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XSIP1, a Member of Two-Handed Zinc Finger Proteins, Induced Anterior Neural Markers in *Xenopus laevis* Animal Cap

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We characterized *Xenopus SIP1* (*XSIP1*), Smad interacting protein, from activin-treated animal caps by differential screening. The *XSIP1* is very similar to mouse *SIP1* in the protein coding region including the zinc finger domain and homeodomain. The expression pattern was analyzed by RT-PCR and whole mount *in situ* hybridization. *XSIP1* expression was initially restricted to the dorsal marginal zone in the late gastrula and was subsequently expressed at the lateral edge of neural plate and, in the tailbud stage, in the forebrain, neural tube, and eye. Overexpression of *XSIP1* at the animal caps resulted in activation of anterior neural markers without mesodermal markers. Ectopic expression of *XSIP1* induced enlargement of neural cells and disordered eye formation. In addition to abnormal head phenotypes, many embryos were short-tailed. Our findings suggest that *XSIP1* is a transcriptional repressor, which may be involved in the activin-dependent signal pathway.

***Key Words: SIP1;* neural induction; *Xenopus laevis;* zinc finger.**

Early embryonic differentiation is governed by two principles. One is unequal distribution of maternal information and the other is signaling molecules mediating between cell–cell communication. In *Xenopus,* the Spemann organizer is formed in the dorsal mesoderm by maternal information and then secretes molecular signals, which play important roles in the induction and patterning of neural and mesodermal tissue. Many of these signal molecules are expressed especially in the organizer region and they are sorted into two main signaling activities. One is the activin-dependent signal transudation pathway, involving, for example chordin (1) and noggin (2); the other is the Wnt signaling pathway exemplified by siamous (3) and nr3 (4). Activin, which is a member of the transforming factor-β superfamily (TGF-β), can induce several mesodermal and neural tissues in animal caps depending on its concentration (5). On the other hand, BMPs, which are also members of TGF-β superfamily, can induce ventral formation through expression of signaling molecules in the ventral side and can also induce ventral mesoderm (6). The TGF-β superfamily includes activin and BMPs which can transmit their signals through Smad proteins and TGF-β signaling plays very important roles in *Xenopus* embryogenesis.

Because activin treatment of animal caps induce neural tissues to form sandwiching animal caps, we anticipated that certain molecules induced by activin dependent signals suppress mesoderm formation and promote neural formation. By differential screening, we identified *Xenopus SIP1* (*XSIP1*) as a candidate for this neural induction molecule. The *SIP1,* a Smad-interacting protein, was identified as a gene encoding two separated clusters of zinc fingers, one N-terminal and one C-terminal, and a homeodomain. Like other two-handed zinc finger/homeodomain proteins, *SIP1* binds to different promoters, including the 5′-CACCT sequence (7). *SIP1* is a member of the *δEF1* family and the zinc fingers of these two genes are bound with two hands to a two-target site (8). *SIP1* binds to the Smad MH2 domain with its Smad binding domain (SBD) and may act as a regulation factor of the immediate response gene for the activin-dependent signal pathway.

Here we analyze the expression and activity of the *XSIP1* gene. We show that *XSIP1* expression demarcates the presumptive neural plate very early, by the middle of gastrulation, and later defines the anterior neural tube. Overexpression of *XSIP1* induced enlargement of neural tissue in anterior region and some

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FIG. 1. (A) Nucleotide sequence of *XSIP1*. Predicted ORF is boxed. (B) Amino acid sequence comparison between mouse *SIP1* and *Xenopus XSIP1*. Gray boxes represent identical or biochemically similar amino acids. The position of the C2H2 type zinc finger in STP1 is indicated by bold underlining, other zinc fingers in SIP1 are indicated by thin double underlining. The dotted line indicates the Smad binding domain (SBD).

