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Gene profiling during neural induction in *Xenopus laevis*: regulation of BMP signaling by post-transcriptional mechanisms and TAB3, a novel TAK1-binding protein

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SUMMARY

The earliest decision in vertebrate neural development is the acquisition of a neural identity by embryonic ectodermal cells. The default model for neural induction postulates that neural fate specification in the vertebrate embryo occurs by inhibition of epidermal inducing signals in the gastrula ectoderm. Bone morphogenetic proteins (BMPs) act as epidermal inducers, and all identified direct neural inducers block BMP signaling either intra- or extracellularly. Although the mechanism of action of the secreted neural inducers has been elucidated, the relevance of intracellular BMP inhibitors in neural induction is not clear. In order to address this issue and to identify downstream targets after BMP inhibition, we have monitored the transcriptional changes in ectodermal explants neuralized by Smad7 using a *Xenopus laevis* 5000-clone gastrula-stage cDNA microarray. We report the identification and initial characterization of 142 genes whose transcriptional profiles change in the neuralized explants. In order to address the potential involvement during neural induction of genes identified in the array, we performed gain-of-function studies in ectodermal explants. This approach lead to the identification of four genes that can function as neural inducers in *Xenopus* and three others that can synergize with known neural inducers in promoting neural fates. Based on these studies, we propose a role for post-transcriptional control of gene expression during neural induction in vertebrates and present a model whereby sustained BMP inhibition is promoted partly through the regulation of TGFβ activated kinase (TAK1) activity by a novel TAK1-binding protein (TAB3).

Supplemental data available on-line

Key words: *Xenopus laevis*, Neural induction, Smad7, Microarray

INTRODUCTION

The first step in neural development involves the initial specification of a neuronal fate from undifferentiated ectoderm. Neural fate acquisition in vertebrate development is thought to occur through inhibition of the BMP signaling pathway, in what has been termed the 'default model' for neural induction (Hemmati-Brivanlou and Melton, 1994; Wilson and Hemmati-Brivanlou, 1995; Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000). In *Xenopus laevis*, neural fate acquisition in the dorsal ectoderm results from the activity of a variety of BMP antagonists secreted from the organizer (Harland and Gerhart, 1997; Weinstein and Hemmati-Brivanlou, 1999).

Xenopus blastula ectodermal explants (animal caps) develop into epidermis when cultured in isolation. In the presence of BMP inhibitors, or when dissociated in culture, they develop into neural tissue (Hemmati-Brivanlou and Melton, 1994). A variety of molecules block the BMP pathway and display direct neural inducing activities; that is, the ability to induce neural tissue in the absence of concomitant dorsal mesoderm or organizer induction. Other molecules reported to have neuralizing activity, such as FGFs, Wnts or retinoids, induce mesoderm, act on pre-specified neural tissue to change the type of neural tissue formed, or mediate BMP downregulation, and therefore are not direct neural inducers (Cox and Hemmati-Brivanlou, 1995; Baker et al., 1999; Weinstein and Hemmati-Brivanlou, 1999; Wilson et al., 2000; Wilson et al., 2001).

Among the many different molecules with direct neural inducing activity, two subgroups can be discerned. The first category includes the secreted BMP antagonists noggin (Lamb et al., 1993), follistatin (Hemmati-Brivanlou et al., 1994), chordin (Sasai et al., 1994), cerberus (Bouwmeester et al., 1996) and Xnr3 (Hansen et al., 1997). The second category comprises molecules that act in a cell-autonomous manner to block BMP signaling intracellularly. The inhibitory Smads, Smad6 and Smad7 belong to this group (Massague and Chen, 2000). Smad6 inhibits the BMP pathway exclusively (Hata et al., 1998; Nakayama et al., 1998), whereas Smad7 has been shown to block both the TGFβ and BMP signaling pathways (Nakao et al., 1997; Casellas and Brivanlou, 1998). Other molecules known to display neural inducing properties are the Smurfs, ubiquitin-conjugating enzymes that target the TGFβ

receptors for degradation (Kavsak et al., 2000; Ebisawa et al., 2001). Presently, it is unclear whether the activities of these neural inducers have completely redundant functions in the embryo in neural fate specification.

Smad7 remains the most powerful neural inducer described to date, as assayed in animal cap ectodermal explants (Casellas and Brivanlou, 1998). Smad7 is thought to act intracellularly at multiple levels to inhibit signaling from both the BMP and the TGFβ pathways, through its ability to block receptor phosphorylation of the effector Smads (Hayashi et al., 1997; Nakao et al., 1997). In addition, Smad7 has been shown to target type II receptors for degradation by the ubiquitin pathway through the recruitment of the UBC-ligases Smurfs to the receptor complex (Kavsak et al., 2000; Ebisawa et al., 2001). The relationship between the inhibition of both branches of the TGFβ superfamily and the potency of this neural inducing molecule remains poorly understood, as are the mechanisms that lead to permanent, transcription-mediated changes downstream of BMP inhibition in the ectoderm. Independently of its role in the TGFβ pathway, Smad7 has also been shown to activate the JNK pathway, in the absence of interaction with TGFβ receptors (Mazars et al., 2001). Whether JNK activation through Smad7 activity plays a role in neuralization remains to be addressed.

In this report, using a *Xenopus laevis* 5000-clone gastrula cDNA microarray, we describe the first large-scale analysis of the transcriptional changes in a neuralized ectodermal cell population following expression of Smad7. We aim to understand patterns of gene expression that might be relevant during neural induction as well as early neural development. Therefore, we pursued genes that are expressed both during mid-gastrulation and neurula-staged embryos, at the time when these important cell fate decisions occur in response to signaling. Previous studies by Sasai's group and others have identified a variety of genes in neuralized animal caps that have been implicated in neural induction (Mizuseki et al., 1998; Song et al., 1999). However, our work represents the first example of a global genomics approach to study neural induction. Overall, we have identified 142 different genes, the expression of which changed in response to neuralizing signals mediated by Smad7. These studies can also be interpreted as global transcriptional profiling in response to TGFβ inhibition, and as such expands beyond embryology. We report the initial characterization of some of these genes, based on their expression profiles and sequence identity as potential candidates in neural induction and early nervous system development. As a first test of the involvement of selected clones during neural induction, we performed gain-of-function experiments in ectodermal explants. We present the results on several clones that display direct neural inducing activities. This study highlights the prominent role of translational control of gene expression during neural induction and the signaling integration of multiple pathways after BMP inhibition in the ectoderm, mediated in part by the regulation of the activity of the TGFβ-activted kinase (TAK1) by a novel TAK1 binding protein, TAB3.

MATERIALS AND METHODS

Gastrula array

DNA microarrays containing 5000 gastrula stage (stage 10.5) cDNAs were prepared as described (Altmann et al., 2001). These arrays contained the set of clones previously printed in the prototype array (Altmann et al., 2001), which can be found at our web site (http://arrays. rockefeller.edu/xenopus). The remaining clones have not been sequenced, except for clones described in this report. For cDNA sample preparation, *Xenopus* embryos were injected at the two-cell stage in both animal poles with 1 ng Smad7 RNA (Casellas and Brivanlou, 1998). Six hundred ectodermal explants were isolated at stage 9 (blastula) from both Smad7-injected and control uninjected embryos, and cultured until stage 15 (neurula). PolyA$^+$ mRNA was isolated using the Total mVader kit (Activemotif), and 1 μg of each mRNA population was used for cDNA synthesis (Atlas Glass Fluorescent Labeling Kit, Clontech). For the competitive hybridization experiments each cDNA (uninjected and Smad7-injected) was labeled with either Cy3 and Cy5 dyes (Amersham Pharmacia Cy3 and Cy5 mono reactive dyes) in independent coupling reactions. Inverse labeling was performed to account for any differences in dye fluorescence and to generate a second data point to increase confidence in the analysis. Labeled cDNAs were purified by first passing the samples through 2 Amersham Pharmacia ProbeQuant G-50 micro columns, followed by centrifugation in a Millipore YM-30 column. Samples were measured in the spectrophotometer for dye incorporation and elimination of unbound dyes. The cDNA populations were mixed as follows: (1) uninjected-Cy3 with Smad7-Cy5; and (2) Uninjected-Cy5 with Smad7-Cy3, and dried in a SpeedVac. Samples were resuspended in 30 μl of hybridization buffer (25% formamide, 5×SSC, 10 μg/ml salmon sperm DNA and 0.1% SDS), boiled for 5 minutes and cooled to room temperature. The arrays were hybridized in a sealed-chamber at 42°C overnight, and washed as follows: 10 minutes at 42°C in 2×SSC/0.1% SDS; 5 minutes in 0.1×SSC/0.1% SDS at room temperature, followed by four 5 minute washes in 0.1×SSC at room temperature. Array image files were gridded and analyzed using Scanalyze (Eisen, Stanford) and the data was uploaded to the TANGO database system (Altmann et al., 2001).

