Epidermal induction and inhibition of neural fate by translation initiation factor 4AIII

Daniel C. Weinstein, Eric Honoré* and Ali Hemmati-Brivanlou†

Department of Vertebrate Embryology, The Rockefeller University, 1230 York Avenue, New York, New York, 10021, USA
*Current address: IPMC-CNRS, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France
†Author for correspondence (e-mail: brvlou@rockvax.rockefeller.edu)

INTRODUCTION

During vertebrate gastrulation, the cells of the dorsal ectoderm give rise to the central nervous system; ventral ectoderm differentiates into epidermis. Over the past two years, a large body of evidence has come to support a ‘default’ model of neural induction (see Weinstein and Hemmati-Brivanlou, 1997). This model argues that the differentiation of epidermis requires inductive signals, while the neuralization of the dorsal ectoderm requires only an inhibition of this signaling. Recent work from several labs suggests that BMP-4, a ligand of the Transforming Growth Factor-

Bone Morphogenetic Protein-4 (BMP-4) is a potent epidermal inducer and inhibitor of neural fate. We have used differential screening to identify genes involved in epidermal induction downstream of BMP-4 and report here evidence of a novel translational mechanism that regulates the division of the vertebrate ectoderm into regions of neural and epidermal fate. In dissociated Xenopus ectoderm, addition of ectopic BMP-4 leads to an increase in the expression of translation initiation factor 4AIII (eIF-4AIII), a divergent member of the eIF-4A gene family until now characterized only in plants. In the gastrula embryo, Xenopus eIF-4AIII (XeIF-4AIII) expression is elevated in the ventral ectoderm, a site of active BMP signal transduction. Moreover, overexpression of XeIF-4AIII induces epidermis in dissociated cells that would otherwise adopt a neural fate, mimicking the effects of BMP-4. Epidermal induction by XeIF-4AIII requires both an active BMP signaling pathway and an extracellular intermediate. Our results suggest that XeIF-4AIII can regulate changes in cell fate through selective mRNA translation. We propose that BMPs and XeIF-4AIII interact through a positive feedback loop in the ventral ectoderm of the vertebrate gastrula.

Key words: epidermal induction, neural induction, translation, XeIF-4AIII, Xenopus laevis
MATERIALS AND METHODS

Isolation of XeIF-4AIII

20 animal caps explants were dissociated and cultured for 4 hours in the presence or absence of 100 ng/ml BMP-4 (Genetics Institute), as described in Wilson and Hemmati-Brivanlou (1995). Parallel cultures were incubated until control embryos reached stage 19, and assayed for the expression of neural and epidermal markers, with results as expected (data not shown). RNA was extracted as previously described (Wilson and Hemmati-Brivanlou, 1995). One-twentieth of this RNA was used as source material for a subtractive screen. Library construction and screening was performed using a modified protocol originally described in Dulac and Axel (1995). Briefly, RNA was derived from cells cultured in the presence or absence of BMP-4. Reverse transcription and Polymerase Chain Reaction (PCR) was used to generate first-strand cDNAs, under conditions favoring uniformly sized molecules. cDNA libraries were constructed from the two pools and plated at 500 plaques/plate. Two radiolabelled probes were made from the remainder of the PCR reactions and used to screen one set of lifts from each library. A total of 5,000 plaques from each pool were screened. Ten clones were isolated that hybridized more strongly to radiolabelled probes generated from the BMP-4 pool. Two of ten cDNAs showed high sequence identity to distinct regions of Nicotiana plumbaginifolia eIF-4A3 (Owttrim et al., 1991). We used these partial cDNAs as probes in subsequent library screens to isolate a full-length cDNA.

RT-PCR

RT-PCR was performed as described in Wilson and Hemmati-Brivanlou (1995). Primers constructed for this study are as follows:

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>XeIF-4AIII: U</td>
<td>5'-GGGGTTTGGCCTAATACTTTTGTTC-3'</td>
</tr>
<tr>
<td>D</td>
<td>5'-CCTGGCAGCATGAAATCTCTGG-3'</td>
</tr>
<tr>
<td>cSRC</td>
<td>5'-GAgTCGCGAAGCTGATTTGCG-3'</td>
</tr>
<tr>
<td>D</td>
<td>5'-TGGAA TGTAGCCAGTCTGCC-3'</td>
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All other primer sequences are listed in Hemmati-Brivanlou and Melton (1994), Hemmati-Brivanlou et al. (1994) and Wilson and Hemmati-Brivanlou (1995). All reactions were performed for 25 cycles except for EF1-a, muscle actin and XeIF-4AIII, which were performed for 21 cycles.