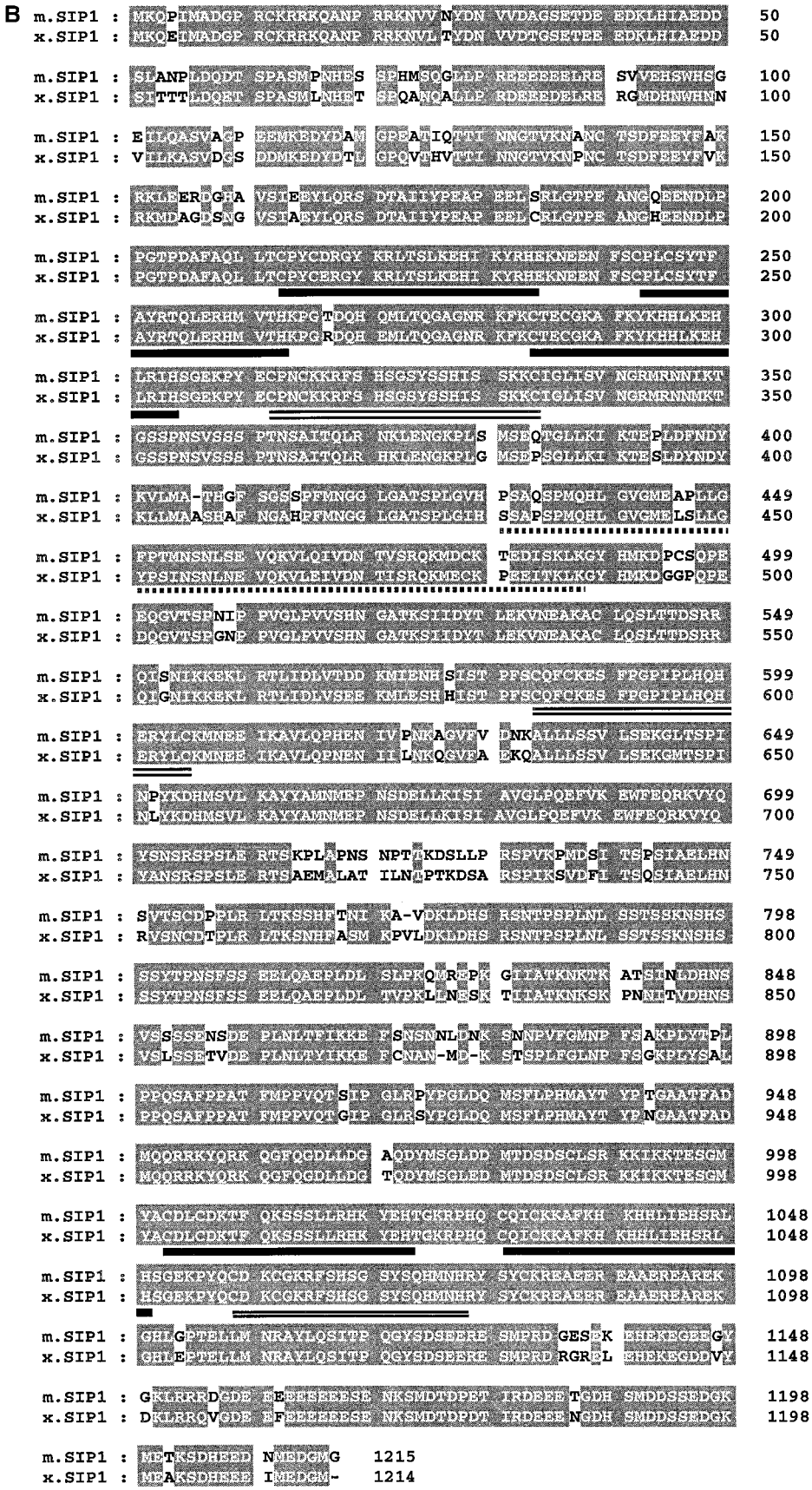

FIG. 1—*Continued*

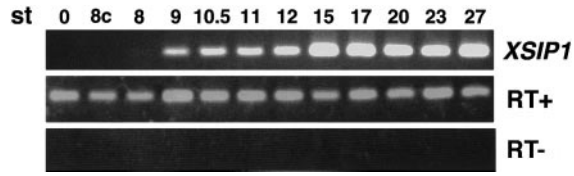

FIG. 2. RT-PCR analysis was performed at various stages. Stages according to Niewcoop and Faber (1956) are shown over the lanes. ODC (lower panel) is indicated as a loading control.

embryos failed to form eye vesicles. Ectopic expression of *XSIP1* induced anterior neural markers suggesting that *XSIP1* plays a role in early neurogenesis.

MATERIALS AND METHODS

Eggs and embryos. Xenopus eggs were obtained by injecting adult males and females with human chorionic gonadotropin (Gestron) at a dose of 600 U. Fertilized eggs were dejellied by treatment with 4.5% cysteine hydrochloride in Steinberg's solution (pH 7.8) with kanamycin sulfate (100 mg/l; Banyu Pharmaceutical Co.), then washed thoroughly with Steinberg's solution (pH 7.4). Embryos were transferred to culture dishes containing Steinberg's solution according to Nieukoop and Faber (12).