Sequencing and sequence analysis

Clones identified by the array were sequenced on ABI 3700 sequencers using Big Dye chemistry. Sequences were blasted against public and private databases, and were analyzed and assembled using AutoAssembler software.

Reverse-transcriptase Polymerase-chain-reaction (RT-PCR) analysis

The RT-PCRs on isolated animal cap explants were performed according to previous protocols (Wilson and Hemmati-Brivanlou, 1997). Primers were designed encompassing either the 5′ or 3′ UTRs. Amplification was performed for 21 or 25 cycles, depending on the primers and transcript abundance. Primers used were as follows (given 5′ to 3′; S, sense primer; AS, antisense primer):

45-A6, (S)TCGACTGAAGGACTTTGTTC,(AS)CACCCTGACTCTTCCCTAGT;

45-D3, (S)ATGATGGCACACTGAGCGAGG,(AS)GTCTCATTTGTCAGCATCGTG;

45-H5, (S)TGGTGACTTATTTCCATCCA,(AS)CACCCTCACCTTCTACATCA;

46-E2, (S)CCCTTCAAAGAGCTAGACAGA;(AS)GTATTTTGCAACCAGACTCCT;

47-A4, (S)GAACTTGGTGTAGGAGACTCG,(AS)ACTCACTACCATTTCCTCCTG;

47-B11, (S)TCACTACCAGGACCAACAGAT,(AS)AGCGCAGAGTAACTGTACTGG;

47-F3, (S)GCTGGATTCCTCATGTAAATA,(AS)TAACTGAGGTTTAGGGGTTTC;

47-G3, (S)TTCGCGAAATGCTGCAGAGC,(AS)CATCTTCCATCATCTCCTGC;

47-G6, (S)AATCCCCTTCTTCTCAGGTAT,(AS)AGGGGATTAGGGAGAGATTTA;

48-A2, (S)TGCAGATCTCTCCACTGTAAA,(AS)AGCCAACATGGAGTCATAGAT;
48-F11, (S)CTTGGGTGTAGGCTTTATGTT,(AS)AAGACCAGGTATAAGGCTGAA;
49-B2, (S)CTAAGCGGTGTTCATTTGTTT,(AS)GTCCACAGACTTTCCATTCAT;
50-E8, (S)CTCCACACTTAGCGAACAATA,(AS)GTCATACCTCACTTTGTCTGC;
51-A10, (S)CCAGATTTTGAAATGGAACTT,(AS)GTTCCATCATAATTCCAAAGC;
51-B6, (S)TACGACAGGAATACGGTGGAG,(AS)CGTCGGTCAGAACTGTTCATTC;
51-E6, (S)AAAACCAACAGGATGAAGGTA,(AS)TCGTTTGTTTCAAGAACTTTG;
52-E10, (S)GTCGTCTTGTGAAGTTGTTTC,(AS)GCGCGAAACACAATTGGCC;
52-G11, (S)GAGAGGTTAAACATGGCACTC,(AS)ATTGATTCATTCCCAGTCTGT;
54-B3, (S)GAGAGGTTAAACATGGCACTC,(AS)ATTGATTCATTCCCAGTCTGT;
55-H2, (S)CCACAGCAAACTAATAACTC,(AS)ATAGCAGCGGTCTTTGCAGAG;
56-G6, (S)GCTGTATAAATTGTGCTGAAGC,(AS)AATGCACCAGGTCTATAGGTC;
57-D10, (S)ATTGAGATAAAATGGCTGGAG,(AS)TAAGGTCTTTGGAAGCATTTC;
57-G10, (S)GGTACCTTACTTAAGTCAGATG,(AS)CAGCAACATCACTTCATTTGCC.

Ornithine decarboxylase (ODC) was used as a loading control. Primers for marker genes are as described (Hemmati-Brivanlou and Melton, 1994; Weinstein et al., 1998).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed with digoxigenin labeled probes (DIG-UTP from Boehringer-Mannheim) as described by Harland (Harland, 1991). Embryos were post-fixed in 4% PFA. Sectioned embryos were embedded in 20%gelatin/PBS and cut at 50 μm or 100 μm.

Plasmid constructs

The long 3′UTR of clone 57-G10/TAB3 was deleted by cutting it with *Kpn*I. The xTAB3C construct was made in pCS2++ by conventional PCR using Cloned Pfu Polymerase™ (Clontech) with the following primers: 5′ CATCGAATTCCACCATGCGGGGAATACCTACCCAACC 3′ and 5′ CATGCTCGAGTCATGTGAATCGTGGCATCTC 3′.

RESULTS

Microarray analysis of gene expression changes in ectodermal explants neuralized by Smad7

In order to gain insights into events that follow BMP inhibition in the ectoderm, and to reveal mediators of Smad7-activity in neural specification, we monitored the transcriptional changes after neuralization with Smad7. To this aim, we used a *Xenopus laevis* 5000 cDNA gastrula microarray (developed in our laboratory) to perform competitive hybridizations from RNA populations isolated from early neurula-stage naive ectodermal explants, which form epidermis, or explants neuralized by injection of Smad7 RNA at the two-cell stage (Fig. 1). Overall, we identified 185 clones corresponding to 142 different genes, the expression of which changed in the neuralized explants. We have categorized the regulated genes into three sets, according to the fold changes in expression upon Smad7 exposure (Table 1). The first set encompasses clones whose expression changed more than threefold and within three standard deviations of the background for both data points in the inverse-dye hybridization experiments [for array analysis, see Altmann et al. (Altmann et al., 2001)]. In this class, we identified 47 clones, of which 35 correspond to different genes (Tables 1 and 2). The second set encompasses genes whose expression changes are greater than twofold on both data points. Seventy-eight clones belong to this class, corresponding to 56 different genes (which are also distinct from the previous set; Table 1). Finally, set 3 corresponds to clones whose expression was

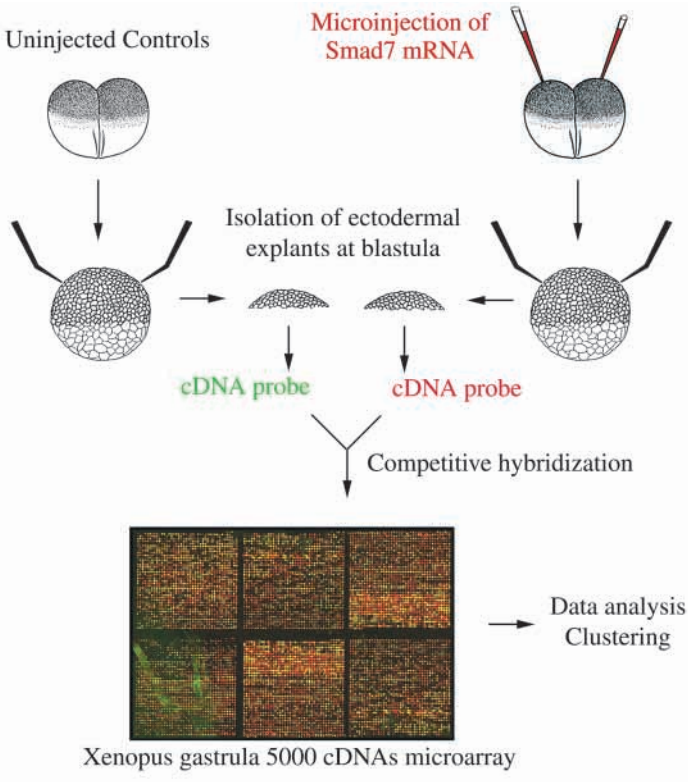

Fig. 1. Experimental approach. Embryos were injected in both cells at the two-cell stage in the animal pole with 1 ng Smad7 RNA. Ectodermal explants were isolated at stage 9 from both Smad7-injected and uninjected embryos and cultured until stage 15 (neurula). Transcriptional differences were analyzed on a 5000 clone gastrula (stage 10.5) cDNA microarray in duplicate as inverse-dye labeled competitive hybridization (Altmann et al., 2001).