RESULTS

Isolation of Xenopus eIF-4AIII

Ectodermal (‘animal cap’) explants form epidermis when cultured intact; these explants neutralize if subjected to prolonged dissociation (Grunz and Tacke, 1989; Godsaver and Slack, 1989). Soluble BMP-4 can induce epidermis in dissociated ectoderm, substituting for the epidermal inducer presumably lost by dilution (Wilson and Hemmati-Brivanlou, 1995). To identify factors involved in epidermal induction downstream of BMP-4, we dissociated late blastula animal cap explants in the absence or presence of 4 μM BMP-4 protein. Libraries were constructed from dissociated cells cultured with or without BMP-4, using a reverse-transcription PCR-based strategy (Dulac and Axel, 1995). Ten clones whose expression was elevated in the presence of BMP-4 were selected for sequence analysis. Two of the ten showed highest homology to distinct regions of Nicotiana plumbaginifolia (tobacco) eukaryotic translation Initiation Factor 4A3 (NeIF-4A3) (Owttrim et al., 1991). We used these partial cDNAs as probes in subsequent library screens to isolate a full-length cDNA. This clone shares higher identity with the tobacco gene than with other vertebrate 4A family members (Nielson et al., 1985; Nielson and Trachsel, 1988); therefore, we refer to it as XeIF-4AIII (Fig. 1, Table 1).

In order to confirm that BMP-4 enhances XeIF-4AIII expression in dispersed ectoderm, we performed additional dissociation experiments and assayed for XeIF-4AIII expression by RT-PCR. Phosphorimaging analysis indicated that XeIF-4AIII transcripts were enriched approximately 3-fold in cells dissociated in the presence of BMP-4. (data not shown).

Distribution of XeIF-4AIII in Xenopus embryos

To analyze the expression of XeIF-4AIII during the period in which ectoderm is competent to differentiate into either neural tissue or epidermis, RT-PCR analysis was performed on embryos harvested between blastula and neural plate stages (Fig. 2). While expression can be detected at all stages assayed, XeIF-4AIII expression increases dramatically after the mid-blastula transition (MBT, stage 9.5), the initiation of zygotic transcription.

Whole-mount in situ hybridization was used to further study the expression of XeIF-4AIII at several stages of Xenopus development. mRNA was synthesized in vitro in the presence of cap analog using the mMessage mMachine kit (Ambion). The coding regions of XeIF-4AIII and XeIF-4AI (Nielson et al., 1985) were subcloned into pCS2 (Rupp et al., 1994; Turner and Weintraub, 1994). The coding regions of XeIF-4E (Klein and Melton, 1994) and the tBR construct (Suzuki et al., 1994), were in pSP64T (Krieg and Melton, 1984). RNA from all constructs was synthesized using the Sp6 promoter. Microinjection, explant dissection and dissociation cultures were performed as described in Wilson and Hemmati-Brivanlou (1995).

<table>
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<th>Table 1. Percentage identity between XeIF-4AIII and selected members of the eIF-4A gene family</th>
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<tr>
<td>XeIF-4AIII</td>
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<tr>
<td>XeIF-4AIII</td>
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<tr>
<td>MeIF-4AII</td>
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<td>MeIF-4AIII</td>
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For oocyte translation, 10 ng of RNA was injected into the marginal zone of stage VI oocytes. Oocytes were cultured, and protein was harvested and analyzed as described previously (Hemmati-Brivanlou et al., 1994).
development. At blastula and gastrula stages, XeIF-4AIII is expressed throughout the animal pole and marginal zone (data not shown). RT-PCR analysis revealed that XeIF-4AIII expression was enriched in the ventral versus dorsal animal pole at gastrula stages (see below). By early neural plate stages, XeIF-4AIII expression begins to show a more restricted pattern of expression. Transcript is relatively abundant around the remnant of the blastopore and is also enriched in the anterior neural plate (Fig. 3A). While the posterior domain of XeIF-4AIII is transient, anterior neural expression is maintained following closure of the neural tube (Fig. 3B). XeIF-4AIII expression persists in anterior neural structures throughout neurula stages. At late neurula stages, staining can be seen throughout the developing brain, to the approximate caudal boundary of the otic vesicle (Fig. 3C-F). Diffuse expression is also observed throughout the craniofacial structures. Finally, we observe a curious, mid-trunk domain of XeIF-4AIII expression during early tailbud stages (Fig. 3C,D). This expression is first visible at stages 23-24, and is gone by stage 27. In section, this stain appears to be concentrated in lateral regions of the somites (data not shown).

