# *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. © 2000 Academic Press

*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 activindependent 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- $\beta$  superfamily (TGF- $\beta$ ), 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- $\beta$  superfamily, can induce ventral formation through expression of signaling molecules in the ventral side and can also induce ventral mesoderm (6). The TGF- $\beta$  superfamily includes activin and BMPs which can transmit their signals through Smad proteins and TGF- $\beta$  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 Smadinteracting 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  $\delta EF1$  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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caggtetega catgtatget catacaggaa aacattegeg tgggtettac gcagagaggg aaagcataac attggagcat gaggaagagg agaatggtga aggataatg acttgettga atgaactgaa atgcattata	agtatcagcg agatatgaca tgtgacttat aaaggccaca actgcactct tcacagcaca agcacgtga tcctcagggc gaaaggagg ccattcaatg gaggatggca aaacactgct ggggcagtt tttgtacagt	caacaagga gattctgact gtgataagac ccaatgtcaa ggcgagaagc tgaatcatag gaagggacat tattctgact gggacgatgt tgaaaataaa gacgatagtt tgtaa ttgac gttttaagat ctgccaaaag attaaggcct aacaaatag	titcagggtg cctgtctgtc attccagaaa atctgtaaaa cgtatccatg gtattcctac ttggaaccca cagaggaaag ttatgacaaa agcatggaca cggaagatgg ttactgcattt gtttatgcac tcaaataaag aaaactgtg ttaattgta	actgyagety actgetega caggaaaaag agcagetete aagcatteaa tgacaaatgt tgtaagaggg cggagetaet agaaagtatg ttgaggagac cagateegga cagaategga gtaaatggaa taagetteet gtgeetgatg ttgagttgag	ccacattigc tggtacacag attaagaaga acacaaacat ggcaaacgct aggcagagga gatgaataga ccaagagaca aggttggtga cacaattaga gccaaatcag ttgtttccag cttccaggaa ttattgtgaa gactaaaccc ctacttatat	agatatgcag gattacatgt cagaaagtgg caaatatgaa catttaatag tctcgcattc agggaagct gcgtattgc gaggaagaga tgaggagtt gatgaagaag tcatgaagaag tagtattgtt gcctgtatag gaactgcatt tgtgtttaat	3080 3120 3220 3390 3360 3430 3500 3570 3640 3780 3780 3780 3920 3990 4060 4130 4270
caggtetega catgtatget catacaggaa aacattegeg tgggtettac gcagagaggg aaagcataac attggagcat agggaagagg agaatggtga agaatggtga acttgettga atgaactgaa atgcataatt agcattata acttaaaaaa	agtatcagcg agatatgaca tgtgacttat aaaggccaca actgcactct tcacagcaca aggcacgtga tcctcagggc gaaaggag ccattcaatg gaggatggca aaacactgct gggggcagtt tttgtacagt ctttaagcac aagaagatat	caacaagga gattctgact gtgataagac ccaatgtcaa ggcgagaagc tgaatcatag gaagggacat tattctgact gggacgatgt tgaaaataaa gacgatagtt tgtaa Ltgac gttttaagat ctgccaaaag attaaggcct aacaaatag	titcagggtg