Table 1. Criteria for selection of analyzed clones from the Smad7 microarray

	Number of clones	Number of genes	Up/Down	Set criteria
Set 1	47	35	33/2	Changes greater than threefold or 3 s.d. in both channels in inverse hybridization
Set 2	78	56	51/5	Greater than twofold changes in both channels
Set 3	60	51	30/21	Greater than twofold changes in one channel

Number of different genes among the sequenced clones, which do not include genes already assigned to the previous set. Sequences with no hits or small inserts are considered here as different genes.

changed greater than twofold at one data point. To this class belong 60 clones corresponding to 51 distinct genes. We provide sequence information on all 142 genes (see http://xenopus.rockefeller.edu/Smad7/Development_supplement.htm). Overall, the number of genes whose expression was upregulated was much higher than those downregulated by Smad7 exposure (114 versus 28, respectively; Table 1). The majority of these genes were full length. The gastrula library employed for array printing was an expression library (Weinstein et al., 1998), which we use to assay the bioactivities of candidate genes in neural induction.

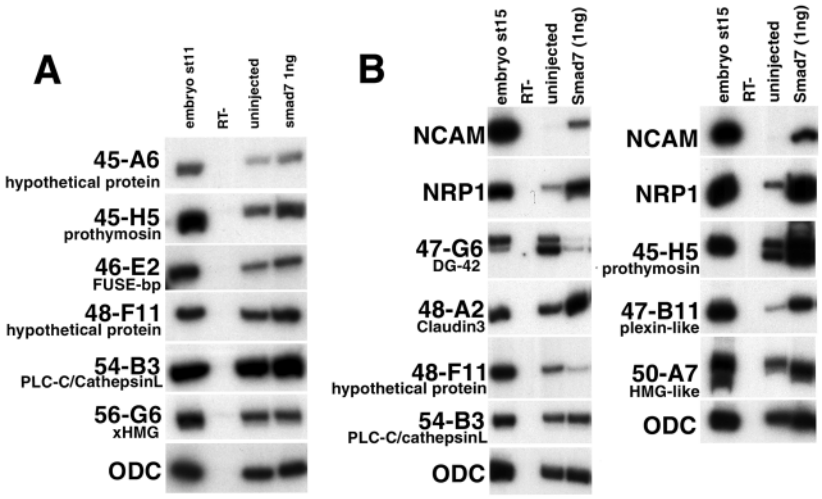

Fig. 2. Confirmation of selected clones by RT-PCR. Individual clones were analyzed by RT-PCR in animal caps injected with 1 ng of Smad7 RNA and harvested at either stage 10.5 (gastrula; A) or stage 15 (neurula; B). Primers were designed to include either 5′ or 3′ UTR sequences. Ornithine decarboxylase (ODC) is used as a loading control and NCAM and NRP1 to control for neural induction by Smad7.

Confirmation of array results by RT-PCR

To confirm the results obtained in the array, we verified the directionality of the expression changes by radioactive RT-PCR (Fig. 2). At neurula stages (stage 15), the majority of genes identified by the array were upregulated in response to Smad7, and included three main categories: (1) known and previously published genes, such as clone 48-A2/Claudin (Brizuela et al., 2001), 54-B3/cathepsin L (Miyata and Kubo, 1997) and 47-G6/DG42 (Rosa et al., 2001); (2) known genes with no characterized function in *Xenopus*, such as 45-H5/prothymosin; and (3) completely novel clones with no known function, such as 48-F11/hypothetical. The majority of the genes analyzed by RT-PCR confirmed the results of the array at this stage (Fig. 2B; 64%). The rest of the clones showed no change as judged by RT-PCR. We attribute these differences to the distinct nature of the specificity of both techniques, namely a precise PCR-based amplification versus the medium-stringency conditions of the hybridization performed in the array, which might allow for highly homologous sequences to hybridize to the printed cDNAs (Heller et al., 1997). However, some clones showed a highly dynamic temporal regulation following Smad7 expression. For example, clone 48-F11 was upregulated in response to Smad7 during gastrula stages (stage 10.5; Fig. 2A), but downregulated at neurula stages (Table 2 and Fig. 2B). Therefore, although the majority of clones showed similar regulation at both gastrula and neurula stages (Fig. 2), some clones might be subject to dynamic temporal regulation after Smad7 expression.

Sequence-based classification of the clones identified in the Smad7 array

Initially, we grouped the different genes according to the functional classifications outlined in EGAD (Expressed Gene Anatomy Database, TIGR) (Altmann et al., 2001) (Fig. 3), to gain a global appreciation of the types of molecules implicated in the earliest steps of neural development. The challenge remains to distinguish clones that act in a mechanistic fashion after BMP inhibition from those whose expression correlates with intrinsic differences among ectodermal fates. Importantly, the neural ectoderm in *Xenopus* shows a higher mitotic index (twofold) than does the non-neural ectoderm during early neurulation (Saka and Smith, 2001), although this alone cannot account for the differences observed in transcript abundance, nor the restricted nervous system expression of the genes thus far characterized.

Overall, we find that the majority of the genes encode proteins belonging to four main groups (Figs 2-4): (1) predicted, hypothetical proteins (19%); (2) post-transcriptional and translational control (18%); (3) signal transduction (18%); and (4) transcription/chromatin remodeling (9%). In addition, there are a number of clones with no hits in database searches (15.5%), which are likely to be partial cDNAs. Smaller subsets of genes are implicated in cell structure, cellular processes, nucleotide metabolism, transport and binding, DNA metabolism and amino acid biosynthesis (Fig. 2 and complete list of clones). A few of the upregulated genes have been previously identified in a microarray-based analysis of neural progenitors in mice (Geschwind et al., 2001), such as hnRNP-

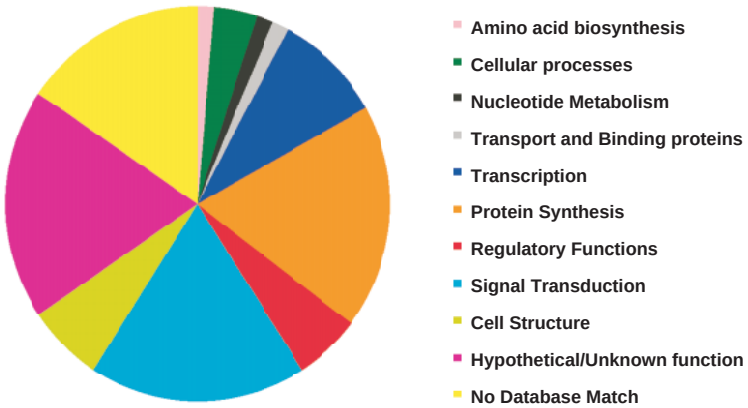

Fig. 3. Microarray analysis of RNAs isolated from ectodermal explants neuralized by Smad7. Pie chart of the classification of clones identified in the array based on EGAD categories (TIGR, Maryland).