Differential screening. Animal caps were cut from the late blastula and treated with 100 ng/ml activin A (supplied by Dr. Yuzuru Eto, Central Research Laboratories of Ajinomoto Co. Inc., Japan) for 1 h, then cultured for 7–10 h in Steinberg's solution. Poly(A)-tailed RNA was extracted from 2000 animal caps cultured in Steinberg's solution for 7–10 h after activin A treatment. In brief, total RNA was extracted according to the guanidine thiocyanate method (10). Then Poly(A)-tailed RNA was extracted from total RNA by binding to oligo d(T) cellulose. A cDNA library was constructed using this RNA as a template. The library, consisting of approximately 100,000 clones, was screened using ^{32}P-labeled probes generated from stage-10 whole embryo cDNA. Negative clones were sequenced and compared with known sequences, and novel clones were examined for their mRNA localization in embryos by hole-mount *in situ* hybridization.

In vitro transcription and microinjection. Capped sense RNAs for microinjection were synthesized (11) by using Ambion Megascript (Austin, TX) SP6 and T7 kits. The clone used was as follows: *XSIP1* linearized by *Not*I and transcribed by Sp6; with embryos fertilized *in vitro*, dejellied, cultured, and injected with solutions as described (12). Embryos were injected in the animal cap with 100 pg to 1 ng at the 2-cell stage into both or one blastomere or at the 4-cell stage into the left side blastomeres.

Whole mount in situ hybridization. Whole mount *in situ* hybridization was performed as described in Harland (13): Embryos obtained from albino females were used. Digoxigenin (DIG)-labeled antisense RNA corresponding to 4953 bp of XSIP was synthesized *in vitro.* Antidigoxigenin antibodies were purchased from Boehringer Mannheim. To prepare sections, embryos stained by whole mount *in situ* hybridization were dehydrated in ethanol, transferred to xylene and embedded in paraffin and sectioned at 10 μm.

RT-PCR. Total RNA was isolated from various developmental stages and mRNA was injected animal caps using ISOGEN (NIPPON GENE). Two micrograms of total RNA were used as a template to generate first strand cDNA. One tenth of this cDNA was used as a template in subsequent RT-PCR (Reverse Transcription-polymerase chain reaction) analysis. The primers used were: XSIP (forward, 5′-GGGAGCCTCACCTACTCTCCT-3′; reverse, 5′-ATCCGCCAGATCT-CTTGCT-3′), ODC (14), ms-actin (15), F-spondin (16), XAG1 (17, 18), NCAM (19) and X1HBOX6 (20).