cctgtctgtc attccagaaa atctgtaaaa cgtatccatg gtattcctac ttggaaccca cagaggaaag ttatgacaaa agcatggaca cggaagatgg tactgcattt gtttatgcac tcaaataaag aaaactgtg ttaattgtat tattaataca	actgyagetg actgetega agcagetete aagcatteaa tgacaaatgt tgtaagaggg cggagetaet agaaagtatg ttgaggagac cagateegga caaatggaa taagetteet gtgeetgatg tttgagttgg tggtteaaga gaattgeaet	ccacattigc tggtacacag attaagaaga acacaaacat ggcaaacgct aggcagagga gatgaataga ccaagagaca aagttggtga cacaattaga gccaaatcag ttgtttccag cttccaggaa ttattgtgaa gactaaaccc ctacttatat gaataaaag	agatatgcag gattacatgt cagaaagtgg caaatatgaa catttaatag tctcgcattc aagggaagct gcgtattgc gaggaagaga tgaggagagt gatgaagaga tagtattgtt gcctgtatag gaactgcatt tgtgtttaat atcactacaa tgcccgcact	3080 3150 3220 3390 3360 3430 3500 3570 3640 3770 3780 3780 3780 3850 3990 4060 4130 4200 4240
caggtetega catgtatget catacaggaa aacattegeg tgggtettae gcagagaggg aaagcataac attggagcat gaggagagag agaatggtga aggataatg acttgettga atgaactgaa atgeaatttata acttaaaaaa gttgtacace	agtatcagcg agatatgaca tgtgacttat aaaggccaca actgcactct tcacagcaca aagcacgtga tcctcagggc gaaaaggagg cattcaatg ggggaggg gaggatggca aacactgct ggggcagtt tttgtacagt ttttaagcac aagaagatat agtattccta	caaacaagga gattctgact gtgataagac ccaatgtcaa ggcgagaagc tgaatcatag gaagggacat tattctgact gggacgatgt tgaaaataaa gacgatagtt tgtaa ltgac gttttaagat ctgccaaaag attaaggcct aaacaaatag ttctaattta	titcagggtg cctgtctgtc attccagaaa atctgtaaaa cgtatccatg gtattcctac ttggaaccca cagaggaaag ttatgacaaa agcatggaca cggaagatgg ttattgcattt gtttatgcac tcaaataaag aaaactgtg ttaattgtat tattaataca atttatggg	actgyagetgy actggaaaag agcagetete aagcatteaa tgacaaatgt tgtaagaggg cggagetaet agaaagtatg ttgaggagae cagateegga cagateegga gactgetggt gtgeetgatg tggeteaag gaattgeaet tttttaaaa etegeaetae	ccacatttgc tggtacacag attaagaaga ttctgagaca acacaaacat ggcaaacgct aggcagagga gatgaataga ccaagagaca aagttggtga cacaattaga gccaaatcag ttgtttccag attatgtgaa gactaaaccc ctacttatat gaataaaaag	agatatgcag gattacatgt cagaaagtgg caaatatgaa catttaatag tctcgcattc aagggaagct gcgtatttgc gaggaagaga tgaggagtt gatgaagaag tagtattgtt gcctgtatag gaactgcatt tgtgtttaat atcactacaa tgcccgcact	3080 3150 3220 3390 3360 3430 3570 3640 3710 3780 3780 3780 3920 4060 4130 4200 4270 4340
caggtetega catgtatget catacaggaa aacattegeg tgggtettae gcagagaggg aaagcataac attggagcat gaggaagagg agaatggtga aggataatg acttgettga atgcaaaatt agcattataa acttaaaaaa gttgtacace cctgcattet	agtatcagcg agatatgaca tgtgacttat aaggccaca actgcactct tcacagcaca aggcacgtga tcctcagggc gaaaggagg ccattcaatg ggggcagtt tttgtacagt tttgtacagt aggaagatgt aggaagatgt tttgtacagt cattcaatgct ggggcagtt tttgtacagt tttgtacagt