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SpSoxB1, a maternally encoded transcription factor asymmetrically distributed among early sea urchin blastomeres

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SUMMARY

We have identified a Sox family transcription factor, SpSoxB1, that is asymmetrically distributed among blastomeres of the sea urchin embryo during cleavage, beginning at 4th cleavage. SpSoxB1 interacts with a *cis* element that is essential for transcription of *SpAN*, a gene that is activated cell autonomously and expressed asymmetrically along the animal-vegetal axis. In vitro translated SpSoxB1 forms a specific complex with this *cis* element whose mobility is identical to that formed by a protein in nuclear extracts. An anti-SpSoxB1 rabbit polyclonal antiserum specifically supershifts this DNA-protein complex and recognizes a single protein on immunoblots of nuclear proteins that comigrates with in vitro translated SpSoxB1. Developmental immunoblots of total proteins at selected early developmental stages, as well as EMSA of egg and 16-cell stage proteins, show that SpSoxB1 is present at low levels in unfertilized eggs and progressively accumulates during cleavage. SpSoxB1 maternal transcripts are uniformly distributed in the unfertilized egg and the protein accumulates to similar, high concentrations in all nuclei of 4- and 8-cell embryos. However, at fourth cleavage, the micromeres, which are partitioned by asymmetric division of the vegetal 4 blastomeres, have reduced nuclear levels of the protein, while high levels persist in their sister macromeres and in the mesomeres. During cleavage, the uniform maternal *SpSoxB1* transcript distribution is replaced by a zygotic nonvegetal pattern that reinforces the asymmetric SpSoxB1 protein distribution and reflects the corresponding domain of *SpAN* mRNA accumulation at early blastula stage (~150 cells). The vegetal region lacking nuclear SpSoxB1 gradually expands so that, after blastula stage, only cells in differentiating ectoderm accumulate this protein in their nuclei. The results reported here support a model in which SpSoxB1 is a major regulator of the initial phase of asymmetric transcription of *SpAN* in the nonvegetal domain by virtue of its distribution at 4th cleavage and is subsequently an important spatial determinant of expression in the early blastula. This factor is the earliest known spatially restricted regulator of transcription along the animal-vegetal axis of the sea urchin embryo.

Key words: Sox, Animal-vegetal axis, SpAN, Sea urchin, Gene regulation

INTRODUCTION

Transcription of the sea urchin gene, *SpAN*, begins shortly after fertilization, before third cleavage. *SpAN* mRNAs accumulate asymmetrically along the primary, maternally specified, animal-vegetal (A-V) axis of the embryo (Reynolds et al., 1992; Kozlowski et al., 1996). *SpAN* transcription is activated cell autonomously and *SpAN* mRNA accumulates in the nonvegetal ~85% of blastomeres during cleavage, but not in cells at the vegetal pole. These properties of early, spatially restricted, cell-autonomous activation suggest that *SpAN*'s transcription is regulated by asymmetrically distributed maternal activities and that synthesis or activation of these regulators must be closely linked to the mechanism that establishes the primary, animal-vegetal (A-V) axis of the embryo.

To pursue these regulatory activities, we have previously characterized the *SpAN* promoter by functional tests of altered transgenes in vivo and by assays of protein-DNA interaction in vitro. These experiments showed that 300 bp upstream of the *SpAN* transcription start site are sufficient to regulate nonvegetal transcription and they identified two small regions in the promoter, sites II and V, that are essential for high level activity (Kozlowski et al., 1996). Because no negative *cis*-acting elements could be identified that repressed promoter activity in vegetal cells, these results strongly suggested that spatial regulation of *SpAN* along the animal-vegetal axis is mediated through strong positive factors that interact with sites II and V. The experiments reported here identify the factor that functions through the quantitatively more important region, site V.

Site V was originally defined as a protected region of ~30 bp

in footprinting assays with nuclear extracts from embryos at early blastula stages (Kozlowski et al., 1996) when *SpAN* is very actively transcribed (Reynolds et al., 1992). Electrophoretic mobility shift assays demonstrated that several different DNA-protein complexes can be formed with a site V probe sequence (Kozlowski et al., 1996). Interestingly, site V contains several sequence motifs known to be recognized by members of the Sox (SRY-related box) transcription factor family.

Sox factors contain a highly conserved ~80 amino acid DNA-binding domain (Sox box) related to those of high mobility group (HMG) proteins. Members of the HMG superfamily bind AT-rich motifs in the minor groove and can regulate gene transcription by altering chromatin structure (reviewed by Grosschedl et al., 1994; Landsman and Bustin, 1993; Pevny and Lovell-Badge, 1997). Although classical HMG proteins contain multiple HMG domains and bind DNA with marginal sequence specificity (Bustin et al., 1990; Jantzen et al., 1990; Soullier et al., 1999), relatives in the Sox subfamily have single DNA-binding domains that are sequence-specific. Some Sox proteins are thought to function primarily as architectural factors in assembly of multiprotein-DNA complexes through their ability to bend DNA (Ferrari et al., 1992; Pevny and Lovell-Badge, 1997), while others have been shown to behave as classical transcription activators by virtue of their activation domains (van de Wetering et al., 1993; Kamachi et al., 1995). Recent studies demonstrate that members of the Sox transcription factor family play pivotal developmental roles in a number of different systems (reviewed by Pevny and Lovell-Badge, 1997).

Sox factors recognize very similar, if not identical, DNA sequence motifs so that it is not currently possible to predict which *trans* factors are most likely to bind a particular Sox *cis*-regulatory element. Consequently, we searched for candidate Sox regulators of *SpAN* that are expressed in very early sea urchin embryos using a PCR strategy with degenerate Sox box primers and cDNAs representing mRNAs from ovaries and early embryos. We show here that one Sox gene, *SpSoxB1*, encodes a protein that binds specifically to the functional *cis* elements in site V of the *SpAN* promoter. SpSoxB1 protein is asymmetrically distributed among nuclei of embryos at the stage when the nonvegetal and vegetal domains are first separated during cleavage. It is significantly more concentrated in nonvegetal (macromere and mesomere) blastomeres of the 16-cell embryo than it is in vegetal micromeres. This nonvegetal pattern is maintained throughout embryogenesis. SpSoxB1 is the earliest known transcription factor that marks the nonvegetal transcriptional domain.

MATERIALS AND METHODS

Embryo culture

Strongylocentrotus purpuratus (Sp) adults were obtained from Marinus Co. (Westchester, CA) and eggs were fertilized and embryos cultured at 15°C as previously described (Angerer and Angerer, 1981). Fertilization was carried out in the presence of 5 mM para-aminobenzoic acid to prevent hardening of fertilization membranes that were subsequently removed at stages before hatching by passage through 50 μm Nitex membrane.