XeIF-4AIII expression is elevated in the ventral ectoderm

In the ectoderm of the Xenopus gastrula, BMP signaling, and consequently epidermal induction, is thought to occur on the ventral side only; in the dorsal ectoderm, BMP signaling is inhibited by secreted factors from Spemann’s organizer (see Weinstein and Hemmati-Brivanlou, 1997). In order to more precisely examine the localization of XeIF-4AIII expression in the ectoderm during gastrula stages, we isolated midgastrula dorsal and ventral ectoderm explants (Fig. 4A), and assayed for XeIF-4AIII expression by RT-PCR (Fig. 4B). Epidermal keratin, a marker of epidermis, was detected in the ventral explants only, as expected (Jonas et al., 1985). While XeIF-4AIII is expressed throughout the midgastrula ectoderm, phosphorimaging data indicated that XeIF-4AIII transcripts are approximately 2-fold more abundant in the ventral explants (Fig. 4B and data not shown). This result is consistent with our data from dissociated cells: active BMP signaling correlates...
with elevated levels of XeIF-4AIII transcript, both in cell culture and in vivo.

**XeIF-4AIII can induce epidermis in dissociated ectoderm**

In order to examine the function of XeIF-4AIII, dissociated cell assays were performed on embryos injected with synthetic XeIF-4AIII RNA at early cleavage stages. Cells were reaggregated at midgastrula stages and cultured until midneurula stages. RNA was extracted and assayed for the expression of cell-type-specific molecular markers by RT-PCR. Cells from control embryos dissociated in this manner show strong normalization to EF1-α levels (Krieg et al., 1989).

Interestingly, another component of eIF-4F, unrelated to eIF-4A, has also been shown to affect cell fate in *Xenopus* ectoderm: eIF-4E overexpression induces mesoderm in intact caps (Klein and Melton, 1994; Fig. 5B, lane 2). Thus, it was important to determine if epidermal induction could also be mediated by other members of the translation initiation complex. This does not appear to be the case: our results indicate that the inductive capacities of XeIF-4AIII and XeIF-4E are distinct. XeIF-4E does not induce epidermal keratin in dissociated cells, (Fig. 5A, lane 2), while XeIF-4AIII does not induce mesoderm in intact animal caps (Fig. 5B, lane 1).

To further assess the specificity of XeIF-4AIII, we examined the inductive ability of a related eIF-4A molecule in the dissociated ectoderm assay. Mouse eIF-4AI (MeIF-4AI) (Nielson et al., 1985) and XeIF-4AIII were subcloned into the same expression vector, and produced similar levels of protein in a rabbit reticulocyte lysate translation system (data not shown). MeIF-4AI, however, did not induce epidermis (Fig. 5C, lane 2), nor did it block induction by XeIF-4AIII when injected at equimolar amounts (Fig. 5C, lane 3).

XeIF-4A has been extensively characterized as a component of the general translation initiation machinery. To determine if XeIF-4AIII overexpression results in a general increase in translation rates, we injected stage VI oocytes with XeIF-4AIII and cultured the cells in the presence of [35S]methionine (Fig. 5D). Exogenous XeIF-4AIII can be seen as a dark band (of approximately 50 kDa) in the cell fraction from the injected cells. Overexpression of XeIF-4AIII did not lead to an increase in the overall levels of protein synthesis; moreover, no increase was seen in the levels of any specific protein band.

