

SpZ12-1, a negative regulator required for spatial control of the territory-specific *CyIIIa* gene in the sea urchin embryo

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SUMMARY

The *CyIIIa* cytoskeletal actin gene of the sea urchin *Strongylocentrotus purpuratus* is activated in late cleavage and expressed exclusively in the aboral ectoderm territory of the embryo. Previous gene transfer studies defined a 2.3 kb *cis*-regulatory region that is necessary and sufficient for correct temporal and spatial expression of a *CyIIIa*•CAT fusion gene. In this paper, a negative regulatory element within this region was identified that is required for repression of the *CyIIIa* gene in skeletogenic mesenchyme cells. The repression mediated by this regulatory element takes place after initial territorial specification. A cDNA clone

encoding a DNA-binding protein with twelve Zn fingers (SpZ12-1) was isolated by probing an expression library with this *cis*-element. Deletion analysis of the SpZ12-1 protein confirmed that a DNA-binding domain is located within the Zn finger region. SpZ12-1 is the only DNA-binding protein in embryo nuclear extract that interacts with the specific *cis*-target sites required for repression of *CyIIIa*•CAT in skeletogenic mesenchyme and is likely to be the *trans* factor that mediates this repression.

Key words: transcription factor, sea urchin gene regulation, *CyIIIa*

INTRODUCTION

In the sea urchin embryo, cleavage of blastomeres is invariant and gives rise to five polyclonal territories. Each of these embryonic territories develops into specific larval structures and those that have been studied are identified by unique spatial patterns of gene expression. The territories are: the aboral ectoderm, the oral ectoderm, the skeletogenic mesenchyme, the vegetal plate and the small micromeres. Territory-specific gene expression begins shortly after the segregation of lineage founder cells from one another at 3rd to 6th cleavage (reviewed by Davidson, 1986, 1989; Cameron and Davidson, 1991).

A well-characterized molecular marker for the aboral ectoderm is the *CyIIIa* cytoskeletal actin gene, which is transcriptionally activated late in cleavage and is expressed exclusively in the aboral ectoderm throughout embryonic development (Shott et al., 1984; Cox et al., 1986; Lee et al., 1986; Hickey et al., 1987; Akhurst et al., 1987). In earlier gene transfer experiments, a 2.3 kb *CyIIIa* regulatory region was found to direct correct spatial and temporal expression of a linked CAT reporter gene in the embryo (Flytzanis et al., 1987; Livant et al., 1988, 1991; Hough-Evans et al., 1987, 1988, 1990; Franks et al., 1990). At least 20 sites within this regulatory region where nuclear proteins bind with high specificity *in vitro* have been mapped in detail, as indicated in Fig. 1 (Calzone et al., 1988; Thézé et al., 1990). Several gene regulatory factors that interact at these sites with high specificity have been isolated and characterized (Calzone et al., 1991; Höög et al., 1991; Char et al., 1993; and unpublished data from

this laboratory). This report concerns the regulatory function of interactions that occur at two adjacent sites within the *CyIIIa* regulatory domain, and a DNA-binding factor which binds specifically at these sites (SpZ12-1). By mutating the SpZ12-1 sites, we showed that interactions at these sites are required specifically for spatial repression of the *CyIIIa* gene expression in skeletogenic mesenchyme cells at the gastrula stage. SpZ12-1 is the only factor interacting with this regulatory element in embryo nuclear extracts, and thus it is likely that it is a mesenchyme repressor for the *CyIIIa* gene.

MATERIALS AND METHODS

Embryos

Eggs of *Strongylocentrotus purpuratus* were prepared for injection as previously described (McMahon et al., 1985). The eggs were microinjected with approx. 2 pl of injection solution after fertilization. The embryos were cultured to mesenchyme blastula stage (30 hours), or late gastrula stage (54 hours) for whole-mount *in situ* hybridization. The injection solution contained 40% glycerol, 0.2 M KCl, 1500-3000 molecules per pl of plasmid, and a 5-fold molar excess of carrier DNA.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridizations were carried out according to the method of Ransick et al. (1993), but using a modified fixation procedure. Briefly, embryos were fixed in Streck Tissue Fixative (Streck Laboratories) at 4°C for 2-6 days. After a brief wash with filtered sea water and PBST buffer (PBS containing 0.1% Tween-20) the embryos were equilibrated with hybridization buffer (50%