In vivo transcription activity assays

Measurements of promoter activity in vivo were made using chimeric constructs carrying wild-type or mutated *SpAN* promoters linked to a bacterial chloramphenicol acetyltransferase (CAT) reporter gene as described previously (Wei et al., 1995). Four new replacement mutations were made in the site V region of the *SpAN* promoter with the PCR strategy described previously (Kozlowski et al., 1996) to create the original site V replacement mutation. Their sequences are listed below:

wt	AAAAGGAGAACAATAACAATGATGGCGACTGCT-TTGTTCCTTAAAGGAGG
V-R	AAAAGttggcaacgtaggaattccgtccagtacgatgacaTTAAAG-GAGG
V-5′R	AAAAGttggcaacgtaggaattcGGCGACTGCTTTGTTCC-TTAAAGGAGG
V-3′R	AAAAGGAGAACAATAACAATGATGGaattccaacaacc-aagccttaaAGG
V-MR	AAAAGctcAACAATAACAATtcgtgaattctCTTTGTTC-CTTAAAGGAGG
V-SR	AAAAGGAGgcactggaattccATGGCGACTGggcattcCC-TTAAAGGAGG

Electrophoretic mobility shift assay (EMSA)

EMSA was conducted using in vitro translated proteins, 9-hour nuclear extracts or whole cell proteins from eggs and 16-cell blastomeres. For extracts made from purified 16-cell blastomeres, EMSAs were carried out as described previously (Kozlowski et al., 1996). The other reactions were carried out in 20 mM Hepes adjusted to pH 7.9 with KOH, 5 mM $MgCl_2$, 10 mM KCl, 10 mM DTT, 0.05% NP40 at 15°C. Probes containing site V (base pairs from –106 to –77) or only the 5′ Sox motifs (site V5′; base pairs from – 106 to –88) were end-labeled with γ-[^{32}P]ATP to a specific activity of 2500-5000 cts/minute/fmol. For each reaction, ~2-4 fmols of probe were incubated at 15°C for 15 minutes with either an aliquot of the in vitro translation reaction (2 μl; see below), or whole cell extracts in the amounts indicated or nuclear extract protein (3 μg); the last was prepared as described previously (Calzone et al., 1988) omitting the dialysis step. To test for binding specificity, unlabeled probe and heterologous competitor DNAs were added in the indicated molar excesses. The reactions were then fractionated by electrophoresis through non-denaturing 4.5% acrylamide gels.

For supershift assays, α-SpSoxB1 antibody or preimmune serum, either undiluted or diluted 1:10 in 1× PBS, was included before the addition of probe. The mixtures were incubated at 15°C for 30 minutes, and an additional 15 minutes after adding probe and analyzed as described above.

Isolation of Sox cDNAs

Degenerate PCR primers were designed that are complementary to conserved regions of the SRY and Sox HMG boxes:

5′-ATGAACGC(C/T)TTCATG(G/A)T(C/T)TGG-3′

coding for MNAFM(V/I)W and

5′-(G/T)GGG(C/T/A)(T/C)(G/T)GTACTTGTA(G/C)T(T/C)(T/G)-GG-3′

coding for P(E/N/D)YKY(Q/R/K)P.

PCR was performed under standard conditions (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate) with 5 μM of each upstream and downstream degenerate primer. Cycling conditions were [(94°C, 1 minute; 42°C, 1 minute; 72°C, 1 minute) × 5 cycles] and then [(94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute) × 30 cycles]. Products of the correct size were obtained from ovary and very early blastula stage randomly primed libraries and cloned using the TA cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's protocol. To obtain full-length cDNAs, a library of randomly primed cDNA inserts in λZAP, representing RNAs from ovary tissue (gift of Dr Kathy Foltz, UC Santa Barbara) was screened by hybridization at high stringency (~T_m

–12°C) with a ^{32}P-labeled, randomly primed probe representing multiple Sox box-containing sequences obtained from the original PCR screen. Plasmids were excised from positive phage according to the manufacturer's protocol (λZAP, Stratagene), rescreened by colony hybridization and partially sequenced using T3 and T7 primers.

Northern blot

5 μg of total RNA isolated from embryos at selected stages were fractionated by electrophoresis through 0.8% agarose-formaldehyde denaturing gels and transferred to Genescreen Plus (Dupont-NEN, Boston MA) as described previously (Hurley et al., 1989), omitting baking in vacuo. After UV crosslinking, blots were prehybridized and then hybridized at 60°C with HMG-box probes [1×10^6 cpm ^{32}P-labeled randomly primed probe per ml of a solution containing 2× SSC, 50 mM sodium phosphate, 0.1% sodium pyrophosphate, 5× Denhardt's solution (0.1% Ficoll, 0.1% Fraction V bovine serum albumin [BSA], 0.1% polyvinylpyrrolidone), 1% SDS, 10% dextran sulfate, 20% formamide and 200 μg calf thymus DNA/ml (pH 6.8)]. Blots were washed successively in 4× SSC, 2× SSC, 1× SSC and 0.2× SSC, each containing 1% SDS at 60°C (the final wash is stringent, corresponding approximately to T_m −10°C).

In vitro translation of SpSox proteins

The *SpSoxB1*, *SpSoxB2* and *SpSoxD1* cDNA sequences were inserted into pSP64T.Clon derived from pSP64T (Promega). pSP64T was altered by replacing the *Bgl*II site with additional restriction sites (*Sma*I, *Kpn*I, *Sac*I, *Eco*RI, *Not*I, *Xma*III) that are between the Sp6 promoter and poly(T) stretch (a gift from L. Dale, University College, London). Protein was synthesized using 1 μg of linearized plasmid and the TNT Coupled Reticulocyte Lysate kit (Promega) in the presence of [^{35}S]methionine (0.8 μM; 1 Ci/μmol). Labeled products (1 μl of the 50 μl reaction) were assayed for size by SDS-PAGE and corresponded to expected molecular masses (SpSoxB1, 36 kDa; SpSoxB2, 31 kDa; SpSoxD1**,** 24 kDa).

In situ hybridization

Probe preparation and in situ hybridization were carried out as described previously (Angerer and Angerer, 1991). Antisense and sense HMG-box riboprobes labeled with [^{33}P]UTP to 0.5-2× 10^8 disints/minute/μg were transcribed in vitro. These probes were hybridized in situ to 5 μm sections of embryos of selected stages. Unhybridized probe was removed by a stringent wash (60°C; 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl and 1 mM EDTA, pH 8.0), incubation with RNase A and washes as described previously (Angerer et al., 1987). Sections were exposed to Kodak NTB-2 liquid track emulsion for 1-2 weeks.

Preparation of 16-cell blastomeres

Blastomeres were isolated in large quantities as described previously (Harkey and Whiteley, 1980) with some minor modifications. The embryos were collected by centrifugation (300 ***g***, 5 minutes), and were subsequently handled at 0-4°C to inhibit cell division. Demembranated 16-cell embryos (3×10^7) were collected on 25 μm Nitex membrane and gently washed two times in one liter CDM I (1:1:0.5 mixture of calcium, magnesium-free sea water (CMFSW), 1 molal dextrose, H_2O, respectively) and one time in one liter CDM II (6:4 mixture of CMFSW: 1 molal dextrose containing 2.4 mM EDTA and 2.4 mM EGTA). After this final wash, embryos were fully dissociated and the cells were collected by centrifugation and resuspended in two liters of solution containing 1: 1: 0.1 mixture of CDMI, CMFSW, 1 M $MgCl_2$, respectively. Micromeres were separated from mesomeres and macromeres by 1 ***g*** sedimentation for 2-3 hours through large gradients built in a circular tank 20 cm in diameter [7-30% sucrose in calcium-free sea water (CFSW)]. Fractions were collected by siphoning with a wide bore (6 mm) pipette embedded in a round 5 cm plastic disk to minimize gradient disturbance similar to that described previously (Harkey and Whiteley, 1980). Experiments in which the micromere purities were >90% were used for the experiments reported here. The remaining blastomeres, which constitute 85-90% of the total embryo volume, were collected from the remainder of the gradient.

Preparation of whole cell extracts from eggs and 16-cell blastomeres

The procedure is similar to that described previously (Calzone et al., 1997). Eggs, dissociated 16-cell embryos, purified micromeres or mesomere-macromere fractions were washed in homogenization buffer (250 mM NaCl, 25 mM EGTA, 5 mM $MgCl_2$, 110 mM glycine, 250 mM glycerol, 10 mM Pipes, pH 6.8, 1 mM DTT, 10 mM PMSF (Sigma), 1 μM leupeptin (Sigma), and 1 μM pepstatin (Roche Molecular Biochemicals)). The cells were suspended in 1-3 cell volumes of buffer and homogenized briefly in a Tissumizer (Tekmar) until nuclei were not visible by DAPI staining. The homogenate was cleared by centrifugation at 20,000 ***g*** for 15 minutes and the supernatant was quick frozen in liquid nitrogen and stored at −80°C.