**Epidermal induction by XeIF-4AIII requires extracellular signaling**

Previous work has suggested that enhanced activity of translation initiation factors preferentially leads to the translation of mRNAs with a complex secondary structure in their 5' untranslated regions (UTR) (see Sonenberg, 1996; Brown and Schreiber, 1996); thus, XeIF-4AIII could mediate the preferential translation of a factor in the epidermal induction pathway. As a first step towards identification of RNA targets for XeIF-4AIII, we performed dissociation experiments with and without subsequent reaggregation. If ectopic XeIF-4AIII mediates the increased production of an intracellular protein, the resulting epidermal induction should not be dependent on reaggregation. If, however, XeIF-4AIII overexpression leads to the increase of a secreted protein, reaggregation might be required to avoid diluting this factor to non-functional levels. In dissociated cultures harvested without reaggregation, ectopic XeIF-4AIII did not induce epidermis or inhibit the neural fate (Fig. 6, lanes 1-8). Neither neutralization nor epidermal induction, per se, requires cell-cell contact: epidermal induction by BMP-4 protein is not dependent on reaggregation (Fig. 6, compare lanes 6,14), while dissociated cells do not require reaggregation to express neural markers (Fig. 6, compare lanes 5, 13). These results suggest that XeIF-4AIII overexpression induces epidermis through an extracellular intermediate.
Our data suggest that XeIF-4AIII may induce epidermis through the preferential translation of an mRNA encoding an extracellular factor. BMP-4 has been described as a soluble epidermal inducer (Wilson and Hemmati-Brivanlou, 1995); thus, BMP-4 mRNA is a candidate target for preferential translation by XeIF-4AIII. To address this possibility, we co-injected XeIF-4AIII RNA with RNA for a truncated type I BMP receptor (tBR), shown to inhibit signaling of BMP-4 and BMP-2 (Graff et al., 1994; Suzuki et al., 1994). Overexpression of tBR neuralizes intact caps (Xu et al., 1995; Sasai et al., 1995; Suzuki et al., 1995). Dissociated cells expressing tBR are distinguishable from uninjected controls (Fig. 7, compare lanes 2, 4), but are unresponsive to addition of BMP-4 protein, again expressing only neural, and not epidermal, markers (Fig. 7, compare lanes 5, 6). Dissociated cells from animal caps injected with XeIF-4AIII express epidermal keratin (Fig. 7, lane 1). Dissociated cells that co-express XeIF-4AIII and tBR neuralize (Fig. 7, lane 3), indicating that overexpression of tBR blocks XeIF-4AIII-mediated epidermal induction. This result suggests that XeIF-4AIII requires active signaling through the wild-type BMP receptor to induce epidermis in dissociated cells.

**DISCUSSION**

In an attempt to identify factors involved in the epidermal induction pathway, we have isolated the translation initiation factor XeIF-4AIII. XeIF-4AIII transcripts are elevated in dissociated cells cultured in the presence of BMP-4, a soluble epidermal inducer; furthermore, XeIF-4AIII transcripts are elevated, albeit modestly, in the ventral ectoderm of the
gastrula, the region fated to give rise to epidermis and a site of active BMP signaling. Ectopic XeIF-4AIII acts as an epidermal inducer and neural inhibitor in dissociated ectoderm via an extracellular intermediate. This induction occurs without a concomitant general increase in protein synthesis and requires an active BMP signaling pathway.

Our results imply a positive feedback loop between a soluble epidermal inducer and XeIF-4AIII (Fig. 8). Ectopic XeIF-4AIII selectively mediates the production of a secreted factor in the epidermal induction pathway that acts, directly or indirectly, upstream of the BMP receptor. This factor may be BMP-4 or a related protein. Conversely, ectopic BMP-4 stimulates expression of XeIF-4AIII. The newly synthesized XeIF-4AIII protein may differ from preexisting molecules in the type or levels of post-transcriptional modification, or in the degree of association with putative, inhibitory 4A-binding proteins, as have been described for eIF-4E (Sonenberg, 1996). In maize, eIF-4A phosphorylation occurs during oxygen starvation and correlates with translational suppression (Webster et al., 1991). The proposed autoregulatory loop offers a means by which XeIF-4AIII could enhance the signal for epidermal induction.

Epidermal induction is inhibited in the dorsal ectoderm by direct binding of neuralizing factors in the dorsal ectoderm to BMPs (see Weinstein and Hemmati-Brivanlou, 1997). The loop would only be maintained, then, in the ventral ectoderm, sharpening the boundary between the neurogenic ectoderm and the future epidermis.

XeIF-4AIII induces epidermis via a soluble intermediate...
that signals through the endogenous BMP receptor. There are, at present, several candidates for this factor. The wild-type BMP receptor has been shown to bind both BMP-2 and BMP-4, and may bind other, related factors as well (Graff et al., 1994; Suzuki et al., 1994). BMP-4 induces epidermis in dissociated ectoderm (Wilson and Hemmati-Brivanlou, 1995; this study). BMP-2 and BMP-7 are also active in this assay (Suzuki et al., 1997). BMP-4 RNA is excluded from the cells of the dorsal blastopore lip and a small region of neighboring dorsal ectoderm, but is strongly expressed in the remaining gastrula ectoderm (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995). Both BMP-2 and BMP-7 are expressed throughout the animal pole at blastula stages, and throughout the ectoderm and marginal zone at gastrula stages (Hemmati-Brivanlou and Thomsen, 1995; Hawley et al., 1995). Thus, the expression patterns and activities of BMP-2, BMP-4 and BMP-7 are consistent with a role for any of these factors, or a combination of them, in eIF-4AIII-mediated epidermal induction.