tttgtacagt agaagatgta agaagatat agtattccta	caaacaagga gattctgact gtgataagac ccaatgtcaa ggcgagaagc tgaatcatag gaagggacat tattctgact ggacgatgt tgaaaataaa gacgatagtt tgtaa Ltgac gttttaagat ctgccaaaag attaaggcct aaacaaatag ttctaattta ttccacttta cttccacttta	tttcagggtg cctgtctgtc attccagaaa atctgtaaaa cgtatccatg gtattcctac ttggaaccca cagaggaaag ttatgacaaa agcatggaca cggaagatgg ttattgcactt gtttatgcac tcaaataaag aaaactgtg ttaattgat tattaataca atttatggtg tccaaatgaa	actgyagetgy actggaaaag agcagetete aagcatteaa tgacaaatgt tgtaagaggg cggagetaet agaaagtatg ttgaggagae caaatggaa taagetteet gtgeetgatg tggeteaaga gaattgeaet ttttttaaaa etegeaetae	ccacattgc tggtacacag attaagaaga ttctgagaca acacaaacat ggcagagga gatgaataga ccaagagaca aagttggtga cacaattaga gccaaatcag ttgtttccag cttccaggaa ttattgtgaa gactaaaccc ctacttatat gaataaaaag gatatgtcag tttaggcctg	agatatgcag gattacatgt cagaaagtgg caaatatgaa catttaatag tctcgcattc aagggaagct gcgtatttgc gaggaagaga tgaggagtt gatgaagaag tagtattgtt gcctgtatag gaactgcatt tgtgtttaat atcactacaa tgcccgcact tattatgatt	3080 3150 3220 3390 3360 3430 3570 3640 3710 3780 3780 3990 4060 4130 4200 4270 4340 4410
caggtetega catgtatget catacaggaa aacattegeg tgggtettae gcagagaggg aaagcataac attggagatga aggaatggtga agaatggtga acttgettga atgaactgaa atgeattata agcatttata agttgtacace ectgeattet ttataatgea	agtatcagcg agatatgaca tgtgacttat aaggccaca actgcactct tcacagcaca tcctcagggc gaaaggagg cattcaatg gaggatggca aacactgct gggggcagtt tttgtacagt tttaagcac cattaagga gggggggggg	caacaagga gattctgact gtgataagac ccaatgtcaa ggcgagaagc tgatcatag gaagggacat tattctgact ggacgatgt tgaaaataaa gacgatagtt tgtaa ltgaa gttttaagat ctgccaaaag attaaggcct aaacaaatag ttctaattta ttccacttta cttcatgatt	tttcagggtg cctgtctgtc attccagaaa atctgtaaaa cgtatccatg gtattcctac ttggaaccca cagaggaaag ttatgacaaa agcatggaca cggaagatgg ttattgcattt gtttatgcac tcaaataaag aaaactgtg ttaattgtg tattatgatg atattatggtg tcaaatgaa	actgyagetgy actggtega caggaaaaag agcagetete aagcatteaa tgacaaatgt tgtaagaggg cggagetaet agaaagtatg ttgaggagae cagateegga cagateegga ttgagttgg tggeteaaga gaattgeaet ttttttaaaa etegeaetae accacacaat gtettaaaag	ccacattige tggtacacag attaagaaga ttetgagaca acacaaacat ggcagagga gatgaataga ccaagagaca aagttggtga cacaattaga gccaaatcag ttgtttecag ettecaggaa ttattgtgaa gactaaacce ctacttatat gaataaaaag gatagtecggat	agatatgcag gattacatgt cagaaagtgg caaatatgaa catttaatag tctcgcattc aagggaagat gcgtatttgc gaggaagaga tgaggagtt gatgagagat tagtattgtt gcctgtatag gaactgcatt tgtgtttaat atcactacaa tgcccgcact tattatgatt atttatgat atttattt	3080 3150 3220 3390 3360 3570 3640 3710 3780 3780 3920 3920 3920 4060 4130 4200 4270 4340 4410 4480