Homogenate supernatants were fractionated by Biogel 1.5m chromatography to obtain a crude purification of transcription factors. Previous work demonstrates that all factors tested, of which there are many, were substantially purified by this method (Calzone et al., 1997). Macromere/mesomere and micromere homogenates were applied to columns whose diameter/height was 1.5 cm/22 cm and 0.5 cm/10 cm, respectively, equilibrated with a buffer containing 250 mM NaCl, 25 mM EGTA, 5 mM $MgCl_2$, 360 mM glycerol, 10 mM Pipes, pH 6.8, 1 mM DTT, 10 mM PMSF, 1 μM each leupeptin and pepstatin. Excluded fractions containing ribosomes were discarded and the remainder of the fractionated and completely included proteins that contain transcription factors were precipitated with 30% ammonium sulfate and resuspended in a buffer containing 20 mM Hepes adjusted to pH 7.9 with KOH, 40 mM KCl, 0.1 mM EDTA, 20% glycerol, 0.1% NP-40, 1 mM DTT, 0.1 mM PMSF (Buffer C; Calzone et al., 1988). Protein concentrations in these extracts were determined by Bradford assay (Biorad) and extracts were stored at −80°C after quick freezing in liquid nitrogen.

Antibody preparation

A GST (glutathione S-transferase)-SpSoxB1 fusion was prepared as follows. Sequence encoding amino acids 135 to 325 was obtained by PCR with primers containing *Eco*RI tails and inserted in frame into *Eco*RI-restricted pGEX-KG vector [derived from pGEX-2T(Pharmacia); (Guan and Dixon, 1991)]. The protein was expressed in a 2 liter culture of *E. coli* ER2508 (NEB; RR1*Ion*::miniTn10-(Tet^r)Δ(malB)Δ(argF-lac)U169Pro$^+$zjg::Tn5(Kan^r)fhuΔ2; Grossman et al., 1983) and purified by glutathione-sepharose chromatography according to the manufacturer's instructions (Pharmacia) with minor modifications. (1) The samples were loaded in a buffer containing 1× PBS, 10% glycerol, 10 mM EDTA, 3 mM DTT (PBS + GET) and (2) the glutathione beads containing bound GST-SpSoxB1 fusion protein were washed sequentially with PBS + GET, 1:1 PBS + GET, PBS and finally PBS. GST-SpSoxB1 protein (~4.5 mg) was eluted with 20 ml of elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) and concentrated under N_2 pressure to 1 mg/ml using an Amicon 3 ml, stirred cell equipped with a PM30 filter. Concentrates were equilibrated in 1× PBS by three cycles of dilution and concentration that reduced the glutathione concentration ~200-fold. Rabbit polyclonal antibodies were raised by Alpha Diagnostics (San Antonio, Texas).

Western blot

Proteins in nuclear extracts (20 μg), in in vitro translates (5 μl TNT reaction) or from 500 embryos of selected developmental stages (~20 μg protein) were analyzed on immunoblots as described previously (McCoon et al., 1998). Blots were incubated with a rabbit polyclonal antiserum, diluted 1:1000, made against the C-terminal half of SpSoxB1 that lacks the DNA-binding domain and represents nonconserved sequences in the protein (α-SpSoxB1). The primary

antibody was detected with horseradish peroxidase-conjugated goat anti-rabbit antibody (Zymed) diluted 1:1000. Chromogenic detection was carried out using the RENNAISSANCE 4CN Plus system (NEN; Boston, MA).

Immunohistochemistry

Embryos of selected stages were fixed in 2% paraformaldehyde, 1× PBS for 10 minutes, at 25°C, washed in 1× PBS three times and blocked with 1× PBS containing 3 mg BSA/ml, 0.2% Tween-20. They were incubated with 1:1000 dilution of primary antibody for 2 hours in blocking solution, washed 2 times with 1× PBS for 10 minutes each, 1 time with blocking solution for 15 minutes and stained with FITC-conjugated goat anti-rabbit secondary antibody (1:1000) for 1 hour (Zymed). Secondary antibody was removed and nuclei were simultaneously stained by a wash in solution containing 1× PBS and 2 μg DAPI/ml. After 4 brief washes in 1× PBS, the last containing 0.02% sodium azide, the embryos were mounted on polylysine-coated coverslips for confocal microscopy.

RESULTS

Sox *cis* elements are required for *SpAN* promoter activity

Previously, we have shown that replacement of sequences between –72 and –106 of the *SpAN* promoter (site V) results in a 20-fold reduction of promoter activity in sea urchin embryos (Kozlowski et al., 1996). EMSA analyses identified multiple DNA-protein complexes that form at site V in vitro. Examination of the sequence revealed several SRY/Sox consensus motifs in this region, as shown in Fig. 1. To determine whether the strong transcription activation mediated by site V was conferred by the Sox motifs, we created a series of mutations within the site V region of the *SpAN* promoter and tested their effects on the expression of the CAT reporter gene. CAT enzymatic activities were determined in substrate excess and normalized for transgene DNA content, as measured by Southern blotting.

As previously observed, replacement of the entire site V sequence (VR) resulted in a large (>20-fold) reduction in promoter activity. When only the Sox motif regions were replaced, the reduction in activity was essentially the same (V-SR). In contrast, replacement of the middle region of site V had no significant effect on promoter activity (V-M). Therefore, site V function is conferred by the Sox motifs. In addition, mutation of either the 5′ or 3′ Sox sites showed that both contribute to site V activity (V-5′R and V-3′R). However, the 5′ Sox motifs consistently confer significantly more positive activity than does the 3′ Sox element.

Sox *cis* elements bind a factor in nuclear extracts of early sea urchin embryos

To determine whether sea urchin embryo nuclei contain proteins at the developmental stage when *SpAN* is transcribed that can bind to the 5′ Sox motif, we carried out EMSAs with 9-hour nuclear extracts. Several complexes usually were observed, although the lowest mobility complex was consistently the predominant species formed. Addition of unlabeled sequences in 250- or 1000-fold molar excess that contain the 5′ Sox elements (Fig. 2, competitors 1, 4 and 5) competed complex formation, while other sequences from site V did not (Fig. 2, competitors 2, 3 and 6). Although competitor 2 contains a sequence that fits the Sox element consensus, it is not nearly as effective a competitor as is the 5′ element. Presumably sequence differences within the core (AACAAT<u>A</u>/G vs. AACAA<u>A</u>G) and/or flanking sequences contribute to differences in Sox-binding affinities.