Table 2. Set 1 genes regulated by Smad7 in isolated ectoderm

Clone	Identity	Increase in array	RT-PCR analysis*	Whole-mount in situ hybridization†
28-A12	Cytokeratin- type II‡	Four- to fivefold	Downregulated	Epidermis
45-A6	Hypothetical protein. DKFZp586 G1123	Sixfold	Upregulated	Dorsal-neural
45-G5	Steroidogenic acute regulatory protein-related (STAR) protein	Fourfold	N/D	N/D
45-G6	*Xenopus* cold-inducible RNA-binding protein 2 (CIRP-2)	Three- to sevenfold	Unchanged	Neural
45-H5	Pro-/Para-thymosin alpha homolog	Eightfold	Upregulated	Neural
46-E2	FUSE/KH-binding protein	Fourfold	Upregulated	Dorsal-neural
46-G6	No matches	Threefold	N/D	N/D
47-A4	CTP synthase II	Fourfold	Upregulated	N/D
47-B11	Plexin/Semaphorin receptor	Fourfold	Upregulated	Dorsal-neural
47-F3	No matches	Fivefold	Unchanged	Neural
47-F5	High-mobility-group (HMG)-like protein	Four- to fivefold	Upregulated	N/D
47-G3	Human hypothetical protein.	Threefold	Upregulated	Dorsal/anterior neural
47-G6	DG-42‡	4.5-fold	Downregulated	Ventral/epidermal
47-G10	*N*-acetyl glucosamine transporter	Fourfold	N/D	N/D
48-A2	*Xenopus* tight junction TM-protein; Claudin 3	Fourfold	Upregulated	Published neural
48-F11	Hypothetical protein.	Fourfold	Downregulated	Dorsal-neural
49-B2	*Xenopus* cold-inducible RNA-binding protein 1 (CIRP-1)	3.5-fold	Unchanged	N/D
51-A10	Hypothetical protein	3.5-fold	Unchanged	Neural
51-E6	Human, mouse hnRNP-D-like protein	3.5-fold	Upregulated	Neural
52-E10	No matches	Fivefold	Upregulated	N/D
52-G11	Non-receptor protein phosphatase	Fourfold	Unchanged	N/D
54-B3	βPLC-C/cathepsin-L	Sixfold	Upregulated	Neural
55-H2	IkB-ε	Sixfold	N/D	Neural
56-G6	Xenopus High-mobility-group (HMG)-X	Fourfold	Unchanged	Published neural
57-D10	Histone H2A-Z12	Fivefold	Upregulated	Neural
57-E10	Clathrin coat adaptor protein complex AP-2	3.5-fold	N/D	Published neural
57-G10	TGFβ activating kinase binding protein 3	Fourfold	Upregulated	Dorsal/Anterior neural
61-B12	EF1α	Fourfold	N/D	N/D
61-G10	Microtubule-associated protein	Threefold	N/D	Published neural
69-F3	No matches	Sixfold	N/D	N/D
M13-1-G10	PNK-2	Fourfold	N/D	N/D
pBS-G7	Activin-Bc	Fivefold	N/D	Published neural
pBS-G8	Alk5	Fivefold	N/D	N/D

*RT-PCR analysis performed during neurula stages (stage 15) (see also Fig. 2 and text for discussion).

†Whole-mount in situ hybridization data are based on neurula-stage embryos. Neural implies expression detected in the nervous system, although not necessarily restricted to it (see also Fig. 4).

‡Genes identified as downregulated genes. All other genes represent upregulated clones identified in the microarray.

The nearest homologs of these clones are listed. Five clones showed no matches in database searches. Four clones have been described in genome-wide sequencing projects as hypothetical proteins. The majority of known clones were full length.

N/D, not determined.

D-like (clone 51-E6), thymosin (45-H5), histone H2A.Z (clone 57-D10), and ubiquitin-like genes (clones 45-D7 and 48-D6).

Surprisingly, we found a higher percentage of differentially regulated genes implicated in post-transcriptional and translational control than genes acting in transcriptional regulation of gene expression (Fig. 2). This finding suggests that post-transcriptional mechanisms play a critical role during neurulation. Among these factors are several initiation factors previously linked to fate decisions in vertebrate development, such as clone 51-D5/eIF4-AIII (Weinstein et al., 1997) and clone 52-G7/eIF4-AII (Morgan and Sargent, 1997). Additionally, other RNA-binding proteins not previously implicated in developmental processes show restricted nervous system expression, such as clone 46-E2 (FUSE-binding protein-2) (Fig. 4A). We also identified a variety of presumed ubiquitous factors, such as hnRNP and snRNP proteins (clones 13-A10/hnRNP A/B; 46-B2/snRNP B; 54-G3/RNA helicase; Table 2). Among these latter clones, a large subset display neuralizing activities in our assays.

Among the genes implicated in transcription, we have identified a variety of chromatin-remodeling, HMG-containing genes (Fig. 2 and Table 2) with nervous system expression. Several HMG-containing genes have previously been described in *Xenopus* nervous system development (Konig et al., 2000; Liu et al., 2001), supporting their putative role in neuronal fate acquisition.

We also identified several molecules belonging to various signaling pathways not previously linked to neural induction. Among these are those known to participate in TGFβ signaling, such as activin B, Alk5, clone 51-F6/MARCKS-related and 57-G10/TAB3 (Table 2). Among the secreted factors is a distantly related member of the cerberus/gremlin family of BMP inhibitors (clone 51-B6), suggesting that Smad7 also promotes the negative regulation of the BMP pathway by inducing the expression of an extracellular BMP inhibitor. In addition, several clones are part of the NFkB or JNK pathways (see list), implicating these pathways in early neural development. Additional molecules implicated in signaling are the small GTPases, G-proteins and plexin-like receptors (clones 53-E12, 51-E10, 47-B11 and 47-E8).

A large proportion of genes share homology to predicted, hypothetical proteins (n=27/142; 19%; see Fig. 4). Some are remarkably conserved in protein-coding regions, and they probably play fundamental roles in metabolism or basic

signaling processes. A third of all the hypothetical proteins within the first two sets have homologs in *Drosophila* or *C. elegans* (*n*=9/27), and many share homologies to *S. pombe* or *S. cerevisiae* (*n*=5/27; 19%). For a full description of these genes, see http//xenopus.rockefeller.edu/smad7/Development_supplement.htm.

Analysis of the temporal and spatial expression patterns of the array clones by whole mount in situ hybridization

As a second means to assess the array results, and to characterize the expression patterns of clones identified in the array, we determined their mRNA distribution during development by whole-mount in situ hybridization (Fig. 4). Overall, all the upregulated genes had neural expression domains (Fig. 4A-H) and those downregulated were excluded from the neural plate (Fig. 4I). Smad7 mRNA expression is widespread in gastrula embryos, although is restricted to the nervous system, the heart and ventral-most mesoderm at neural plate and tadpole stages (Casellas and Brivanlou, 1998). Similarly, the clones analyzed were expressed in the animal pole and the mesoderm of the dorsal marginal zone (DMZ) and ventral marginal zone (VMZ) of gastrula-stage embryos, as detected by in situ hybridization (Fig. 4A-E and G-I, left panels) and RT-PCR (not shown).

All of the clones analyzed were strongly expressed at gastrula (Fig. 4A-E,G-I left panels). For example: clone 45-H5/prothymosin is expressed throughout the outer epithelial layer but not within the sensorial layer or deep zone cells by the dorsal blastopore lip (Fig. 4B); clone 47-B11, a plexin-like molecule (Fig. 4C), and 47-G3, a hypothetical gene (Fig. 4E), were highly expressed within the blastopore lip (see asterisks in Fig. 4); by contrast, clone 47-F3, a novel hypothetical gene (Fig. 4D), and 47-G6/DG42 (a downregulated clone; Fig. 4G),