**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).

В

m.SIP1	:	MKQ <mark>P</mark> IMADGP RCKRRKQANP RRKNVVN	YDN VVDAGSETDE EDKLHIAEDD	50
x.SIP1		MKQ <mark>E</mark> IMADGP RCKRRKQANP RRKNVLT	YDN VVDTGSETEE EDKLHIAEDD	50
m.SIP1	:	SLANPLDODT SPASMPNHE <mark>S SPHM</mark> SOG	LLP REFEREELRE <mark>SV</mark> VEHSWHS <mark>G</mark>	100
x.SIP1		ST <mark>TTT</mark> LDORT SPASMLNHE <mark>T SPOA</mark> NOA	LLP RDEREDELRE <mark>RG</mark> MDHNWHN <mark>N</mark>	100
m.SIP1	:	ETLQASVACP BENKEDYDAM GPEATIQ	TTI NNGTVKN <mark>a</mark> nc TSDFEEYF <mark>a</mark> k	150
x.SIP1		VILKASVDCS DDMKEDYDIL GPOVTHV	TTI NNGTVKN <mark>P</mark> NC TSDFEEYF <mark>V</mark> K	150
m.SIP1	:	RKLE <mark>ERDGRA</mark> VSIEEVLORS DTAITYP	eap eel <mark>S</mark> rlgtpe ang <mark>o</mark> bendlp	200
x.SIP1		RKMDAGDSNG VSIAEVLORS DTAITYP	Eap eel <mark>C</mark> rlgtpe ang <mark>b</mark> eendlp	200
m.SIP1	:	PGTPDAFAQL LTCPYCDRGY KRLTSLK	EHI KYRHEKNEEN FSCPLCSYTF	250
x.SIP1		PGTPDAFAQL LTCPYCERGY KRLTSLK	EHI KYRHEKNEEN FSCPLCSYTF	250
m.SIP1	:	AYRTQLERHM VTHKPGTDQH QMLTQGA	GNR KFKCTECGKA FKYKHHLKEH	300
x.SIP1		AYRTQLERHM VTHKPGTDQH EMLTQGA	GNR KFKCTECGKA FKYKHHLKEH	300
m.SIP1	••••	LRIHSGEKPY ECPNCKKRFS HSGSYSS	HIS SKKCIGLISV NGRMRNNIKT	350
x.SIP1		LRIHSGEKPY ECPNCKKRFS HSGSYSS	HIS SKKCIGLISV NGRMRNNMKT	350
m.SIP1	••	GSSPNSVSSS PTNSAITQLR NKLENGK	PL <mark>S</mark> MSE <mark>Q</mark> TGLLKI KTE <mark>P</mark> LDFNDY	400
x.SIP1		GSSPNSVSSS PTNSAITQLR HKLENGK	PL <mark>G</mark> MSE <mark>P</mark> SGLLKI KTE <mark>S</mark> LDYNDY	400
m.SIP1	00 OF	KVIMA-TH <mark>G</mark> F SGS <mark>S</mark> PFMNGG LGATSPL	GVH PSAQSPMQHL GVGMEAPLLG	449
x.SIP1		KLIMA <mark>A</mark> SH <mark>A</mark> F NGA <mark>H</mark> PFMNGG LGATSPL	GIH SSAPSPMQHL GVGMELSLLG	450
m.SIP1	:	FPTMNSNLSE VQKVLQIVDN TVSRQKM	DCK TEDISKLKGY HMKD <mark>PCS</mark> OPE	499
x.SIP1		YPSINSNLNE VQKVLEIVDN TISRQKM	ECK PEEITKLKGY HMKD <mark>GGP</mark> OPE	500
m.SIP1	:	EQGVTSP <mark>NI</mark> P PVGLPVVSHN GATKSII	DYT LEKVNEAKAC LQSLTTDSRR	549
x.SIP1		DQGVTSP <mark>GN</mark> P PVGLPVVSHN GATKSII	DYT LEKVNEAKAC LQSLTTDSRR	550
m.SIP1	:	QI <mark>S</mark> NIKKEKL RTLIDLVIDD KNIENH S	IST PFSCQFCKES FPGPIPLHQH	599
x.SIP1		QI <mark>G</mark> NIKKEKL RTLIDLVSEE KMLESH	IST PFSCQFCKES FPGPIPLHQH	600
m.SIP1	:	ERYLCKMNEE IKAVLOPHEN IV PNKA	OVF <mark>V</mark> D <mark>NK</mark> ALLLSSV LSEKGLTSPI	649
x.SIP1		ERYLCKMNEE IKAVLOPNEN II LNKO	AVE <mark>A</mark> D <mark>KQ</mark> ALLLSSV LSEKGMTSPI	650
m.SIP1		N <mark>P</mark> yrdhmsvl kayyammep nsdellr	ISI AVGLPQEFVK EWFEQRKVYQ	699
x.SIP1		N <mark>l</mark> yrdhmsvl kayyammep nsdellr	ISI AVGLPQEFVK EWFEQRKVYQ	700