Cloning of *SpSoxB1*

Because these studies suggested that a major positive regulatory activity of the *SpAN* promoter is mediated by a Sox factor, we searched for candidate Sox cDNAs using a PCR-based screen with degenerate primers selecting for Sox family DNA-binding domain (HMG) sequences. Of the five different Sox box sequences recovered in this screen, three were found by RNA blot analysis to be expressed both in the maternal population and during stages when the *SpAN* gene is transcribed (Fig. 3). Similar loads of total RNA from unfertilized eggs, and from blastula, gastrula and pluteus stage embryos, were hybridized under moderately stringent conditions to probes representing the different DNA-binding domains. Each probe detects an RNA of similar length that is present at approximately equal levels in the egg and throughout embryogenesis. The faint, faster migrating species detected in the SpSoxB2 hybridization probably results from cross reaction to the very closely related and more abundant SpSoxB1 species. Comparison of the Sox box sequences shows that SpSoxB1 and B2 are most closely related to members of the SoxB class as defined previously (Wright et al., 1993). At the amino acid level they are 95%

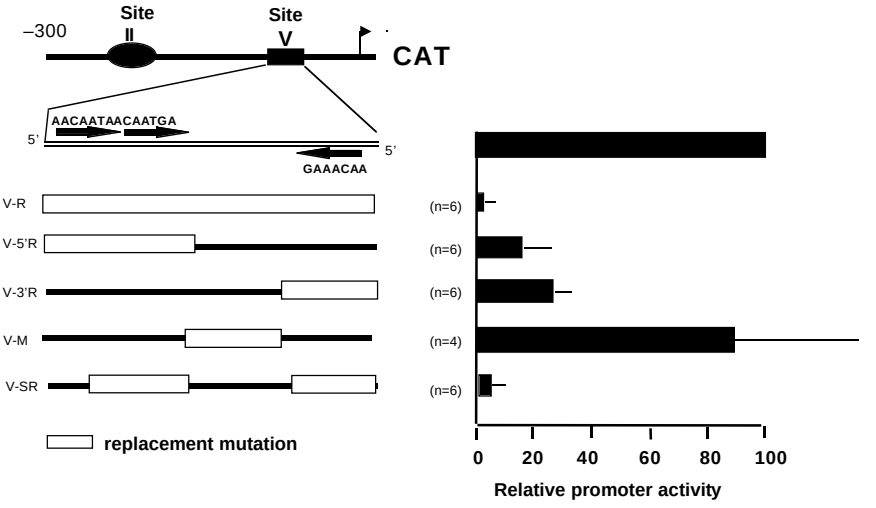

Fig. 1. The positive activity of *SpAN* site V is exerted through the Sox motifs. *SpAN* promoter activity was measured in vivo using CAT reporter transgenes. Constructs included the complete –300 promoter, which has been demonstrated to drive correct nonvegetal expression (Kozlowski et al., 1996). Regions containing Sox motifs and the central non-sox region were mutated by exact-length replacements (open boxes), as indicated in the diagram. CAT enzyme activities were normalized for transgene content, as quantitated by Southern blots (not shown) and the average values from multiple experiments are plotted in the histogram; s.d. is indicated by error bars.

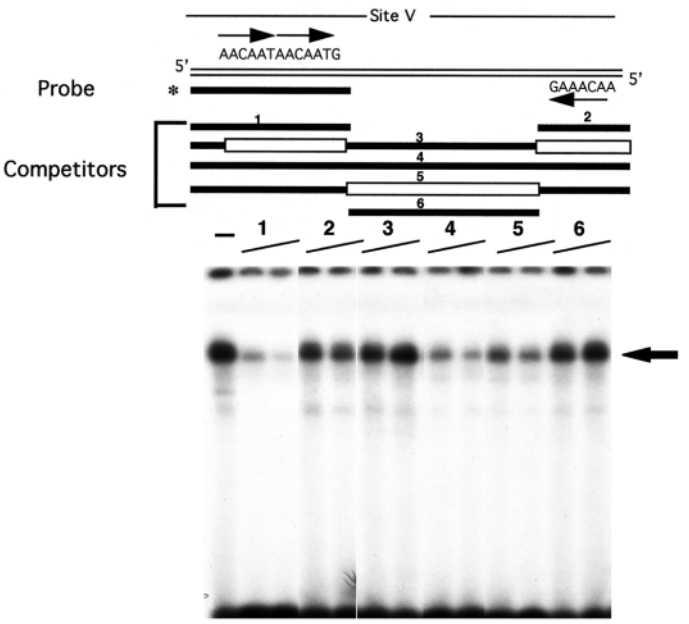

Fig. 2. The Sox motifs of siteV form a single major complex in EMSAs. *SpAN* site V is diagrammed at top. EMSAs were carried out using a probe that contained the 5′ Sox motifs (Probe*). Specificity of interaction was demonstrated by competition with a 250- or 1000-fold excess (left and right of each numbered pair of lanes, respectively) of unlabeled DNA fragments including the correspondingly numbered sequences diagrammed above the lanes (competitors). Competitors contain sequences that are either wild type (black line) or mutated by replacement (open boxes) as described in Materials and Methods. The arrow at right indicates the position of the complex formed specifically with Sox motifs. (–), no competitor.

identical to each other and ≥90% identical to HMG domains of the mouse and human *Sox-1*, *Sox-2* and *Sox-3* genes. SpSoxD1 is most closely related to Sox5, a member of the SoxD class (88% and 93% amino acid identity and similarity, respectively). A recent, rigorous phylogenetic analysis (Soullier et al., 1999) yields similar, but not identical groupings for Sox family members. For the subfamilies containing SpSoxB1, SpSoxB2 and SpSoxD1, there is excellent agreement between the two studies (group B corresponds to Sox2/3; group D corresponds to Sox5/6). These sea urchin Sox genes have been named according to the letter classification to avoid confusion since two are within the same subfamily but their exact homologs are not clear.

Full-length open reading frames were obtained from an ovary cDNA library. Each open reading frame initiates with a methionine that is in a good translation initiation context (Kozak, 1987) and the inferred open reading frames for SpSoxB1, SpSoxB2 and SpSoxD1 encode proteins with molecular masses of 36, 31 and 24 kDa, respectively, which are in good agreement with the observed molecular masses of the corresponding in vitro synthesized proteins. Furthermore, western blotting with anti-SpSoxB1 antibody shows that it recognizes a protein of the same size in nuclear extracts (see below). Outside of their HMG domains, the sequences of these three proteins are quite divergent, as is often the case for different vertebrate Sox proteins (e.g. see van de Wetering et al., 1993). Consequently, they do not reveal homology to a particular vertebrate Sox family member. SpSoxB2 contains a polyalanine region that is found in many *Sox-1* and *Sox-3* proteins and a serine-rich region, found in *Sox-2*, *Sox-4* and *Sox-5* proteins (Denny et al., 1992; van de Wetering et al., 1993; Kamachi et al., 1995; Yuan et al., 1995), that may function in transcriptional activation (van de Wetering et al., 1993; Yuan et al., 1995). The SpSoxB1 protein sequence contains several short motifs that are similar to those found in vertebrate *Sox-2* proteins and are specific to members of the *Sox B* subfamily (Vriz and Lovell-Badge, 1995); however, functions of these domains have not been determined. [Accession numbers for SpSoxB1, SpSoxB2 and SpSoxD1 sequences in GenBank are AF157387, AF157388 and AF157389, respectively.]

SpSoxB1 protein binds to the 5′ Sox element of *SpAN* site V

The mobilities of complexes formed between in vitro translated SpSoxB1, SpSoxB2 and SpSoxD1 proteins and the 5′ Sox elements of *SpAN* site V were compared to those formed with 9-hour nuclear extracts using EMSAs. Fig. 4A, lanes PI and B1, show that the SpSoxB1 complex migrates identically with the predominant, slow mobility complex. No complex of mobility similar to that formed with SpSoxB2 (lane B2) was detected. The highest mobility complex formed with nuclear extract protein migrates just slightly faster than that formed with SpSoxD1 (lane D1).