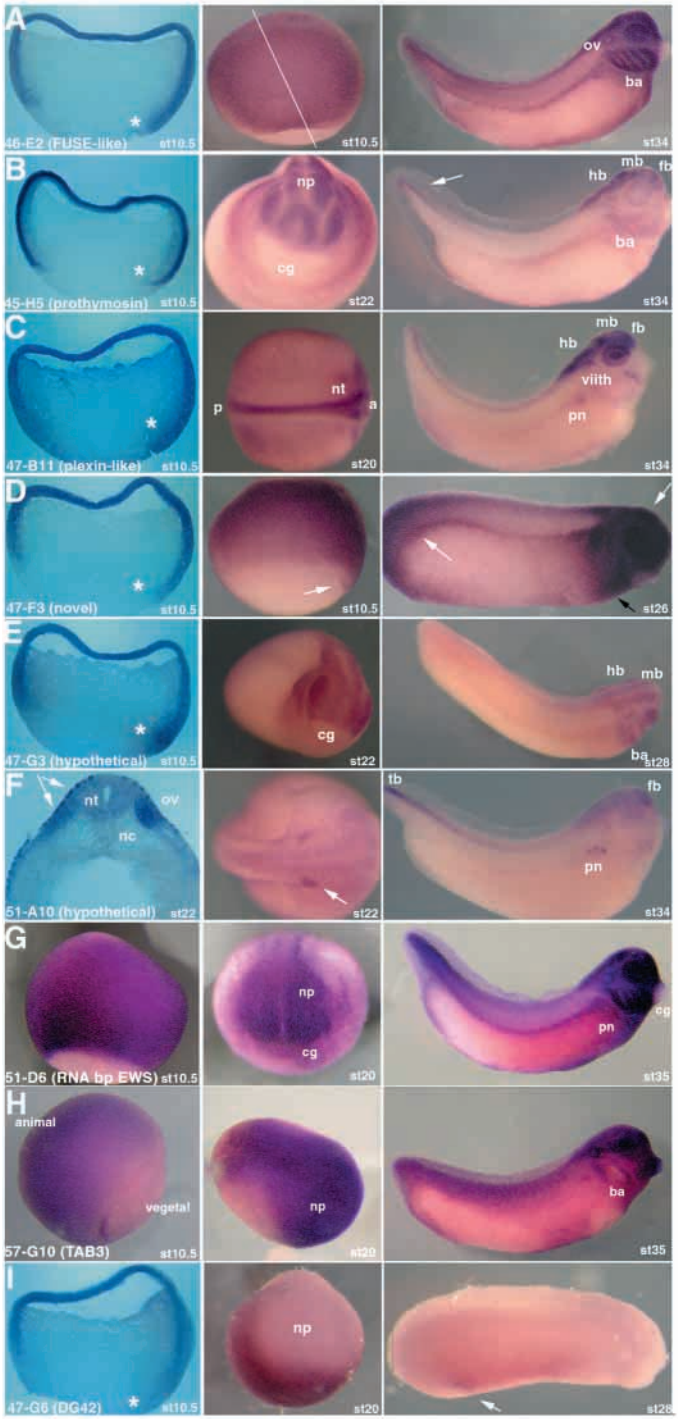

Fig. 4. Whole-mount in situ hybridization of selected clones. (A) Clone 46-E2/FUSE-binding protein is expressed throughout the animal pole of the gastrula embryo (left and center) and at tadpole stages in the branchial arches and the brain (right panel). (B) Clone 45-H5/prothymosin is expressed at high levels throughout the epithelial layer of the animal pole (left panel) and in anterior neural tissue both at neurula (middle panel) and tadpole stages (right panel). It is also seen in the tailbud (arrow in right panel). (C) Clone 47-B11/plexin, is expressed at gastrula stages throughout the animal pole (left panel). At neurula transcripts are detected throughout the spinal cord and neural tube. At tadpole stages, it is detected in the brain, spinal cord, the facial and trigeminal ganglia, and the pronephros (right). (D) Clone 47-F3/novel is expressed throughout the gastrula embryo (left and center panels, arrow in middle panel depicts the dorsal blastopore lip). At later stages, expression is very strong in the brain and caudal end of the embryo (white arrows in right panel). It is also detected in ventral tissue (black arrow, right). (E) Clone 47-G3/hypothetical protein is strongly expressed in the animal pole at gastrula stages and in the dorsal blastopore lip (left). High levels are also detected in the nervous system and it is excluded from the cement gland (middle). At tadpole stages (right), it is expressed exclusively in the anterior neural tube. (F) Clone 51-A10/hypothetical protein is expressed at neurula stages in ciliated epidermal cells (arrows, left) and the presumptive otic placodes (arrow in center). In the tadpole (right), notice expression in the nervous system, branchial arches and pronephros. (G) Clone 51-D6/RNA bp EWS is expressed at high levels throughout the gastrula embryo excluding the vegetal pole (left panel), expression is very high in the anterior neural plate at neurula (middle) and is then expressed throughout the brain at tadpole stage (right). (H) Clone 57-G10/TAB3 is expressed throughout both the embryo at both gastrula and neurula stages (left and middle). At tadpole stage, expression is seen in the brain and branchial arches (right). (I) Clone 47-G6/DG42 is expressed throughout the animal pole in the gastrula (left) and is excluded from the neural plate at neurula stages (center); by tadpole stage it is only expressed in ventral regions of the gut (right, see arrow). a, anterior; ba, branchial arches; cg, cement gland; fb, forebrain; hb, hindbrain; mb, midbrain; nc, notochord; np, neural plate; nt, neural tube; ov, otic vesicle; p, posterior; pn, pronephros; tb, tailbud; viith, facial ganglia; *, dorsal blastopore lip.

were weakly expressed in the dorsal lip. Both clones 51-D6/RNA-binding protein EWS and 57-G10/TAB3 are also expressed at very high levels in the animal pole (Fig. 4G,H).

Among the upregulated genes, most had general neural expression domains. However, some genes showed more restricted expression patterns in neurula (4B,C,E-H, middle panels; Fig. 4F, left panel) and tadpole-staged (right panels) embryos. Interestingly, few of these genes had expression domains that overlapped with Smad7 mRNA expression outside of the nervous system, suggesting that these genes might be regulated by Smad7 only in the context of ectodermal patterning. Together, and in a manner consistent with their regulation, this analysis has shown that the majority of clones have overlapping expression domains with Smad7 in the gastrulating embryo, as well as during early neural development.

Functional characterization of identified clones in ectodermal explants

Transcriptional profiling represents a powerful tool in the identification of genes potentially implicated in a particular process. The challenge remains to address whether these genes play a functional role in neural induction. A major advantage of performing these experiments in *Xenopus* is the combination of array technologies with gain-of-function studies, in order to assay the functional involvement of identified genes. To this aim, we microinjected RNAs encoding selected clones into animal caps at the two-cell stage, and tested for their ability to promote cell-fate changes, as judged by a variety of markers (Figs 5 and 6). We have analyzed the involvement of the RNA-binding proteins identified in the array, and showed that clones 56-G6/HMG-X, and 57-G10/TAB3 display direct neuralizing activities.

Of the factors involved in post-transcriptional regulation not previously linked to fate decisions in vertebrates, 12 were full-length and their RNAs were injected alone or in the presence of low doses of noggin in the caps (15 pg). Of these, four clones displayed neuralizing activities: 46-B2/snRNP-D; 54-G3/putative DECD-box RNA-helicase; 47-C4/ribosomal protein XL1a, and 51-D6/RNA-bp EWS (Fig. 5). Only 51-D6/RNA-bp EWS can act as a direct neural inducer, as judged by the expression of NCAM, NRP1, Otx2, Pax6 and XAG (Fig. 5). The product of this gene has been implicated in the aetiology of Ewing's familial tumours (Arvand and Denny, 2001). The remaining clones lacked neuralizing activity on their own, although they synergized with noggin to promote anterior neural fates, as judged by the lack of expression of the spinal cord marker HoxB9 (Fig. 5). By contrast, hnRNP-A1 did not induce neural markers when co-injected with noggin. We never observed posterior neural marker expression in this assay, consistent with neural fate acquisition mediated by BMP inhibition.

Within the genes in the transcription category, clone 56-G6/HMG-X encodes an HMG1/2 homolog previously identified as a gene induced during neurogenesis (Kinoshita et al., 1994). In animal caps, 56-G6/HMG-X displayed direct neuralizing activity, as judged by the expression of NCAM and Otx (Fig. 6A). In a second set of experiments, we found that 56-G6/HMG-X enhanced the expression of the neural markers NCAM and Sox2 induced by low levels of Smad7 RNA (5 pg; Fig. 6B), while it inhibited expression of the cement gland markers XAG and CG. Development of the cement gland is sensitive to levels of BMP signaling (Hemmati-Brivanlou and Melton, 1994; Wilson et al., 1997; Gammill and Sive, 2000), and cement gland markers can be induced following a partial inhibition of BMP signaling in the ectoderm. The type of neural tissue induced by 56-G6/HMG-X was anterior in character, as judged by the lack of expression of En2 (mid/hindbrain marker), Krox20 (hindbrain), Hoxb9 (neural tube) and Twist (neural crest). This anterior neural tissue can be converted to posterior one upon exposure to bFGF (not shown), similar to what has been reported after BMP inhibition (Cox and Hemmati-Brivanlou, 1995). To confirm the results obtained in the explant experiments, we injected 56-G6/HMG-X RNA in the ectoderm (Fig. 6C-H). Overexpression of 56-G6/HMG-X leads to hyperplasia of the anterior neural plate (Fig. 6C-E), and to ectopic expression of anterior markers, such as Pax6 (Fig. 6F-H).

