, indicating a requirement for other zygotically supplied factors. As shown in Fig. 2B, XAP-2 falls into this latter category. In this experiment, embryos were cultured and dissociated in medium lacking divalent cations, a procedure that inhibits signaling by endogenous factors including BMPs (Sargent et al., 1986; Wilson and Hemmati-Brivanlou, 1995). This procedure prevents activation of AP-2, which can be restored by adding recombinant BMP-4 to the medium. However, this recovery is blocked by prior addition of cycloheximide, whereas activation of Msx1, which behaves as an immediate early response, is not prevented.

**RESULTS**

Early Expression of AP-2 in Xenopus

In an earlier study from this laboratory (Snape et al., 1991), expression of an epidermal type I keratin gene (XK81; Jonas et al., 1985) in transient DNA injection experiments was shown to depend upon a specific AP-2 binding site located at –157 in the XK81 promoter region. However, the ability of this factor to regulate the endogenous XK81 gene, or other epidermal genes, has not been directly assessed. To examine this question, we carried out rescue experiments in which epidermal fate was suppressed in ectodermal explants by injection of chordin RNA, and co-injection of XAP-2 RNA was tested for its ability to restore epidermal gene expression. As shown in Fig. 3, under conditions in which BMP signaling is strongly suppressed (Luo et al., 2001) XAP-2 is able to activate expression of at least two keratin genes, XK81 and XK76, a type II epidermal keratin essentially identical to XIKERB2 (Genbank Accession X02895; Hoffmann et al., 1985). An epidermal homeodomain gene, Dlx5, was also activated. The degree of activation varied, but in no case was expression returned to control levels. In contrast, the homeodomain gene Msx1 was not detectably reactivated by XAP-2 overexpression in these assays. Nor were neural plate marker genes, such as Zic-r1 (Mizuseki et al., 1998), repressed. Similar results were obtained with intact embryos by ectopic overexpression of XAP-2 in the neural plate (data not shown).

The presence of a binding site for AP-2 in the XK81 promoter region (Snape et al., 1990, 1991), in conjunction
with the results just described, supports a direct physical interaction between XAP-2 and the XK81 gene. Such direct interaction can be demonstrated by using an inducible fusion protein comprising XAP-2 and the ligand binding region of the human glucocorticoid receptor protein (Kolm and Sive, 1995), in conjunction with cycloheximide treatment to preclude postactivation protein synthesis. The results of such an experiment are shown in Fig. 4A. Fertilized eggs were injected with RNAs encoding chordin, to inhibit keratin gene expression, and the GRXAP-2 fusion. Ectodermal explants were removed at mid to late blastula, then divided into two groups, one of which was incubated in 10 \( \mu \)g/ml cycloheximide, which inhibits at least 95% of protein synthesis (Suzuki et al., 1997; data not shown). Subsequently, each group was split again, and half treated with 10 \( \mu \)M dexamethasone (DEX) to activate the GRXAP-2 factor. Following culture to the equivalent of stage 12 (mid/late gastrula), RNA was isolated and analyzed by northern blotting. Keratin gene XK81 was clearly activated in a DEX-dependent manner, in the presence or absence of cycloheximide. As a control for general metabolic inhibition due to the protein synthesis blockade, XK81 gene activation was normalized to the expression of the translational elongation factor gene EF1\( \alpha \), and the result of this calculation is shown in Fig. 4B. After making this correction, the activation of XK81 by XAP-2 is comparable in magnitude with or without protein synthesis following induction by DEX. Therefore we conclude that XAP-2 binds directly to the XK81 gene in vivo, resulting in its transcription in epidermal cells.