To confirm that the protein forming the low mobility complex in nuclear extracts was SpSoxB1, a rabbit polyclonal antiserum was raised against the nonconserved C-terminal half of the protein, omitting the conserved DNA-binding domain. Fig. 4B (left) shows that immune serum recognizes the in vitro translated SpSoxB1 protein whose mass, 39 kDa, is similar to that inferred from the cDNA sequence (36 kDa). This antibody is highly specific because it recognizes only a single protein of the same mobility in 9-hour nuclear extracts (Fig. 4B, right). The anti-SpSoxB1 antibody was then tested for its ability to interact with the low mobility complex formed in EMSA assays. As shown in Fig. 4A, addition of either a 1:10 dilution of this antiserum or of undiluted antiserum (lanes I) results in loss of the specific complex and appearance of a larger

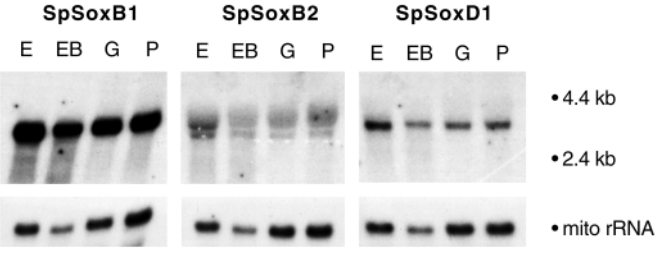

Fig. 3. Transcripts of three different Sox factor genes are present at approximately constant abundance throughout embryonic development. Abundance of transcripts from SpSoxB1, SpSoxB2 and SpSoxD1 was quantitated by RNA blotting as described in Materials and Methods. Total RNA was purified from unfertilized eggs (egg, E) or embryos at 8 hours (mid cleavage, about 100 cells, EB), 36 hours (mid gastrula, G) or 72 hours (differentiated pluteus larva, P) postfertilization. The positions of the ribosomal RNAs are indicated at right. RNA loads were compared using a probe for small mitochondrial rRNA, whose abundance is approximately uniform during development (Wells et al., 1982).

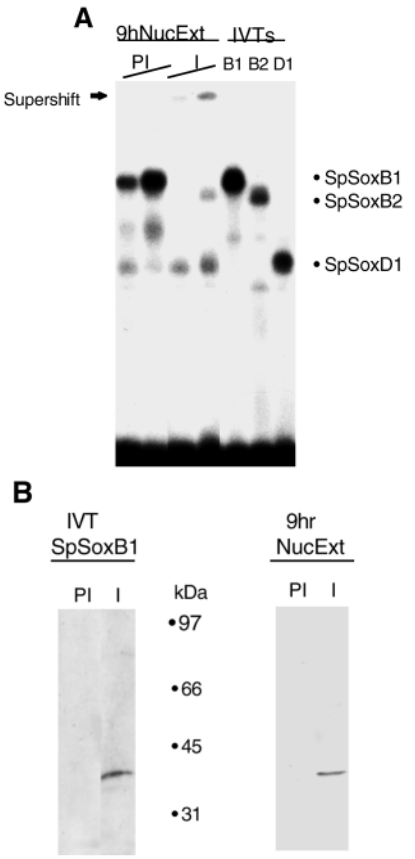

Fig. 4. SpSoxB1 is the major factor interacting with the SpAN site V Sox motifs. (A) EMSAs were carried out using a probe representing only the siteV 5′ Sox motifs and nuclear extracts from 9-hour embryos (9hNucExt) or proteins translated in vitro (IVTs) from cDNA sequences including the complete open reading frames of *SpSoxB1*, *SpSoxB2* or *SpSoxD1*. Complexes formed with nuclear extract were tested for inhibition/supershifting by preimmune serum (PI) or antiserum raised against the C-terminal region of SpSoxB1 (I) at 1:10 dilution or undiluted. Supershift, arrow, indicates the complex formed by this antiserum. (B) Western blot analysis of SpSoxB1 protein translated in vitro (IVT SoxB1) and proteins in nuclear extracts of 9-hour sea urchin embryos (9hrNucExt). PI, preimmune serum; I, immune serum. The positions of size markers are shown between the panels (kDa).

complex, whereas preimmune serum (lanes PI) causes no reduction. The amount of supershifted complex is reduced, probably because the polyclonal antibody also partially interferes with complex formation. At the higher concentration of antibody, a new complex appears that migrates similarly to the SpSoxB2 complex (*cf.* lanes I, right, and B2 in Fig. 4A) suggesting that it or another protein can bind to this probe if SpSoxB1 is removed from the reaction. We conclude that SpSoxB1 is the predominant protein in nuclear extracts that interacts with the 5′Sox *cis* element.

SpSoxB1 transcripts are uniformly distributed in the egg, but restricted to the nonvegetal domain of early blastulae

Because SpSoxB1 binds to the *cis* element that confers the strong positive activity of site V in the *SpAN* promoter, it could be a major spatially restricted regulator of *SpAN* transcription in the nonvegetal domain. To determine the sites of *SpSoxB1* gene expression in the egg and early embryo, in situ hybridization was carried out with ^{33}P-labeled antisense riboprobes to sections of sea urchin embryos of selected stages. Transcripts were observed to be uniformly distributed in the egg (Fig. 5A,B) and 16-cell embryo (Fig. 5C,D), but were absent from the vegetal pole of early blastulae (12-15 hours postfertilization) (Fig. 5E,F) and mesenchyme blastulae (24 hour) (Fig. 5G,H). Signals were similar at each stage in agreement with RNA blot data (Fig. 3); sense control hybridization signals (not shown) were very low and equivalent to those obtained with antisense probe in the vegetal pole regions of blastulae. The change in transcript distribution during cleavage requires that maternal *SpSoxB1* mRNAs turn over in vegetal cells. Whether there is also turnover in nonvegetal cells of these maternal *SpSoxB1* RNAs, which are replaced by new nonvegetal zygotic transcription, is likely but not yet known.

SpSoxB1 protein accumulates during cleavage

SpSoxB1 protein was detectable in immunoblots of whole-cell protein beginning between 2 and 3 hours of development (2- to 4-cell stage) and gradually increased in abundance during cleavage (Fig. 6A). Only one protein was detected with immune, but not preimmune serum, and its mobility was the same as that of the factor detected in nuclear extracts (Fig. 4B). To determine whether there might be some maternal SpSoxB1 protein whose concentration was below the sensitivity of this assay, we performed EMSAs with egg and 16-cell protein extracts prepared identically. Since maternal transcription factors are likely to be stored in the egg cytoplasm (Zeller et al., 1995; Chuang et al., 1996; Xian et al., 1996; Calzone et al., 1997), we used proteins extracted from whole cells for these studies. Using the 5′SoxB1 *cis* element as the probe and increasing amounts of protein, we found that several different complexes were formed that differed in relative amounts compared to those formed with later, 9-hour extracts (Fig. 6B). The slowest migrating complex contained SpSoxB1 since it could be supershifted by the antibody (Fig. 6B, right 4 lanes). Its concentration was at least 5-fold greater in 16-cell than in egg protein extracts, consistent with the immunoblot data showing that SpSoxB1 protein concentration is lower in eggs than in cleaving embryos. Because the SpSoxB1 mRNA concentration is constant between these two stages, it is likely that most of the SpSoxB1 protein present when *SpAN* gene transcription is activated between 4- and 8-cell stages is translated from maternal message. This interpretation is supported by the fact that, in this embryo, the protein synthesis rate increases dramatically after fertilization (~30-fold; Brandis and Raff, 1978). It is also possible that some zygotic transcription contributes to the *SpSoxB1* mRNA pool during early cleavage stages, but this is expected to be relatively minor since maternal *SpSoxB1* mRNA is moderately abundant and the number of embryonic nuclei is low.