Among the genes implicated in TGFβ signaling, clone 57-G10 encodes the *Xenopus* ortholog of a novel TGFβ-activating kinase (TAK1) binding protein, which we have termed TAB3

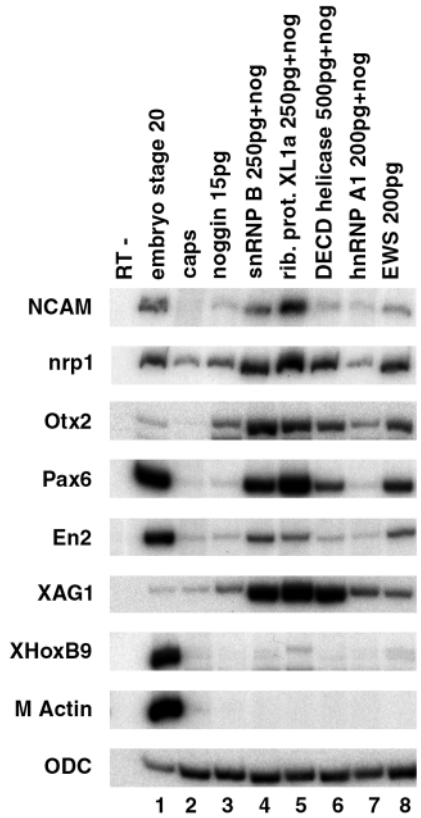

Fig. 5. Functional analysis in animal cap explants of clones implicated in post-transcriptional regulation. mRNAs for 13-A10/hnRNP-A/B, 46-B2/snRNP-B, 51-B11/snRNP-D, 54-G3/RNA helicase and 51-D6/RNA-bp EWS were injected into both animal cap cells at the two-cell stage, either alone or together with 15 pg of noggin RNA. RT-PCR analysis was performed at stage 20 (tadpoles). In all instances, the neural tissue induced is anterior in character, and no posterior (spinal cord) neural markers are detected. Similarly, there is no expression of mesodermal derivatives, as judged muscle actin (muscle) and collagen-type II (notochord) expression. Markers used are: NCAM and NRP-1, pan-neural; Otx2 and Pax6, forebrain/midbrain; XAG, cement gland; En2, midbrain/hindbrain boundary; Hoxb9, spinal cord; muscle actin, muscle.

(Fig. 7). The TAK1 kinase and binding partners have been shown to play crucial roles in signaling crosstalk between various pathways, including the p38 MAP kinase pathway (Kimura et al., 2000; Goswami et al., 2001; McDermott and O'Neill, 2002), the JNK pathway (Takaesu et al., 2000; Wang et al., 2001), the NFkB pathway (Wang et al., 2001) and the Wnt pathway (Ishitani et al., 1999; Meneghini et al., 1999). The activity of TAK1 and its coupling to downstream signaling pathways is largely modulated by the TAK-binding proteins, or TABs (Takaesu et al., 2000). In *Xenopus*, TAK1 can be activated downstream of the BMP receptor, where it can promote ventral fates in association with TAB1 (Shibuya et al., 1998; Goswami et al., 2001). 57-G10/TAB3 is the ortholog of a recently identified mouse and human TAB, which we have termed TAB3 (60% identity at the amino acid level) and is also closely related to TAB2 (35% identity; Fig. 7A). Interestingly, as in the case of TAB2, 57-G10/TAB3 contains a ubiquitin ligase-binding domain (Fig. 7B) suggesting that it might also activate TAK1 through a ubiquitination step (Wang et al., 2001). Because the different TAB proteins appear to modulate the specificity of the kinase activity, and because of its similarity to TAB2, we tested whether 57-G10/TAB3 could play a role in neural induction, promoting neural fates in contraposition to the role of TAB1 in the establishment of ventral fates downstream of BMP signaling.

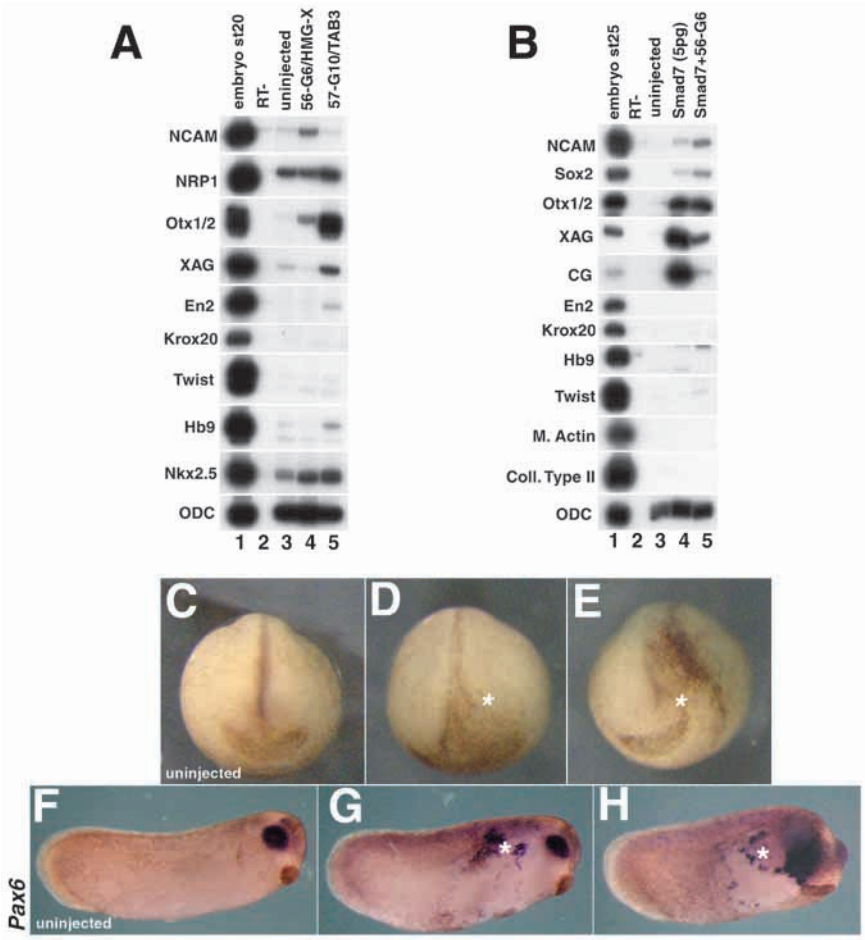

Fig. 6. Functional analysis of clones 56-G6/HMG-X and 57-G10/TAB3 in animal cap explants. (A) Clones 56-G6/HMG-X and 57-G10/TAB3 were injected into animal caps and analyzed for fate changes at stage 20. Both clones induce neural or cement gland marker expression. Notice no induction of posterior neural markers (En-2, mid/hindbrain; Krox-20, hindbrain; twist, neural crest) and weak induction of the heart marker xNkx2.5. (B) 56-G6/HMG-X synergizes with low amounts of Smad7 (5 pg). Notice inhibition of cement gland fates induced by Smad7 in the presence of 56-G6/HMG-X (lane 5). (C-H) Gain-of-function experiments with 56-G6/HMG-X in vivo. Embryos were injected into 1/2 cells in the animal pole and embryos analyzed at tadpole stages. Notice enlargement of the anterior neural plate (C-E), and ectopic expression of the anterior neural marker Pax6 (F-H). Asterisks indicate enlarged anterior neural territory (D,E) and ectopic Pax6 expression (G,H).

In animal cap explants, 57-G10/TAB3 induced expression of Otx1/2 and XAG, and weakly of NRP1 (a pan-neural marker) and En2 (Fig. 6A). Interestingly, as with 56-G6/HMG-X, 57-G10/TAB3 also weakly induced the expression of Nkx2.5, a marker of heart tissue, although we failed to detect other markers of mesoderm in these explants. The expression of Nkx2.5 in the explants might suggest that 57-G10/TAB3 could inhibit the Wnt pathway, which has been shown to regulate Nkx2.5 expression (Bouwmeester et al., 1996). The neuralizing activity of 57-G10/TAB3 prompted us to speculate that TAB3 might form a complex with TAK1/TAB1 and switch the specificity of TAK1 to promote neural versus epidermal fates. In order to test this idea, we made a dominant-negative C-terminal construct of 57-G10/TAB3 (TAB3C; Fig. 7), based on the approach taken by Takaesu et al. (Takaesu et al., 2000) with TAB2. When we injected low doses (250-400 pg) of xTAB3C in the animal caps, there was an increase in epidermal keratin expression in gastrula and tadpole stage caps (Fig. 7C,H), suggesting that xTAB3C can promote BMP signaling. Higher doses of xTAB3C induced apoptosis in the ectoderm, consistent with the activation of TAK1/TAB1 activity in *Xenopus* (Goswani et al., 2001) (not shown). In order to test whether xTAB3C could prevent neuralization, we co-injected it together with 50 pg of either Smad7 or noggin (Fig. 7D-G,H). Explants expressing Smad7 form neural and cement gland tissue (Fig. 7E). The heavily pigmented cement gland tissue does not form in Smad7 caps co-injected with xTAB3C (Fig. 7F). When the caps were analyzed by RT-PCR, we observed a marked inhibition of neural genes normally induced by BMP inhibitors (Fig. 7H). Altogether, these results strongly suggest that TAB3 acts in the neural plate to promote neural fates, and supports the notion that TAK1 complexes might be crucial in the establishment of neural fates.