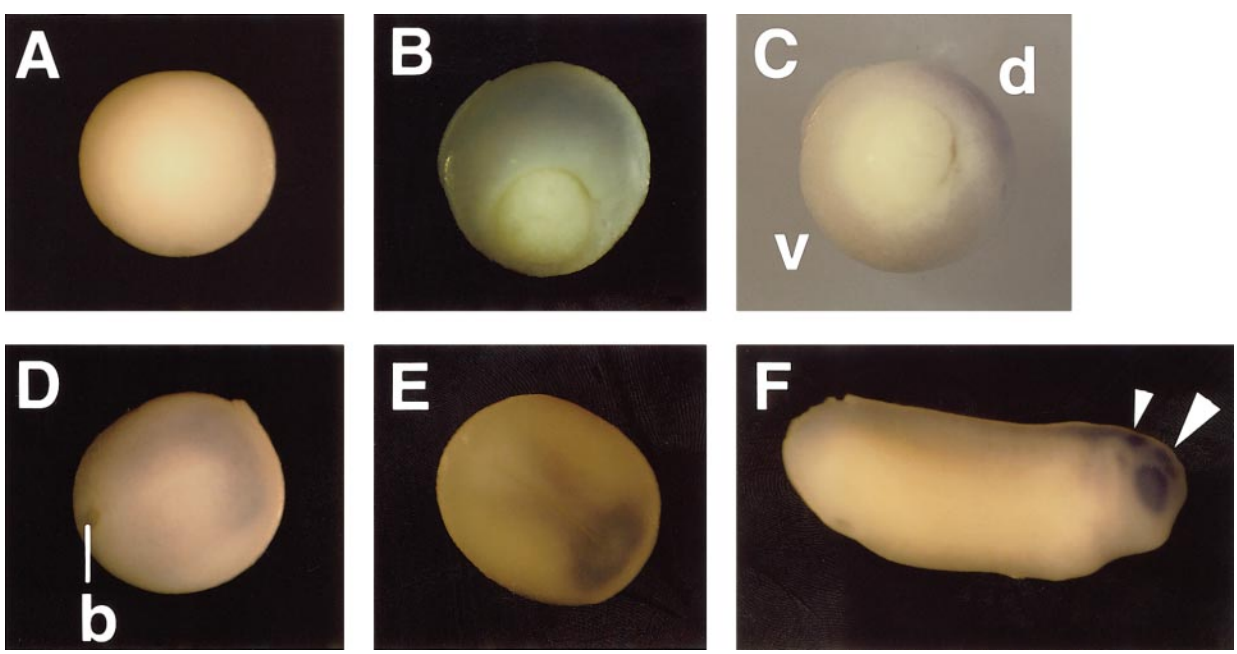

FIG. 3. Whole mount *in situ* hybridization with an *XSIP* antisense probe. (A) Stage 9 embryo, vegetal view. XSIP was not detected. (B and C) Stage 10.5 embryo, dorsovegetal view and vegetal view. *XSIP1* is detected in the dorsal ectoderm. (D) Stage 12 embryo, dorsal view. (E) Stage 17 embryo, dorsal view. (F) Stage 26 embryo. Large white arrowhead and small white arrowhead indicate the telencephalon and mesencephalon. d, dorsal; v, ventral; b, blastopore.