m.SIP1	60 B6	YSNSRSPSLE RTS <mark>KPL</mark> A <b>PNS NPT</b> IKDS	ILLP RSPVK <mark>P</mark> MD <mark>S</mark> I TS <mark>P</mark> SIAELHN	749
x.SIP1		YANSRSPSLE RTS <mark>AEMALAT ILN1977</mark>	IDSA RSPIKSVDF1 TSQSIAELHN	750
m.SIP1	:	SVTSCOPPLR LTKSSHF <mark>T</mark> NI K <b>A-V</b> DKI	.DHS RSNTPSPINL SSTSSKNSHS	798
x.SIP1		RVSNCD <mark>TPLR LTKSNHFA</mark> SM KP <b>VL</b> DKI	.DHS RSNTPSPINL SSTSSKNSHS	800
m.SIP1	::	SSYTPNSFSS EELQAEPLDL SLPK <mark>O</mark> M	OPK GITATKNETE ATSUNGDENS	848
x.SIP1		SSYTPNSFSS EELQAEPLDL TVPK <mark>L</mark> L	NSK TITATENESE PNNITVDENS	850
m.SIP1	:	VS <mark>S</mark> SSE <mark>NS</mark> DE PLNLTFIKKE F <mark>S</mark> NSN <mark>NI</mark>	D <mark>NK SNNPVFGMNP FS<mark>A</mark>KPLYT<mark>P</mark>L</mark>	898
x.SIP1		VS <mark>L</mark> SSE <mark>TV</mark> DE PLNLTYIKKE F <mark>C</mark> NAN-1	D-K S <mark>T</mark> SPLFGLNP FS <mark>G</mark> KPLYS <mark>A</mark> L	898
m.SIP1	:	PPQSAFPPAT FMPPVQT <mark>S</mark> IP GLR <mark>P</mark> YP(	ildo msflphmayt yp <mark>t</mark> gaatfad	948
x.SIP1		PPQSAFPPAT FMPPVQT <mark>G</mark> IP GLR <mark>S</mark> YP(	ildo msflphmayt yp <mark>n</mark> gaatfad	948
m.SIP1	:	MQQRRKYQRK QGFQGDLLDG AQDYMSO	HLDD MTDSDSCLSR KKIKKTESGM	998
x.SIP1		MQQRRKYQRK QGFQGDLLDG TQDYMSO	HLED MTDSDSCLSR KKIKKTESGM	998
m.SIP1	::	YACDLCDKTF QKSSSLLRHK YEHTGKF	PHO COICKKAFKH KHHLIEHSRL	1048
x.SIP1		YACDLCDKTF QKSSSLLRHK YEHTGKF	PHO COICKKAFKH KHHLIEHSRL	1048
m.SIP1	:	HSGEKPYQCD KCGKRFSHSG SYSQHMA	HRY SYCKREAEER EAAEREAREK	1098
x.SIP1		HSGEKPYQCD KCGKRFSHSG SYSQHMA	HRY SYCKREAEER EAAEREAREK	1098
m.SIP1	:	GHL <mark>G</mark> PTELLM NRAYLQSITP QGYSDSB	iere: Smprd <mark>GES</mark> ox Eheregee <mark>c</mark> y	1148
x.SIP1		GHL <mark>E</mark> PTELLM NRAYLQSITP QGYSDSB	Iere: Smprd <mark>RGR</mark> o <mark>L</mark> Eheregdovy	1148
m.SIP1	:	GKLRRR <mark>D</mark> GDE E <mark>E</mark> EEEEESE NKSMDTI	OPET IRDEEE <mark>T</mark> GDH SMDDSSEDGK	1198
x.SIP1		DKLRRQ <mark>V</mark> GDE E <mark>F</mark> EEEEEESE NKSMDTI	OPDT IRDEEE <mark>N</mark> GDH SMDDSSEDGK	1198
m.SIP1 x.SIP1	:	ME <mark>T</mark> KSDHEED NMEDGMG 1215 MEAKSDHEEE IMEDGM- 1214		

### 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 <sup>32</sup>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  $\mu$ m.

*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. 1, 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  $\delta$ -crystalline enhancer binding protein ( $\delta EF1$ ), 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  $\delta EF1$  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 upregulation 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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