DISCUSSION

Our microarray analysis has led to the identification of a large number of genes directly or indirectly regulated at the

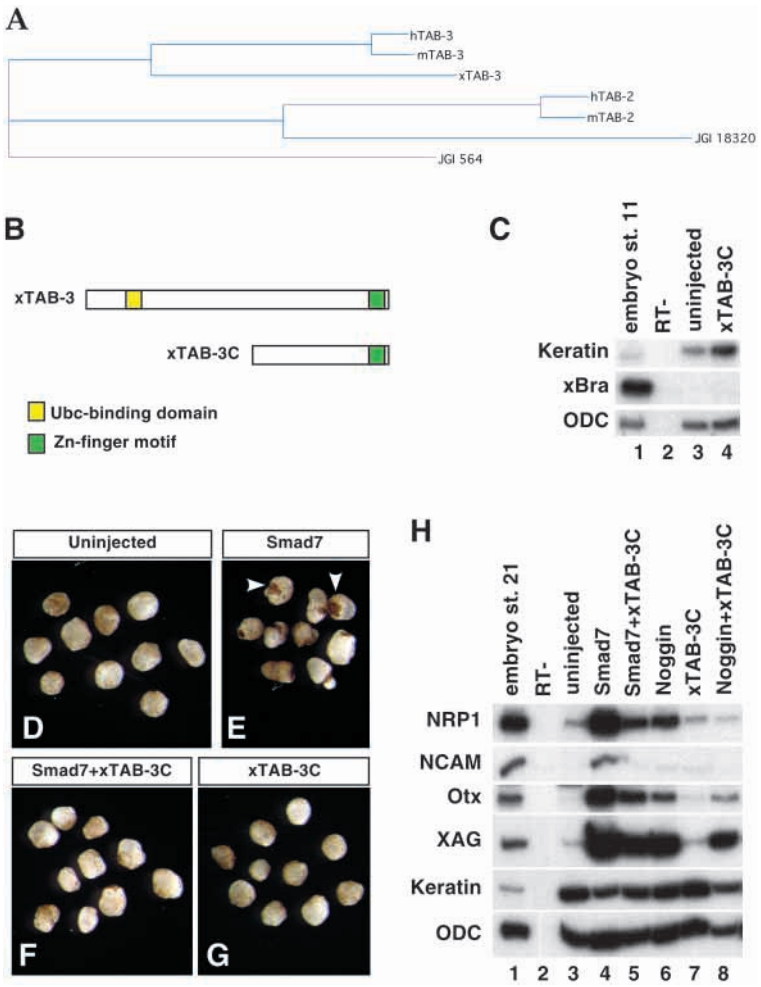

Fig. 7. Analysis of xTAB3 involvement in BMP signaling and neural induction. (A) Dendrogram of the identified TAB2 and TAB3 family. Murine and human TAB3 were found in NCBI searches. Two TAB-related genes found in the fugu database are included (JGI 18320 and JGI 564). (B) Schematic of xTAB3 and xTAB3C proteins. Yellow box depicts the CUE domain (ubiquitin ligase interacting domain). Green box depicts the Zn-finger motif. (C) Injection of xTAB3C RNA into animal caps results in upregulation of epidermal keratin expression in gastrula stage explants, a marker responsive to BMP signaling. There is no mesodermal gene expression in these caps (xBra). (D-G) Animal cap explants (stage 23) injected with 50 pg Smad7 form neural and cement gland tissue (E). Notice pigmented cement glands (arrowheads). Co-expression with xTAB3C largely inhibits cement gland (F). (H) Analysis of explants injected with 50 pg of Smad7 or noggin in the absence (lanes 3, 6) or presence (lanes 4, 8) of xTAB3C shows that xTAB3C can inhibit neural and cement gland fates induced by BMP inhibitors.

transcriptional level by Smad7 and novel candidates in neural induction and early nervous system development. It has provided information on pathways that might be modulated after BMP inhibition, leading to neural fate specification. Because of the large number of potential target genes, we chose to further characterize genes with pronounced expression changes in the array, based on sequence analysis and potential involvement in TGFβ signaling. To validate the robustness of the microarray assay, we have shown that the changes in majority of these genes can be independently confirmed by RT-PCR. In addition, all the upregulated clones show restricted neural expression domains during early development.

From this information, we suggest potential mechanisms by which permanent neural fate acquisition might be imparted on the ectoderm by direct neuralizing agents (Fig. 8). Although the mechanism of action of Smad7 in preventing BMP and TGFβ signaling can explain the final outcome of neural fate acquisition in the developing ectoderm, the molecular mechanisms that promote the differentiation of neural tissue are not clear. Previous studies have suggested that the period of competence during which neural inducing molecules act to prevent BMP is defined (Wilson and Hemmati-Brivanlou, 1997), the outcome being dependent on the total levels of BMP exposure or the intracellular effectors Smad1 and Smad5 (Suzuki et al., 1997; Wilson and Hemmati-Brivanlou, 1997). Fate acquisition appears to be dependent on the concentration and length of exposure to BMP signals, in a mechanism that is seemingly conserved in different vertebrates (Wilson et al., 1997; Barth et al., 1999). Therefore, the morphogenic effects of BMPs in ectodermal fates may be modulated at the level of transcriptional regulation of gene expression (Wilson et al., 1997). However, for permanent neural acquisition, BMP inhibition must be sustained (Hartley et al., 2001).

Role of prolonged BMP inhibition in neural specification

BMP inhibitors have been characterized mostly in the context of the organizer, which acts to impart dorsal/anterior fates in the surrounding germ layers, and neural fates in the overlaying ectoderm (Harland and Gerhart, 1997; Weinstein and Hemmati-Brivanlou, 1999). However, the potential involvement of neural progenitors in promoting neuralization of the ectoderm has not been addressed. The phenomenon of homeogenetic induction (neural tissue induces neuralization in non-neural ectoderm in recombination experiments) (Spemann and Mangold, 1924; De Robertis et al., 1989; Servetnick and Grainger, 1991) can be explained if neural tissue itself produces inhibitors of BMP signaling. In the ectoderm, BMPs activate two biochemical pathways, one mediated by Smads (Wilson et al., 1997) and a second mediated by the p38/MAP kinase pathway downstream of TAK1 (Shibuya et al., 1998; Goswami et al., 2001) (Fig. 7). Our preliminary analysis suggests that Smad7-mediated neural induction operates through an inhibition of both branches of BMP signaling. First, we have identified a secreted factor with weak homology to the cerberus and gremlin-families of BMP inhibitors, expressed in gastrula-stage embryos, with an ability to inhibit BMP and TGFβ ligands (E. B., I. M.-S., C. R. A. and A. H. B., unpublished). This, together with the broad expression of Smad7 itself in the prospective neural plate (Casellas and Brivanlou, 1998) suggests that prolonged BMP inhibition is a requirement for neural development.