Embryonic ectoderm is receptive to numerous inductive signals during gastrulation. This receptiveness is not constant with time, however, such that by the end of gastrulation, ectoderm is no longer competent to respond to mesodermal or neural induction (Servetnick and Grainger, 1991). To determine if the XK81 gene exhibits time-dependent changes in sensitivity to XAP-2, another experiment using

FIG. 2. BMP dependence of XAP-2 expression. (A) Effect of chordin expression. Fertilized eggs were injected with 250 pg of RNA encoding chordin, then ectodermal explants were dissected at stage 7/8 and cultured until sibling embryos reached late gastrula (st12.5). RNA was extracted and probed by Northern blot using cDNAs for XAP-2, Zic-r1, or EF1\( \alpha \) as a control. Chordin injection inhibited XAP-2 and simultaneously induced expression of the neural plate marker Zic-r1. (B) Requirement for protein synthesis. Embryos were cultured in calcium/magnesium-free medium beginning at early cleavage stage (16/32-cell), and vitelline envelopes removed to facilitate continuous dissociation and dispersion, a procedure which blocks cell-cell communication (Sargent et al., 1986). Dispersed cells were divided into four pools. Protein synthesis was inhibited in two of the pools by treatment in 10 \( \mu \)g/ml cycloheximide beginning at the equivalent of stage 7/8 (CHX+). After 15 min, 50 ng/ml recombinant human BMP4 protein (Research Diagnostics, Inc., Flanders, NJ) was added to two pools (BMP+), Ca\(^{2+}\) and Mg\(^{2+}\) restored to 1 mM each, and the cells cultured until stage 11. RNA was prepared and analyzed by Northern blot, using probes for XAP-2, Dlx3, Msx1, and EF1\( \alpha \) as a control for general inhibition of protein synthesis by the CHX treatment. XAP-2 RNA induction by BMP was inhibited by CHX treatment, indicating indirect activation by this signaling pathway.

FIG. 3. Rescue of keratin expression in neuralized ectoderm by XAP-2. Fertilized eggs were injected with 250 pg RNA encoding chordin, with or without 100 pg RNA encoding XAP-2. Ectodermal explants were isolated at stage 7–8 and cultured until sibling embryos reached stage 13, then samples were processed for Northern blot analysis. Probes were for type I (XK81) and type II (XK76) embryo-specific epidermal keratins, two epidermal homeodomain genes, Dlx5 and Msx1, and neural-plate (Zic-r1) or EF1\( \alpha \). Chordin strongly inhibited all epidermal gene expression. The keratin genes and Dlx5 were partially restored by addition of XAP-2 RNA. Msx1, however, was not activated, nor was repression of Zic-r1 observed. Note that under these conditions endogenous XAP-2 expression was inhibited, as shown in Fig. 2A and B.
the GRXAP-2 was carried out. In this case, embryos were injected with RNAs encoding chordin and this fusion protein, ectodermal explants excised at blastula stages, and then treated with DEX beginning at different times ranging...
from the mid blastula transition (stage 8.5) to early neurula (stage 13). Following culture in DEX to tailbud (stage 20), RNA was isolated and analyzed by Northern blot. As shown in Fig. 4 (panels C, D), XK81 gene expression was activated most effectively when DEX was added at the earliest stage, but this response declined rapidly, falling roughly twofold by early gastrula (stage 10.25) and was essentially absent by the end of gastrulation. This reduction was not due to degradation of the injected AP-2 RNA; which were approximately equal in all samples, and the period allowed for transcription of keratin RNA was long relative to the interval between the first and last DEX addition. Therefore the positive regulation of XK81 gene expression by XAP-2 is under some form of inhibitory competence control that begins to be imposed at the very beginning of gastrulation and is essentially complete within a few hours.