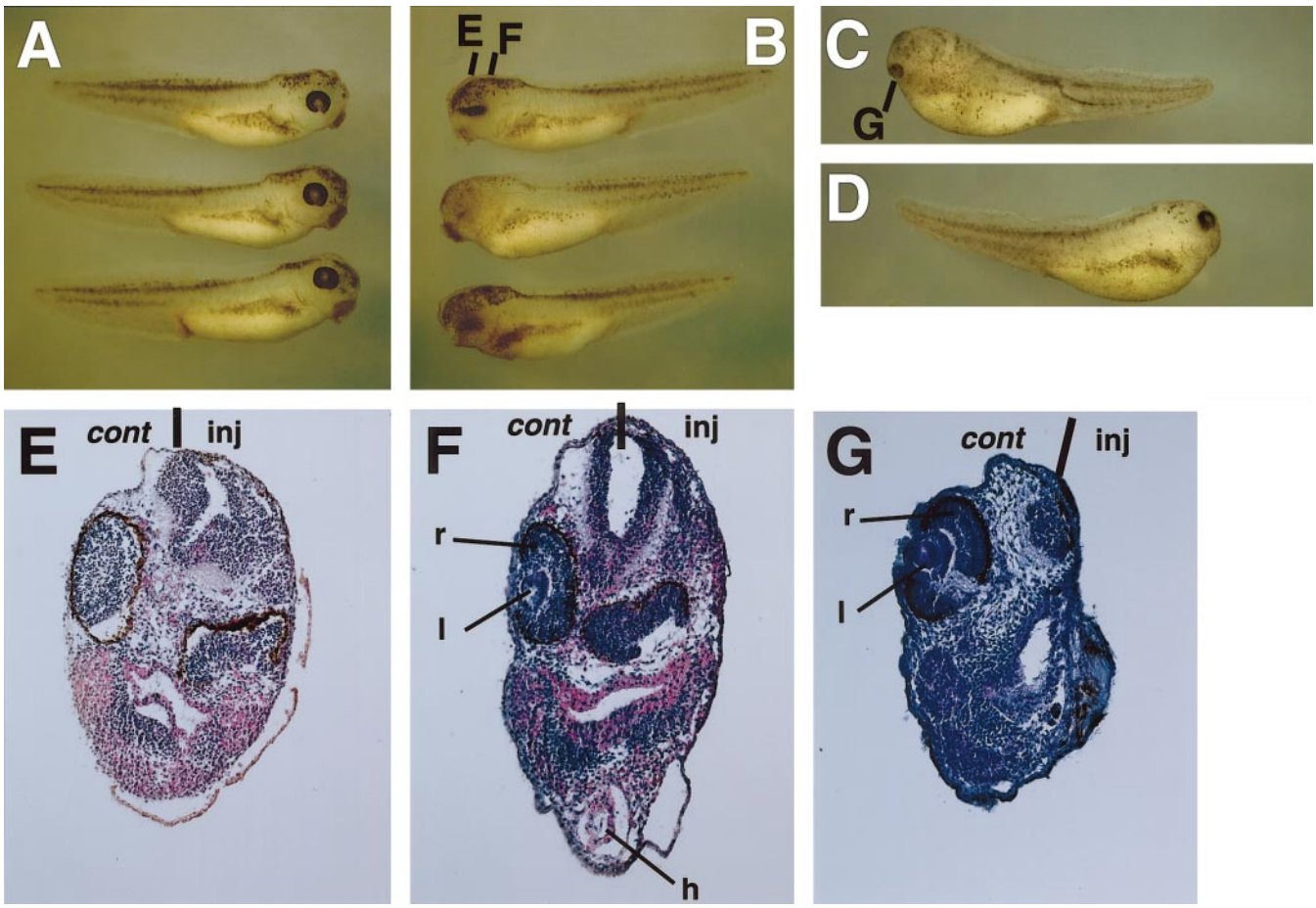

FIG. 4. *XSIP1* overexpressing embryos. (A) Stage 38 embryo, uninjected control side. (B) Stage 38 embryo, *XSIP1* mRNA (500 pg) injected side. (C) Stage 38 embryo, *XSIP1* mRNA (1 ng) injected side. (D) Stage 38 embryo, uninjected side. (E and F) Transverse section of the embryo shown in B. The section levels are indicated in B. (G) Transverse section of the embryo shown in C. The section levels are indicated in C. l, lens; r, retina; nt, neural tube.

RESULTS AND DISCUSSION

Animal caps treated with activin A differentiated into mesodermal or endodermal tissues depending on concentration, and treated animal caps mimic the organizer and induced a variety of tissues in other untreated animal caps with contact. For example, animal caps, treated with 100 ng/ml activin A for 1 h and then cultured for 7–10 h, showed strong induction of neural tissues into sandwiching animal caps (data not shown). In order to isolate the genes responsible for activating neural determination, we carried out differential screening between activin A treated animal caps and late blastula whole embryos. We isolated a number of clones, up-regulated in animal caps treated with activin and cultured but not in whole embryo blastulas. One clone had a two-handed zinc finger/homeodomain class gene, but lacked the 5′ sequence. We screened the gastrula library again and isolated this clone (Fig. 1A). In comparison with other vertebrate zinc finger genes, this clone showed marked similarities to mouse Smad Interaction Protein 1 (*SIP1*), with 77% amino acid identity (Fig. 1B), and to the vertebrate δ-crystalline enhancer binding protein (δ*EF1*), with 39% identity. The homologies between mouse *SIP1* and this gene were 68%, at the nucleotide level, and 83%, at the biochemically similar amino acid level, (Fig. 1B). Notably, these proteins share the same amino acids in the zinc finger domain and certain similarities in their Smad binding domain (SBD). These findings suggest that this clone is a homologue of *SIP1* in *Xenopus*, and for that reason the clone was named *XSIP1*.

The length of *XSIP* was 4529 bp and conceptual translation of the 3642 bp ORF yielded a 1214 amino acid sequence containing a two-handed zinc finger and a homeodomain. This sequence also contains 230 bp of the 5′-untranslated region and 653 bp of the 3′-untranslated region. The homeodomain has conserved arginine and asparagine at the same position, which is

critical for DNA binding. However *mSIP1*, *XSIP1* and *δEF1* do not contain these critical amino acids. This suggests they are not able to bind DNA directly and that they are the same type of two-handed zinc finger/homeodomain class gene.

To examine the *XSIP1* gene expression pattern in embryos at various developmental stages, RT-PCR analysis and whole mount *in situ* hybridization were carried out (Fig. 2). We designed *XSIP1* specific primers to discriminate another clone, which was for several of the first amino acids. This clone had almost the same sequence (97% similarity to *XSIP1*) but there was no effect on embryos with the injection of its mRNA (data not shown), suggesting that it might be a splicing isoform. The single product was not detected from the unfertilized egg to the stage 8 embryo, and then first appeared at stage 9 and its expression was maintained through the progression of development, gradually increasing through the early neurula stage, and was then sustained in the tail-bud stage. *ODC* confirmed equal loading of PCR products in each lane.