SpSoxB1 protein accumulates preferentially in nonvegetal nuclei

Immunostaining of whole embryos shows that SpSoxB1 protein accumulates in nuclei. No nuclear staining was observed with preimmune serum at any stage. The low level of maternal SpSoxB1, which is probably distributed uniformly

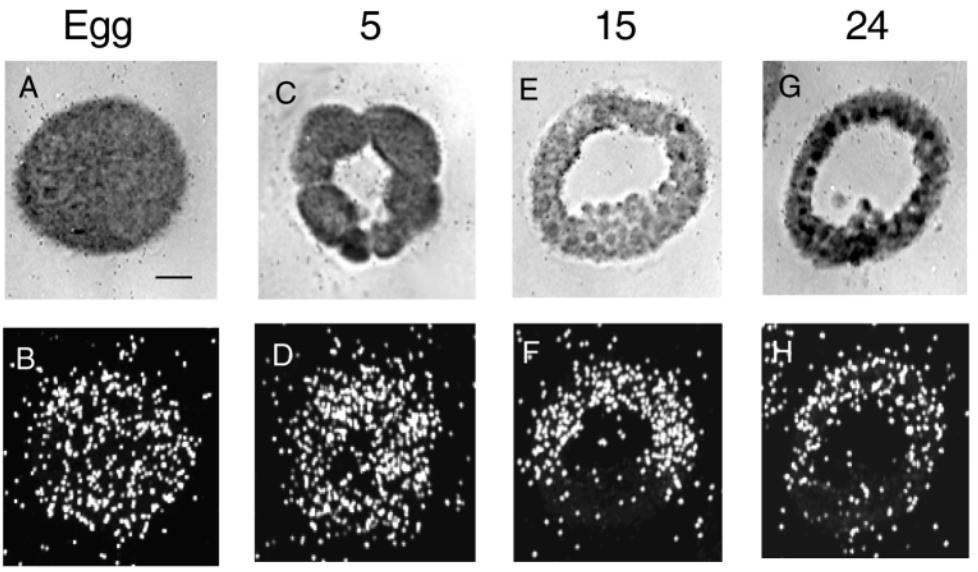

Fig. 5. SpSoxB1 transcript distribution is uniform in unfertilized eggs and modulates to the nonvegetal pattern during cleavage. *SpSoxB1* transcript distribution was monitored by hybridization in situ with ^{33}P-labeled antisense RNA probes to sections of unfertilized eggs (egg), and embryos at the indicated hours of development (5 hours, 16-cell; 15 hours, ~200 cell; 24 hours, mesenchyme blastula). Top row, bright-field illumination; bottom row, dark-field illumination. Except for egg, the sections are aligned with the animal pole at top. The bar in A indicates 10 μm.

throughout the cytoplasm of unfertilized eggs, is not detectable; but by 4-cell stage, each of the nuclei is stained intensely with both anti-SpSoxB1 antibody and DAPI (Fig. 7A,B), as is the case one cleavage division later (not shown). Strikingly, at 16-cell stage, when a major portion of the vegetal domain is separated into the micromeres, the nuclei of these cells contain at least several-fold lower levels of SpSoxB1 than do those of their sister macromeres (Fig. 7C,D). This is clearly evident when the fluorescein (green) and DAPI (blue) signals are combined (Fig. 7E). In this merged image, a ratio of pixel intensities greater than 1 (blue/green) is shown by pseudocolor progressing from yellow to red. The micromere nuclei appear red, indicating lower SpSoxB1 content since DAPI staining intensities are equivalent (Fig. 7F). As cleavage proceeds, the difference in SpSoxB1 concentration between nonvegetal and vegetal nuclei becomes more pronounced (Fig. 7G,H, 32-cell; I,J, ~60-cell) so that, at the time of peak transcription of the *SpAN* gene, SpSoxB1 is localized appropriately to control spatial expression of the gene (Fig. 7K,L, 12-hour VEB stage). As development proceeds, the size of the region lacking detectable SpSoxB1 signal expands and the border sharpens, until the unlabeled region includes the entire vegetal plate at

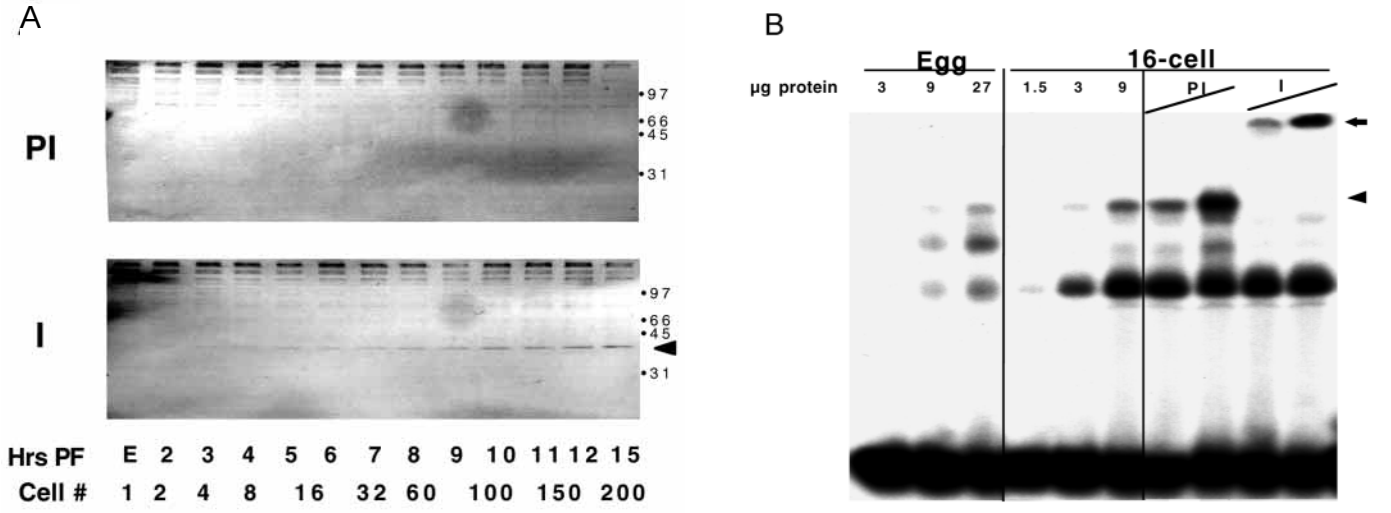

Fig. 6. SpSoxB1 protein accumulates during development. (A) Accumulation of SpSoxB1 was monitored by western blots using protein extracts of whole eggs or embryos and the antiserum raised against the C-terminal nonconserved region of SpSoxB1. Protein extracts were prepared from unfertilized eggs (E) and from embryos at the indicated hours post fertilization (PF). PI, blot probed with preimmune serum; I, blot probed with immune serum. The arrowhead indicates the band produced by SpSoxB1. High M_r bands near the top of each lane are nonspecific, as indicated by their reactivity with preimmune serum. Extracts of nuclear proteins at very early blastula stages showed only the specific band (See Fig. 4B). The positions of size markers are shown at right. (B) The concentration of proteins interacting with the *SpAN* siteV 5′ Sox motifs was compared in eggs and 16-cell embryos by EMSAs using total protein extracts. Protein loads (μg protein) are indicated above the lanes in the left two panels. In the right-hand panel, 9 μg protein from 16-cell embryos was incubated with probe and antiserum raised against the nonconserved C-terminal region of the SpSoxB1 protein (I, immune; PI, preimmune) either diluted 1:10 or undiluted. The arrowhead at right indicates the position of specific complex formed with SpSoxB1 protein and the arrow indicates the supershifted complex.