Our data suggest that XeIF-4AIII is involved in selective translation. A similar role has been proposed for the vasa gene product in the translation of nanos RNA during Drosophila development (see Curtis et al., 1995). vasa shows homology to eIF-4A and has RNA helicase activity in vitro (Lasko and Ashburner, 1988; Hay et al., 1988; Liang et al., 1994). Other initiation factors, unrelated to eIF-4A, have also been shown to translate a subset of RNAs when overexpressed. For example, ectopic eIF-4E selectively increases the translation of activin mRNA in a Xenopus oocyte system (Klein and Melton, 1994). In similar assays, we failed to detect any increase in translation of either BMP-2 or BMP-4 mRNA when co-injected with XeIF-4AIII (data not shown). There are several simple explanations for these results: (1) our BMP constructs may lack the complete 5′ UTR with which the translation machinery interacts in vivo; (2) XeIF-4AIII may act by increasing translation of BMP-7 or other, as yet uncharacterized, epidermal inducers or (3) XeIF-4AIII may increase the production of the soluble inducer indirectly, employing molecular intermediates not present in the oocyte.

This is the first report of an animal homolog of eIF-4AIII. Two related molecules, eIF-4AI and eIF-4AII, have been reported in mammals (Nielson et al., 1985; Nielson and Sargent, 1994, 1997). The expression patterns of the two mammalian genes are distinct: the level of eIF-4AII transcript varies widely among tissues, while eIF-4AI expression is relatively uniform in all tissues examined (Nielson and Trachsel, 1988) and, recently, in Xenopus (Morgan and Sargent, 1997). The expression patterns of the two mammalian genes are distinct: the level of eIF-4AII transcript varies widely among tissues, while eIF-4AI expression is relatively uniform in all tissues examined (Nielson and Trachsel, 1988). In Xenopus, we find that eIF-4AI is not ubiquitous, but exhibits a dynamic pattern of expression during development. Xenopus eIF-4AI (XeIF-4AI) is expressed at all stages and in all tissues examined, while XeIF-4AII is expressed at high levels from stage 11.5 (Morgan and Sargent, 1997). Interestingly, XeIF-4AII expression at midgastrula stages is enriched in the dorsal versus ventral ectoderm, the converse of what we observe for XeIF-4AIII (Morgan and Sargent, 1997; this study). More surprising, Morgan and Sargent demonstrate that both eIF-4AI and eIF-4AII can induce markers of the neural plate border region in intact ectodermal explants. Cells in this region give rise to tissues that include neural crest and cement gland, fates that can be considered intermediate between neural and epidermal along the dorsoventral axis. Thus, eIF-4AII, enriched in the dorsal gastrula ectoderm, can induce dorsal fates, while eIF-4AIII is enriched in the ventral gastrula ectoderm and can induce ventral fate (epidermis) (Morgan and Sargent, 1997; this study). These results clearly point to an important role for the eIF-4A gene family in the early patterning of the vertebrate ectoderm.

Our results, together with those of Morgan and Sargent, indicate that members of the eIF-4A gene family can trigger specific developmental responses. In addition, these data suggest that different eIF-4A family members have different activities. How might related eIF-4A molecules confer distinct biological activities? eIF-4AI and 4AII share high sequence identity, and are incorporated into the eIF-4F complex with similar kinetics (Nielson and Trachsel, 1988; Yoder-Hill et al., 1993). XeIF-4AIII is clearly more divergent from 4AI and 4AII at the sequence level than the two are from each other; differences in incorporation kinetics and/or helicase activity between eIF-4AIII and other family members are intriguing possibilities. Distinct activities and expression domains among related eIF-4A genes suggest an important and previously unsuspected role for these factors in the specific regulation of gene expression. Further progress will require a combination of biochemical and embryological approaches.

We wish to thank P. Klein, N. Sonenberg and A. Suzuki for providing us with the XeIF-4E, MefIF-4AI and tBr cDNA clones, respectively. We would also like to thank A. Suzuki for help with the microdissection schematic, J. Marden for excellent technical assistance, and P. Wilson and G. Lagna for critical reading of the manuscript. Recombinant human BMP-4 was a gift of Genetics Institute. A. H. B. is a Searle and a McKnight scholar. This work was supported by NIH grant HD 32105-01 and by grants from the Klingenstein and Merck Foundations to A. H. B.

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(Accepted 28 August 1997)