To determine localization of *XSIP1* expression, we performed whole mount *in situ* hybridization using a digoxygenin-labeled *XSIP1* sense and antisense RNA probe on whole embryos. There was no detectable signal in stage 9 embryos (Fig. 3A), based on RT-PCR detection. The first detectable expression of *XSIP1* RNA was observed in the dorsal ectoderm at stage 10.5 (Figs. 3B and 3C). From the early neurula stage onwards, *XSIP* expression had entered a second phase with restriction to the lateral edge of the neural plate (Fig. 3D), i.e., the presumptive dorsal neural tube and neural crest. Then, *XSIP1* was expressed in the neural plate and tube along the anteroposterior axis of the developing central nervous system (CNS) (Fig. 3E). After the tailbud stage, expression was seen mainly in the telencephalon and diencephalon of the forebrain, and more posteriorly along the neural tube and eye (Figs. 3F and 3G).

Since many zinc finger genes expressed in the early gastrula through neurula stages induce neural genes like those of the zinc family (21), we induced overexpression of *XSIP1* by microinjection of synthesized *XSIP1* mRNA to test the neuralizing activity. *XSIP1* mRNA and control mRNA (pCS2) were transcribed in vitro, then dissolved in Gurdon's buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris-HCl, pH 7.5). First, we injected *XSIP1* mRNA into one blastomere of 2-cell stage embryos to over express *XSIP1* hemilaterally. In almost all cases, the *XSIP1* injected side was enlarged and showed poor eye formation (Fig. 4B) or ocular defects (Figs. 4C and 4G), whereas the uninjected control side was normal (Fig. 4A). The sections through the head regions of injected embryos showed neurals to be markedly thickened of the injection side (Figs. 4D and 4F). Hyperplastic neural walls, as well as distorted eyes

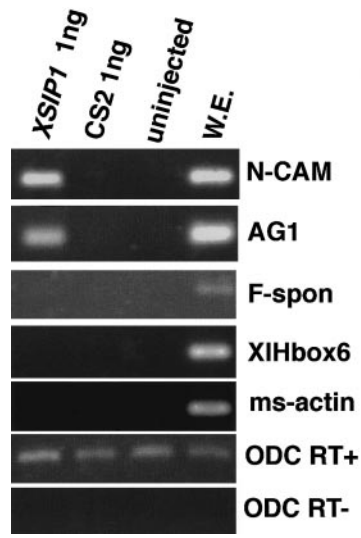

FIG. 5. *XSIP1* induced neural marker genes without mesoderm induction in animal cap explants. Embryos were injected with 1 ng of *XSIP1* or control mRNA into the 2-cell stage animal pole. Animal caps were explanted at stage 9 and cultured for 2 days. N-CAM, general neural marker; XAG1, cement gland marker; F-spondin and X1Hbox6, trunk/tail neural marker; ODC, internal positive control.

and neural tissues characterized the injected side. Particularly, no lens induction occurred on the injected side and retinal pigment cells were variably diminished. In addition to the abnormal head phenotypes, embryos injected with 1 ng of *XSIP1* in both blastomeres developed short tails (Fig. 4G), probably due to inhibition of Xbra (7).

As has been noted, *XSIP1* plays important roles in early neural development. To clarify the effect of injected *XSIP1* mRNA in molecular marker gene expression, after the injection of mRNA into the animal pole of 2-cell stage embryos, the animal caps from these embryos at stage 9 were dissected and total RNA from the caps, which had been cultured for 2 days, was extracted. We carried out RT-PCR analysis, demonstrating that *XSIP1* mRNA injection caused up-regulation of *N-CAM* (a general neural marker), and *XAG1* (cement gland marker), but no up-regulation of *F-spondin*, *XlHbox6* (trunk/tail neutral marker) or *ms-actin* (mesodermal marker) (Fig. 5). The structures induced by *XSIP1* injection suggest that *XSIP1* is an early activin response gene. When animal caps are cultured in a medium containing activin A, neural tissue is secondarily induced, owing to primarily induced mesodermal tissue, resulting in expression of *N-CAM*. Mouse *SIP1* interacts with Smads via the SBD and by binding the *Xbra-2* enhancer to regulate its expression and down-regulating Xbra expression (8). *XSIP1* is induced by activin A (data not shown), thereby inducing the neural marker without mesodermal formation and suppresses *Xbra* expression indicate the possibilities

that *XSIP1* makes neuroectoderm from neural rather than mesodermal tissue. *XSIP* injected animal caps expressed neural marker but did not form neural tissue (data not shown). This, it is probable that other signals, which can induce neural tissue in animal caps with activin/Smad 2 signaling, cooperate with *XSIP1* to induce the formation of neuroectoderm from neural tissue.

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