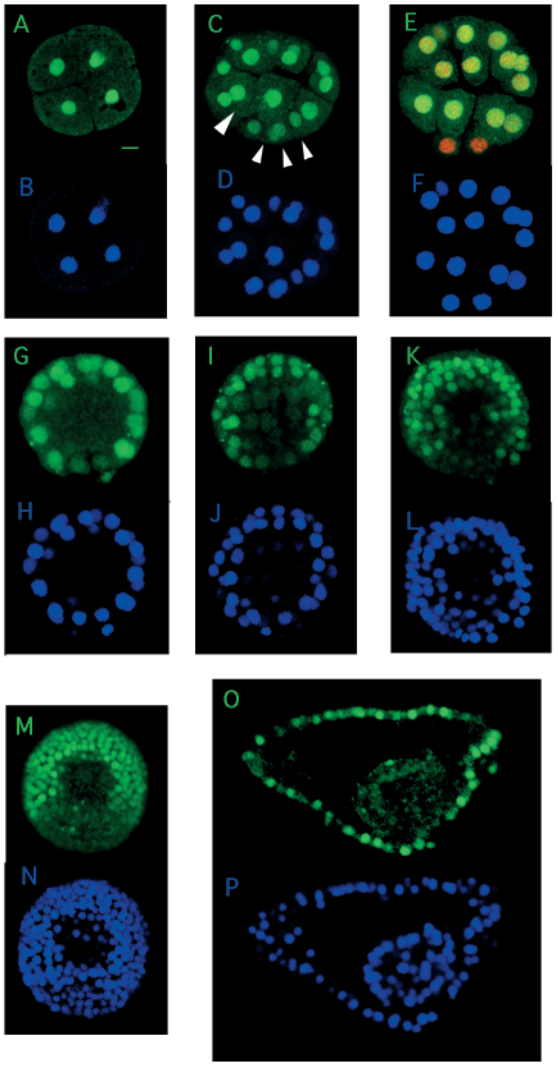

Fig. 7. SpSoxB1 nuclear protein concentration mirrors the spatial regulation of *SpAN*. SpSoxB1 protein distribution was monitored by immmunofluorescence microscopy as described in Materials and Methods. Immunofluorescence (FITC) with the SpSoxB1 antiserum (A,C,G,I,K,M,O); nuclear staining with DAPI (B,D,F,H,J,L,N,P). (E) The FITC and DAPI signals were merged. Lower FITC signals relative to DAPI signals are indicated by pseudocolor progressing from yellow to red. The images shown are reconstructed partial stacks obtained by confocal microscopy; therefore, signals from those nuclei not completely included in the stack are lower, as shown by comparison to the DAPI-stained images (e.g. mesomere nuclei at the top of the embryo illustrated in C and D). Stages shown are: A,B, 4-cell; C,D, 16-cell; E,F, between 16- and 32-cell (some of the mesomeres have divided); G,H, 32-cell; I, J, ~60-cell; K,L, ~200-cell, early blastula; M,N, mesenchyme blastula; O,P, pluteus larva. Embryos in C-N are shown with the animal pole at top. The small arrowheads in C indicate the four vegetal micromere nuclei, for which signals are significantly lower than for the macromere nuclei immediately above them (large arrowhead). All images are shown at the same magnification and the bar in A indicates 10 μm.

the mesenchyme blastula stage (Fig. 7M,N; 24 hours), which contains endoderm and mesenchyme precursors. In the gastrula-pluteus period, labeling is predominantly in ectodermal nuclei (Fig. 7O,P), although SpSoxB1 protein reappears in foregut nuclei (not shown).

We were able to obtain an independent estimate of differences in SpSoxB1 concentration in animal and vegetal blastomeres of the 16-cell embryo. The small micromeres were separated from the larger macromeres and mesomeres by 1 ***g*** sedimentation through a sucrose gradient. The micromere fraction was determined to be >90% pure by microscopy. EMSAs were carried out with equal quantities of protein from whole-cell extracts and a probe (−106 to −77) that includes the 5′ Sox motifs of *SpAN* site V. Again competitions to demonstrate binding specificity were carried out with self (S) and nonself (NS) sequences. The amount of SpSoxB1 complex (arrow) formed with animal blastomeres was estimated with a Phosphorimager to be about 4-fold greater than in the micromeres (Fig. 8). Similar results were observed for three different extract preparations.

CCAAT factors that interact with SpAN site II are also enriched in nonvegetal blastomere

The other *cis*-acting element essential for *SpAN* promoter function is site II, which contains the motif CCAATCA. Replacement of site II reduces *SpAN* promoter activity about 10-fold (Kozlowski et al., 1996). The same motif, which interacts with the same factor, is also present in duplicate in the *SpHE* gene, which has a spatial pattern of expression identical to that of *SpAN*. Furthermore, CCAATCA motifs in *SpHE* have been shown to be sufficient to drive nonvegetal transcription, in combination with the *SpHE* basal promoter (Wei et al., 1997a). To examine the possibility that asymmetric distribution of CCAATCA factor activity could also be involved in spatial regulation of *SpAN*, we carried out EMSAs with this site and whole-cell extracts from micromere and macromere/mesomere populations exactly as described for SpSoxB1. These results show a similar enrichment of factors binding CCAATCA as was demonstrated for SpSoxB1.

DISCUSSION

Here we present evidence that SpSoxB1 is an important positive regulator of *SpAN* transcription in the nonvegetal region of very early sea urchin embryos. First, SpSoxB1 is the major nuclear protein that interacts with Sox *cis*-acting elements in an essential regulatory region of the *SpAN* promoter when it is maximally active. This conclusion is based on the facts that DNA-protein complexes formed with in vitro translated SpSoxB1 and with nuclear extracts have identical mobilities in EMSAs and are supershifted by an antibody that reacts specifically with SpSoxB1 (Fig. 4A). Second, SpSoxB1 functions as a positive regulator of *SpAN* transcription since promoter activity is reduced about 20-fold when the Sox *cis* elements are replaced (Fig. 1). Third, SpSoxB1 protein is much more abundant in nonvegetal than in vegetal blastomeres of the 16-cell embryo: EMSA demonstrates approximately 4-fold more SpSoxB1 per μg of whole-cell protein in macromeres and mesomeres than in micromeres (Fig. 8). Since micromeres are approximately 4-fold and 2.5-fold smaller than macromeres and mesomeres, respectively, the absolute amount of SpSoxB1 is about 10-fold lower in micromeres. These differences result in a significantly lower concentration of SpSoxB1 in nuclei of

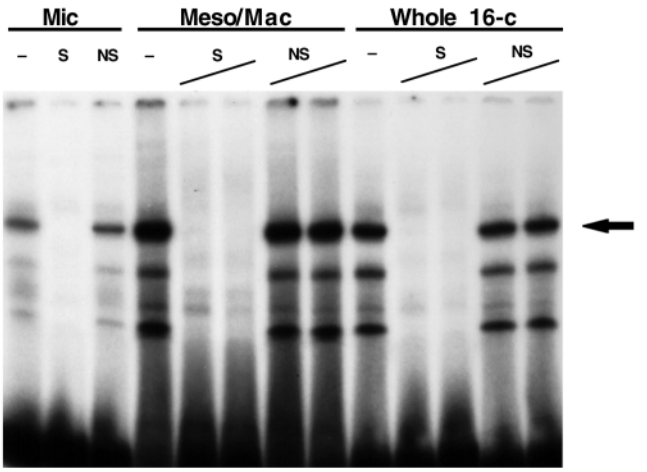

Fig. 8. SpSoxB1 protein that interacts with the Sox *cis* element is present in higher concentration in nonvegetal than in vegetal blastomeres of 16-cell embryos. EMSAs were carried out using the site V probe (diagrammed in Fig. 1) and whole-cell protein extracts from micromeres (Mic), mesomeres+macromeres (Meso/Mac), or whole 16-cell embryos (Whole 16-c). The arrow at right indicates the position of the specific complex formed between this probe and SpSoxB1. Other, higher mobility bands are complexes formed with the middle, non-Sox region of siteV, which has no demonstrable functional significance (see Fig. 1 and text). –, no competitor; S, competition with a 250- to 1000-fold molar excess of unlabeled site V sequence; NS, competition with the same molar excess of an unrelated sequence. For the micromere samples, only one concentration of competitor (1000-fold molar excess) is shown.