**XAP-2 Is Required for Epidermal Gene Expression and Gastrulation**

The data presented above support an obligatory and positive role for XAP-2 in regulating epidermal gene expression. To further test this model, loss-of-function experiments were carried out. Two different approaches were used: Overexpression of a modified XAP-2 in which the activation domain was replaced by a repressor domain from the Drosophila engrailed protein (EnR-ΔAD), and injection of a metabolically stable antisense oligonucleotide (ASO281) that resulted in degradation of endogenous XAP-2 mRNA. Both of these manipulations would be expected to interfere with XAP-2 function in vivo, via different mechanisms. As shown in Fig. 5, expression of several epidermal genes was inhibited in animal cap experiments using these reagents. In general, the engrailed fusion resulted in more pronounced effects; both keratins and two different epidermal localized homeodomain factors (Dlx5 and Msx1) were repressed, and there was a concomitant activation of neural plate marker gene expression (Fig. 5, left panels). ASO281 injection resulted in significant but incomplete destruction of endogenous XAP-2 RNA, and substantially reduced expression of epidermal keratin genes. Dlx5 and Msx1 were not appreciably affected, however, nor were neural marker genes activated. An important advantage of the antisense approach is that the effect can be rescued by co-injection of a mutated XAP-2 that is refractory to cleavage (XAP-2*), ruling out nonspecific effects. The general conclusion from these experiments was that XAP-2 function is required for epidermal identity, with varying degrees of sensitivity for different genetic targets. Complete loss of AP-2 function resulted in neuralization, while partial loss of function had preferential effects on targets such as keratin genes, presumably reflecting a higher sensitivity to XAP-2 levels.

To determine if loss of XAP-2 function has effects on morphogenesis, we carried out similar experiments on whole embryos. Embryos at the 8-cell stage were injected radially in the animal quartet with at total of 600 pg of ASO281, alone or combined with either 100 pg of XAP-2* as a rescue control, or with 50 pg each of synthetic mRNAs for XK81 and XK76, and cultured until sibling embryos had reached stage 20. Typical results are shown in Fig. 6. ASO281 treatment resulted in highly abnormal development, with complete penetrance: the ectoderm collapsed into a small sac. Co-injection of XAP-2* RNA rescued essentially normal development in about 12/31 of embryos, partially rescued another 9/31, and had little effect on the remainder. An example of each is shown in panel B. Keratin mRNA injection had little if effect; 24/27 were essentially identical to embryos injected with ASO281 alone, although the remaining three showed some a more moderate phenotype. One of the three partially normal embryos is shown in panel C (embryo on the right).
for gastrulation in Xenopus, probably functioning as a regulator of ectodermal morphogenesis.

DISCUSSION

Molecular and genetic analysis of the BMP signaling pathway have revealed several steps in the transduction of such signals, including complex antagonism of BMP ligand binding, the nature of the BMP receptors, cytoplasmic transduction by SMAD factors, and interaction with nuclear factors (for recent reviews see Altmann and Brivanlou, 2001; Sasai, 2001). Some directly and indirectly responsive genes have also been identified, but the transcriptional response to BMP signaling, and how this leads to differentiation of ventral cell types, is still mostly unknown. One way to identify regulatory components of this pathway is to perform functional mapping of the control regions of BMP-dependent genes, and use this information to identify transcription factors that mediate this regulation. Using such an approach, the transcription factor AP-2 was identified as an important element in the regulation of the XK81A1 gene, which encodes the major type I cytkeratin expressed in embryonic Xenopus epidermis (Jonas et al., 1985). AP-2 was subsequently shown by studies on zebrafish BMP signaling mutants to be under the control of this pathway in the fish embryo (Nguyen et al., 1998), and has been implicated in the regulation of a diverse array of target genes, including several expressed in epidermis (Hilger-Eversheim et al., 2000). In this report we present evidence from gain and loss of function experiments indicating that AP-2 is a critical transcriptional component of BMP-based specification in Xenopus ectoderm.

Gene-targeted mice lacking functional AP-2 die perinatally with numerous morphological defects including failure of cranial, neural tube and abdominal wall closure (Schorle et al., 1996; Zhang et al., 1996). It is possible that the molecular basis of this phenotype is related to the gastrulation failure we observed in Xenopus embryos with compromised AP-2 function, perhaps reflecting a role for this factor in regulating the shape or migration of epidermal cells during gastrulation and neurulation. On the other hand, the strict dependence of keratin gene expression on XAP-2 function in the frog embryo contrasts with the situation in the AP-2 null mouse, where, at least on the dorsal side, stratified epidermis exhibited essentially normal histology (Talbot et al., 1999). Some alterations in keratin gene expression in such null mice have been reported (Maytin et al., 1999), but nothing as severe as the blockade to type I and type II keratins described here. It is possible that the differences between frog and mouse are due in part to functional redundancy in the AP-2 gene family, since the β and γ genes, although possibly present in the Xenopus genome, do not appear to be expressed in the gastrula stage frog embryo. Alternatively, in view of the fact that they are embryo-specific (Jonas et al., 1985), XK81 and XK76 may not be true homologs of keratin genes expressed in stratifying mouse epidermis, and therefore may be regulated differently. In any case, it is clear that AP-2 is important in development from insect to mammalian phyla, and the direct interaction between this factor and the XK81 keratin gene in Xenopus should provide a useful model for other AP-2 targets, some of which may play regulatory roles in morphogenesis.