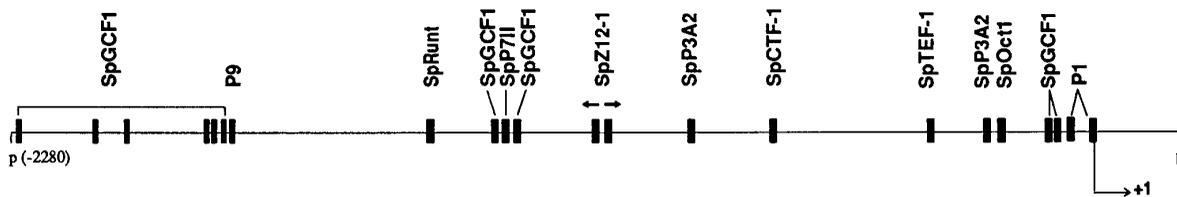


Fig. 1. The 5' regulatory domain of the *CyIIIa* cytoskeletal actin gene. Black boxes represent the specific sites of interaction with nuclear proteins from blastula stage embryos in the 5'-flanking region of the *CyIIIa* gene that extends from -2280 to +235 bp (Calzone et al., 1988; Thézé et al., 1990; Coffman et al., unpublished data). The bent arrow indicates the transcription start site. Note that there are two adjacent SpZ12-1 binding sites in inverse orientation within this region. Restriction sites that flank this region are *Bam*HI (b) and *Pst*I (p). Nuclear factors binding to the sites are indicated: SpGCF-1 is a factor of novel sequence that recognizes a G/C-rich motif (Coffman and Davidson, 1992; R. W. Zeller and E. H. Davidson, unpublished); SpRunt in an *S. purpuratus* factor homologous to the *Drosophila* runt protein (J. A. Coffman and E. H. Davidson, unpublished data); SpP3A2 and SpP7II are both factors of novel sequence, which may be involved in distinct negative control functions of the *CyIIIa* regulatory system (Hough-Evans et al., 1990; Calzone et al., 1991; C. Kirchhamer and E. H. Davidson, unpublished data; J. A. Coffman and E. H. Davidson, unpublished data); SpCTF-1 is a CCAAT binding factor (Calzone et al., 1988; Barberis et al., 1987); SpOct-1 is an octamer factor (Char et al., 1993). SpTEF1 is an *S. purpuratus* factor homologous to the human enhancer factor TEF-1 (J. Xian and E. H. Davidson, unpublished data). P9 and P1 are not yet characterized. All of these sites of interaction appear to have functional roles in control of *CyIIIa* expression (with the possible exception of the P9 interaction, which has not yet been studied).

formamide, 10% PEG, and 0.6 M NaCl, 5 mM EDTA, 20 mM Tris, pH 7.5, 500 µg/ml yeast tRNA, 0.1% Tween-20, and 2× Denhardt's solution). It is worth noting that proteinase K digestion is not necessary when Streck fixative is used. Hybridization was carried out at 45°C overnight with a digoxigenin-labeled CAT antisense RNA probe at 0.02 ng/µl. Three washes of 1× SSC at 65°C were followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin F(ab) (Boehringer Mannheim Biochemicals). The chromogenic reaction was performed with NBT and BCIP (Boehringer Mannheim Biochemicals). After dehydration, the embryos were mounted for observation.

Construction of *CyIIIa*•CAT and *mZ12CyIIIa*•CAT

(1) *CyIIIa*•CAT

The *CyIIIa*•CAT construct contains a 4.5 kb genomic DNA fragment that includes the 5' *CyIIIa* regulatory region, transcription start site, and 5' leader sequence of the *CyIIIa* primary transcript (Akhurst et al., 1987; Flytzanis et al., 1987). The *CyIIIa*•CAT construct was linearized for microinjection at a unique *Not*I site (Fig. 3).

(2) *mZ12CyIIIa*•CAT

Site-directed mutagenesis of the SpZ12-1 binding sites was carried out by PCR. A 1.2 kb *Kpn*I fragment from the *CyIIIa*•CAT plasmid that included the two SpZ12-1 binding sites was subcloned into the pBluescript KS (+) vector at its *Kpn*I site, to generate pBlue HE. The site-specific substitutions were made with two overlapping mutagenic oligonucleotide primers (DS-T7 and DS-T3). The sequence of these primers, with the overlapping region underlined, and the substituted nucleotides indicated in bold type, was as follows.