We have also shown that a novel TAK1-binding protein (TAB3) is upregulated after Smad7 expression and can induce

neural marker expression in isolated explants. TAK1/TAB1 complexes have been shown to promote epidermal fates downstream of BMPs in *Xenopus* ectoderm (Shibuya et al., 1998; Goswami et al., 2001) and inhibition of TAK1 induces neural gene expression in animal caps (Goswami et al., 2001). There is presently little known about how neuralizing molecules modulate TAK1 activity during neural induction, or whether any other TABs associate with TAK1 in the emerging neural plate. This remains an important point, as associated TABs might switch the specificity of TAK1/TAB1 complexes during neural induction, hence effectively blocking BMP inputs. For example, TAB2 has been identified as a key effector in the activation of the NFkB and JNK pathways (Takaesu et al., 2000; Wang et al., 2001). In this report, we have shown that TAB3 can promote neural fates and that a C-terminal dominant-negative form of TAB3 can inhibit neural induction downstream of BMP inhibition. Because a C-terminal dominant negative TAB2 has been shown to bind TAK1, it is likely that TAB3C also acts by binding TAK1 (Takaesu et al., 2000). xTAB3C lacks the ubiquitin ligase-binding domain, and therefore it is likely that signaling through TAB3 is mediated by ubiquitination of TAK1. Therefore, we propose that the activation of TAB3 by neural inducers might be a mechanism for inhibiting epidermal fates mediated by TAK1/TAB1 (Fig. 8). This latter point is important because pathways other than BMP inhibition have been implicated in neural induction, namely the FGF and Wnt pathways (Harland, 2000; Wilson and Edlund, 2001). Whether BMP inhibition is sufficient for neuralization or whether it acts in synergy or in parallel with FGFs and Wnts remains a highly debated issue. Of particular importance is the modulation of p38 MAPK pathways by TAK1. Therefore, the regulation of TAK1 probably plays a role in signaling crosstalk between BMPs and FGFs, and might reconcile some findings in *Xenopus* and chick embryos about the involvement of both BMP inhibition and FGF signaling during neural induction (Wilson and Edlund, 2001).

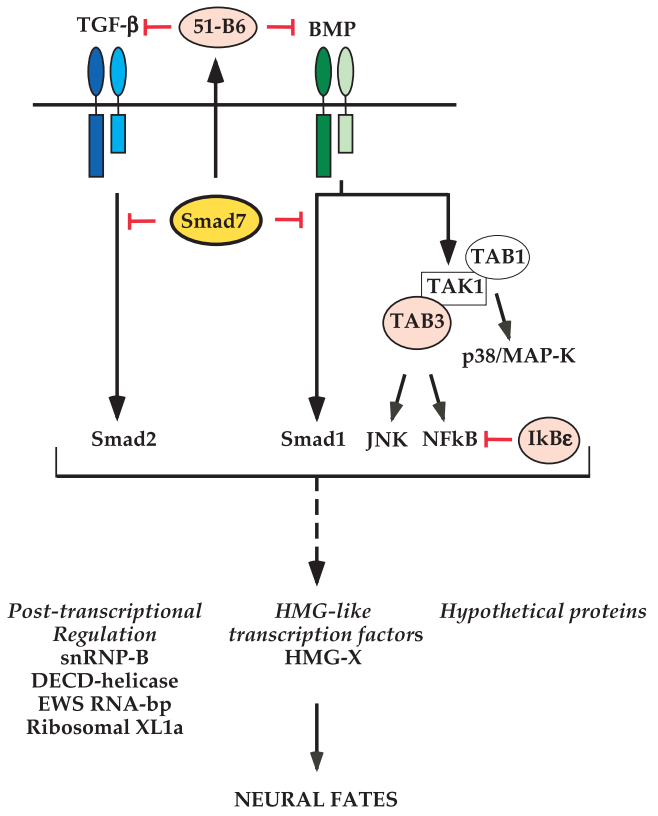

Fig. 8. Model for signal integration mediated by Smad7 in *Xenopus laevis* ectoderm. A proposed model of signaling integration in the context of neuralization might be mediated by Smad7. In this model, BMP signaling leads to the activation of both the Smad and TAK1 pathways. BMP signaling can be inhibited by 51-B6, a cerberus/gremlin-related factor (E. B., I. M.-S., C. R. A. and A. H. B., unpublished). We postulate that the transcriptional activation of TAB3 modulates the activity of TAK1, and might activate the NFkB and JNK pathways, as has been demonstrated for TAB2 in other contexts (Mazars et al., 2001; Wang et al., 2001). In addition, this switch might derepress some MAPK pathways, as TAK1/TAB1 complexes signal through p38 MAPK. Additional mechanisms to activate neural gene expression involve the HMG-X transcription factor and a variety of RNA-binding proteins implicated in post-transcriptional control. Red lines depict inhibitory signaling interactions. Green lines depict stimulatory signaling, either transcriptionally or through biochemical activation. Pink indicates genes identified in the array.

Whether TAB3 links TAK1 activity to NFkB or JNK instead of p38 pathways remains to be addressed. However, the activation of the JNK pathway downstream of Smad7 has been demonstrated in epithelial cells (Lallemand et al., 2001; Mazars et al., 2001) and NFkB homologs have been implicated in early dorsoventral patterning in *Xenopus* (Kao and Lockwood, 1996; Yang et al., 1998; Lake et al., 2001). Intriguingly, we also identified in the array several genes associated with NFkB and interleukin-related pathways, such as IkB-ε, cyclophilin-binding protein, interleukin enhancer binding factor 2 and interferon-related regulator. Whether these genes will be implicated in neural fate acquisition downstream or in parallel with BMP inhibition, and whether JNK or NFkB pathways are regulated by TAK1/TAB3 complexes remains to be explored. However, the regulation of TAK1 activity by Smad7 and possibly other BMP inhibitors suggests that TAK1 might be at the center of signaling crosstalk between BMP inputs and other pathways that may play additional roles during early neural fate acquisition.

Role of post-transcriptional control

A surprising result is the remarkably high number of RNA-binding proteins identified in the array, which are thought to play a role in post-transcriptional control. A few of these genes have been shown to modulate embryonic fate decisions, such as eIF4AI and eIF4AIII in neural and epidermal fate specification, respectively (Morgan and Sargent, 1997; Weinstein et al., 1997). We have extended these observations and demonstrated that a number of RNA-binding proteins can act as direct neural inducers or work in synergy with BMP antagonists to neuralize the ectoderm.

These proteins could form a complex to regulate the translation of neural genes or, alternatively, regulate BMP signaling at the post-transcriptional level. Further work should discern between these possibilities. Regardless of their mode of action during neural development, it is notable that these previously thought ‘ubiquitous’ factors show neural-specific patterns of expression, suggesting that they have specific targets during neural specification. In *Drosophila*, translational repression plays a crucial role in nervous system specification

downstream of Notch signaling (Okabe et al., 2001) and it is likely that similar mechanisms operate in vertebrate neural specification. This regulation might be crucial in the post-transcriptional regulation of the pro-neural genes, most of which show an initial broad RNA expression in the ectoderm that becomes restricted to the emerging neural territory (Bertrand et al., 2002). Therefore, there must be a tight regulation at the level of RNA stability, degradation or translational efficiency. Given the activity of the genes isolated in the array, our work strongly suggests that post-transcriptional control of gene expression is critical for neural fate acquisition.

Additional pathways implicated in early nervous system development

Cell-fate acquisition must extend from signaling to its overall transcriptional response in order to promote and maintain cell-fate decisions. It is not well understood whether permanent changes at the chromatin remodeling level are connected to fate decisions during embryogenesis. For example, the fate competence of the ectoderm to mesoderm-inducing signals is regulated by histone acetylation (Steinbach et al., 1997), and therefore global chromatin remodeling is likely to modulate fate decisions in other contexts as well. A variety of HMG-containing proteins have been implicated in fate changes (Meneghini et al., 1999; Decoville et al., 2001), although their regulation and mechanism of action is poorly understood. We have shown that the HMG-X gene can induce neural tissue downstream of BMP inhibition. Interestingly, cement gland fates are inhibited when HMG-X is co-expressed with Smad7, and therefore HMG-X might function in the patterning of the anterior ectoderm. Consistent with this idea, the expression of HMG-X is confined to the neural plate, and is absent from the cement gland (Kinoshita et al., 1994). In support of our model, we identified another HMG-containing protein (Baf57) (Domingos et al., 2002) that synergized with Smad7 in promoting neural fates in an expression cloning screen. Similarly, expression of Baf57 enhanced neural fates at the expense of cement gland fates (Domingos et al., 2002).

Interestingly, a large number of clones encode hypothetical proteins or have no matches in database searches, several of which are extremely conserved from *C. elegans* to humans. The high frequency of genes of unknown function, coupled to our preliminary analysis of their expression in the developing nervous system, highlights the gap existing in our knowledge of how early neural fate specification and induction is mediated. Overall, we have presented an initial analysis of the global changes in gene expression that occur after exposure to a neural inducer. This analysis has identified potential target genes and pathways implicated in the earliest specification of the nervous system, and highlighted the importance of post-transcriptional control during neural induction. Ongoing and future work will extend the microarray analysis of neural induction in a variety of conditions to refine our understanding of this complex embryological decision.

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