Another interesting feature of AP-2 function in Xenopus is the loss of competence of neuralized tissue to respond to this factor. This competence loss is quite rapid, declining rapidly during gastrulation. This loss suggests that some change takes place in ectodermal cells during this interval which prevents AP-2 from interacting with genomic target sequences. The molecular mechanism for this inhibition is unknown, but one possibility is that a required co-factor for XAP-2 might decay during gastrulation, rendering the over-expressed XAP-2 protein nonfunctional. This seems unlikely, as XAP-2 is expressed in epidermis, as well as numerous other tissues, throughout the life of vertebrates. A more plausible alternative is remodeling of chromatin in the vicinity of the XK81 gene. AP-2 has been shown to associate with hypersensitive sites upstream from the mammalian K14 genes (Sinha et al., 2000). Since the XK81 is closely related in sequence to mammalian K14, something similar could be taking place in Xenopus embryonic epidermis. Developmental alterations in chromatin configuration in the upstream regulatory region of the XK81 gene (Snaple et al., 1990, 1991) could result in the physical exclusion of AP-2. Xenopus ectoderm loses responsiveness to different inducers at different times, with competence for mesoderm induction declining first, followed by that for neural induction (Servetnick and Grainger, 1991). It is interesting that the initial drop in response of the epidermal keratin gene has occurred by the earliest stage of gastrulation (st. 10.25), whereas the neural induction process that resulted in the repression of keratin remains possible until mid/late gastrula. This suggests that epidermal repression, possibly at a chromatin level, is among the earliest steps in neural induction.

An argument supporting a central role for AP-2 in epidermis derives from the activation of neural marker genes by EnR-ΔAD; this neuralization is comparable to what happens when BMP signaling is disrupted, suggesting that most of the downstream response to this inducer is dependent upon XAP-2. It is interesting that the EnR-ΔAD effect includes repression of Msx1; activation of Msx1 by exogenous BMPs can occur in the presence of cycloheximide added before the midblastula transition, indicating that zygotic factors, including XAP-2, are not needed (Suzuki et al., 1997). One model to explain the inhibition of Msx1 expression by EnR-ΔAD suggests that XAP-2 may be necessary for maintenance of the epidermal differentiated state once this has been triggered by BMP signaling. Thus, Msx1 (and other immediate response genes) may be activated by BMP signaling, but in the absence of XAP-2 function the epidermal program fails, and the ectoderm reverts to anterior neural fate. This interpretation would be consistent
with the observation that ectopic XAP-2 can restore keratin and Dlx5 gene expression in neuralized ectoderm, but not Msx1 expression, and that this occurs without concomitant repression of neural genes. The gastrulation failure in embryos injected with AP-2 antagonists also implies more global functions of AP-2 in the early embryo. While keratin gene expression was preferentially affected by the ASO treatment in animal cap assays, the morphological phenotype was probably not due simply to mechanical defects in epidermal cells resulting from loss of the principal cytoskeletal proteins; injection of mRNAs encoding type I and type II keratins failed to rescue normal development, and an identical morphological phenotype was obtained by the more potent antagonist EnR-3AD (data not shown).

Thus, the constellation of XAP-2 target genes may encode a range of epidermal proteins, both structural and possibly also additional regulators, for example, factors controlling morphogenetic movements in gastrula ectoderm. Identification of such targets will be an important goal of future research. It will also be very interesting to examine the role of AP-2 in neural crest, where this factor is intensely expressed following gastrulation. Approaches similar to those used here may be applicable to such questions.

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