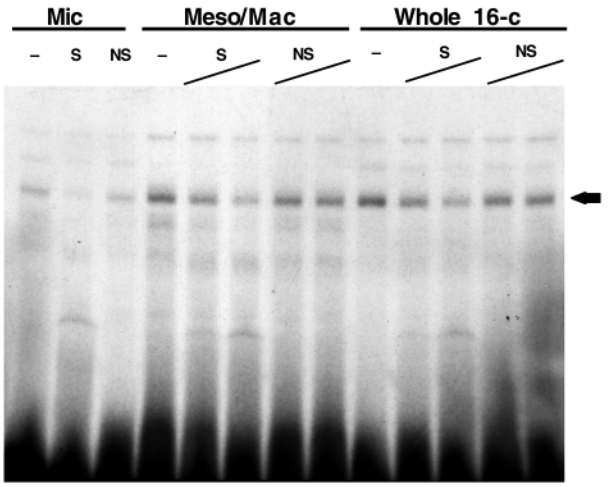

Fig. 9. CCAAT factors interacting with *SpAN* promoter siteII also are present at lower concentration in micromeres of 16-cell embryos. EMSAs were carried out using a probe containing sequences between –220 and –190 of the *SpAN* promoter. Several different complexes can form with sequences in this probe; those complexes containing CCAAT factors as shown by previous EMSA competition assays (Kozlowski et al., 1996) are indicated by the arrow. –, no competitor; S, competition with a 50- to 250-fold molar excess of unlabeled site V sequence; NS, competition with the same molar excess of an unrelated sequence. For the micromere samples, only one concentration of competitor (250-fold molar excess) is shown.

micromeres than in those of other blastomeres at 16-cell stage (Fig. 7). Since the Sox *cis* elements are critical for promoter activity, this difference in SpSoxB1 nuclear concentration alone may be sufficient to account for the fact that micromeres do not accumulate *SpAN* transcripts detectable by a very sensitive RNase protection assay, whereas macromeres and mesomeres do (Reynolds et al., 1992).

However, restriction of activity to the nonvegetal domain appears to be characteristic of a number of different transcription factors. Although SpSoxB1 is necessary for high level *SpAN* promoter activity in the nonvegetal domain, it is not sufficient. Work to be published elsewhere suggests that the positive function of the SpSoxB1 protein is mediated through its ability to bend DNA rather than by direct activation of transcription. Consistent with a requirement for at least one additional, active, positive regulator, deletion of a CCAATCA motif upstream in site II reduces *SpAN* promoter output on transgenes about 10-fold (Kozlowski et al., 1996). The EMSA data presented here (Fig. 9) show that the factor that binds to this element also is present at lower concentration in micromere extracts. Interestingly, the same CCAAT factor also interacts with two copies of the CCAATCA motif (sites V and VI) in the promoter of a second gene, *SpHE* (Wei et al., 1995; Kozlowski et al., 1996) that is expressed in the same nonvegetal pattern as is *SpAN* (Reynolds et al., 1992). Furthermore, this duplicated *cis* element, in combination with the *SpHE* basal promoter, has been shown to be sufficient to regulate nonvegetal expression of transgenes (Wei et al., 1997a) as does an Ets motif that binds SpEts4 recently cloned in our laboratory (Wei et al., 1999).

Our previous detailed analyses of the *SpAN* and *SpHE* promoters showed that these genes are regulated via *cis* elements that interact only with positive factors; no negative element could be found whose deletion led to ectopic expression in vegetal blastomeres. This fact led us to propose that a major component of the animal-vegetal axis of the sea urchin embryo is the establishment of a nonvegetal transcriptional domain that includes a number of different transcription factor activities (reviewed by Davidson et al., 1998; Angerer and Angerer, 1999). The fact that both *SpHE* and *SpAN* are activated cell autonomously indicated that construction of this domain ultimately is under maternal control. However, because functional assays of the promoters in vivo were necessarily carried out at later stages, when detectable CAT or β-gal activity had accumulated, the possibility remained that the factors regulating expression during cleavage were not the same as the maternal factors that initially establish the nonvegetal regulatory domain. *SpSoxB1* provides the first example of a maternally transcribed gene encoding a factor that accumulates differentially in vegetal and nonvegetal domains. This difference is established at 16-cell stage, when the micromeres first partition off a major portion of the vegetal domain.

Although the amount of SpSoxB1 protein is about 10-fold less in micromeres, the concentration of *SpSoxB1* mRNA, as judged by in situ hybridization grain densities, is similar in the three blastomere types (Fig. 5). While the smaller *quantity* of SpSoxB1 mRNA molecules distributed to the micromeres will gradually amplify the difference in protein amounts, it is unlikely that this effect makes a significant contribution in the brief interval between 4th cleavage and the time the SpSoxB1 protein levels were determined in the experiments shown in

Figs 7 and 8. Consequently post-transcriptional mechanisms are also likely to contribute to the asymmetry of SpSoxB1 nuclear concentration and could include its differential translation and/or stability in nonvegetal versus vegetal blastomeres.

A distinct zygotic mechanism assumes control over the expression of *SpSoxB1* mRNA and protein during cleavage. The uniform maternal distribution of *SpSoxB1* transcripts is gradually replaced by a nonvegetal distribution congruent with that of *SpAN* transcripts, as maternal mRNAs turn over and are replaced by zygotic messages. A similar transition has been demonstrated for transcripts from a number of other genes (e.g., Eldon et al., 1987; Grimwade et al., 1991; our unpublished observations). It is most clearly revealed by *SpEts4* transcripts, which encode a spatially restricted regulator of *SpHE*. In that case, an extension of the 3′UTR provides a specific probe that detects zygotic transcripts only in nonvegetal blastomeres (Wei et al., 1999). Thus, the nonvegetal pattern is exhibited both by genes encoding regulators of transcription, such as SpSoxB1 and SpEts4, and by the targets of these regulators. It seems likely that the positively acting factors in this group also activate each other's transcription. This would serve to amplify initial maternal asymmetries, such as that demonstrated for SpSoxB1 nuclear concentration at 16-cell stage. It also could lead to production of additional regulatory activities in the nonvegetal transcriptional domain.

Several factors indicate that there must be a later zygotic mechanism that modulates the vegetal border of *SpAN* (and presumably *SpHE*) transcription. First, in situ hybridization to *SpAN* and *SpHE* mRNAs indicated that the size of the vegetal region lacking these transcripts is variable among embryos at the very early blastula stage (~150 to 200 cells) and does not correspond to any particular lineage border (Reynolds et al., 1992). We originally suggested that this reflects an inherent imprecision in the vegetal-nonvegetal border among embryos and that the nonvegetal region only approximately demarcates the future ectoderm territory. However, the SpSoxB1 distribution described here suggests that only the difference between micromeres and adjacent macromeres may be established maternally, by asymmetric cell division, and thus does represent a lineage border. After this stage, the border may be repositioned in macromere descendents by cell-cell interactions. The rate at which this refinement occurs may vary among embryos, accounting for the variability in *SpAN* and *SpHE* transcriptional domains at very early blastula stages (12-15 hours postfertilization). Consistent with this interpretation, the size of the nonvegetal domain can be altered by experimental treatments that modulate cell signaling pathways (Ghiglione et al., 1993; Emily-Fenouil et al., 1998; our unpublished observations).

The observation that the distribution of nuclear SpSoxB1 progressively withdraws from the vegetal pole until it marks the ectoderm-endoderm border leads to the hypothesis that its zygotic transcription, and probably that of other major regulators of *SpAN and SpHE*, is progressively repressed in the vegetal plate by the signaling cascade that originates in the micromeres and sequentially specifies secondary mesenchyme and endoderm (reviewed by Angerer and Angerer, 1999). A corollary of this model is that the initial, cell-autonomous function of the nonvegetal transcription activators is to specify a primitive type of ectoderm, i.e., autonomously specified to form the classic dauerblastulae that develop from the animal halves of 8-cell embryos. This tissue is then competent to respond to a set of both positive and negative signals from the vegetal pole required for further differentiation.

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