Primer for SpZ12-1 binding site I:

-DS-T7,
5'-ACAAACAATATGCGCTTGACCTATTGATTCCTCTGTACCGTGCATTCTG-3'

Primer for SpZ12-1 binding site II:

-DS-T3,
5'-GTCAAGCCATATGTTTGTGCTATTGGTTCCCTCACTAATGTATGCCGTTTC-3'

The first round of PCR was designed to introduce base substitutions into the SpZ12-1 binding sites I and II in two separate fragments using a combination of mutant and non-mutant primers. The reactions were performed in 25 µl containing 100 ng template DNA (pBlue HE), 0.4 mM DS-T7 or DS-T3 primers, 4 mM T7 or T3 primers, 0.4 mM each dNTP, and one unit of Vent DNA polymerase (New England Biolabs), with 15 cycles of amplification (94°C, 30 seconds; 55°C, 1 minute; 72°C, 1 minute). The second round of PCR reactions

were carried out to rejoin the two purified PCR fragments (100 ng each) using the external T7 and T3 primers. Following *Kpn*I digestion of the final PCR product, the *mZ12CyIIIa*•CAT fusion gene was generated by inserting the 1.2 kb fragment back into the *CyIIIa*•CAT fusion gene at the *Kpn*I site (see Fig. 3).

Cloning of the SpZ12-1 gene

The sequence of the SpZ12-1 binding site I was used to generate the complementary oligonucleotide probe shown below (Calzone et al., 1988; Thézé et al., 1990; Wang et al., unpublished data).

5'-ACATGTTGCTAGGTAGGTCAA-3'
3'-CAACGATCCATCCAGTTTGTA-5'

A λZAP (Stratagene) cDNA expression library of 4-hour embryo poly(A) RNA was screened with this probe as described by Vinson et al. (1988) and Höög et al. (1991). One positive clone was isolated and both strands were sequenced using subclones generated by exonuclease III digestion. Sequencing was performed by the dideoxy method, using Sequenase reagents (US Biochemical Corp.).

Expression, purification and renaturation of bacterial proteins

A T7 RNA polymerase based expression system (Studier and Moffat, 1986) was used to prepare recombinant SpZ12-1 protein. Three different expression constructs (SpZ12-1, SpZ12-1ΔZF and SpZ12-1ΔN) were made in pET vectors. The SpZ12-1 construct contains an *Eco*RI-*Nci*I fragment of SpZ12-1 cDNA. The fragment was end-filled and ligated into the pET3c vector at the *Bam*HI site. The SpZ12-1ΔZF construct, which includes an *Eco*RI-*Mlu*I fragment of SpZ12-1 cDNA, was generated by ligating the end-filled fragment into the pET3c vector at the *Bam*HI site. The SpZ12-1ΔN construct contains an *Esp*I-*Nci*I fragment of SpZ12-1 cDNA, prepared by joining the end-filled fragment into the *Bam*HI site of pET3b vector. Expression of SpZ12-1 proteins in *E. coli* was performed as described by Studier et al. (1986) and Calzone et al. (1991). Briefly, IPTG-induced bacterial cells were harvested by centrifugation, and the pellet was resuspended in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1% Triton X-100, and 1 mM PMSF. Following a 1-hour incubation with 1 mg/ml lysozyme on ice and brief sonication, 15,000 g pellets were washed in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 25% sucrose, and 1 mM PMSF. After centrifugation at 15,000 g, the washed pellets were resuspended in 20 mM Hepes-KOH (pH 7.9), 0.1 mM EDTA, 1 mM DTT, and 5 M guanidine hydrochloride. In order to renature the SpZ12-1 proteins, guanidine hydrochloride was diluted from 5 to 0.5 M with 10 mM Hepes-KOH (pH 7.9),

100 mM KCl, 0.2 mM ZnCl₂, 0.1 mM PMSF, 1 mM DTT, 0.1% NP-40 and 20% glycerol in three serial two-fold dilution steps. At each step the solution was incubated at 4°C for 4-6 hours. After renaturation, insoluble protein was eliminated by centrifugation at 15,000 *g* and the supernatant was stored at -70°C.

Antibodies and immunoblots

SpZ12-1 Δ ZF protein (Fig. 8) was expressed from *E. coli* as described above and further purified by SDS-PAGE. The purified protein was used for the immunization of rabbits. The specificity of the antiserum preparations (Cocalico Biological, Inc.) was tested by using bacterially expressed SpZ12-1 protein and whole embryo lysate. Immunoblots were carried out according to Calzone et al. (1991).

Immunoreactive proteins were visualized by the ECL detection system (Amersham) following procedures recommended by the supplier.

RESULTS

Expression pattern of the control *CyIIIa*•*CAT* fusion gene at late gastrula stage

In this study we utilized a sensitive whole-mount in situ hybridization procedure to locate *CAT* transcripts deriving from injected *CyIIIa*•*CAT* fusion constructs (Ransick et al.,

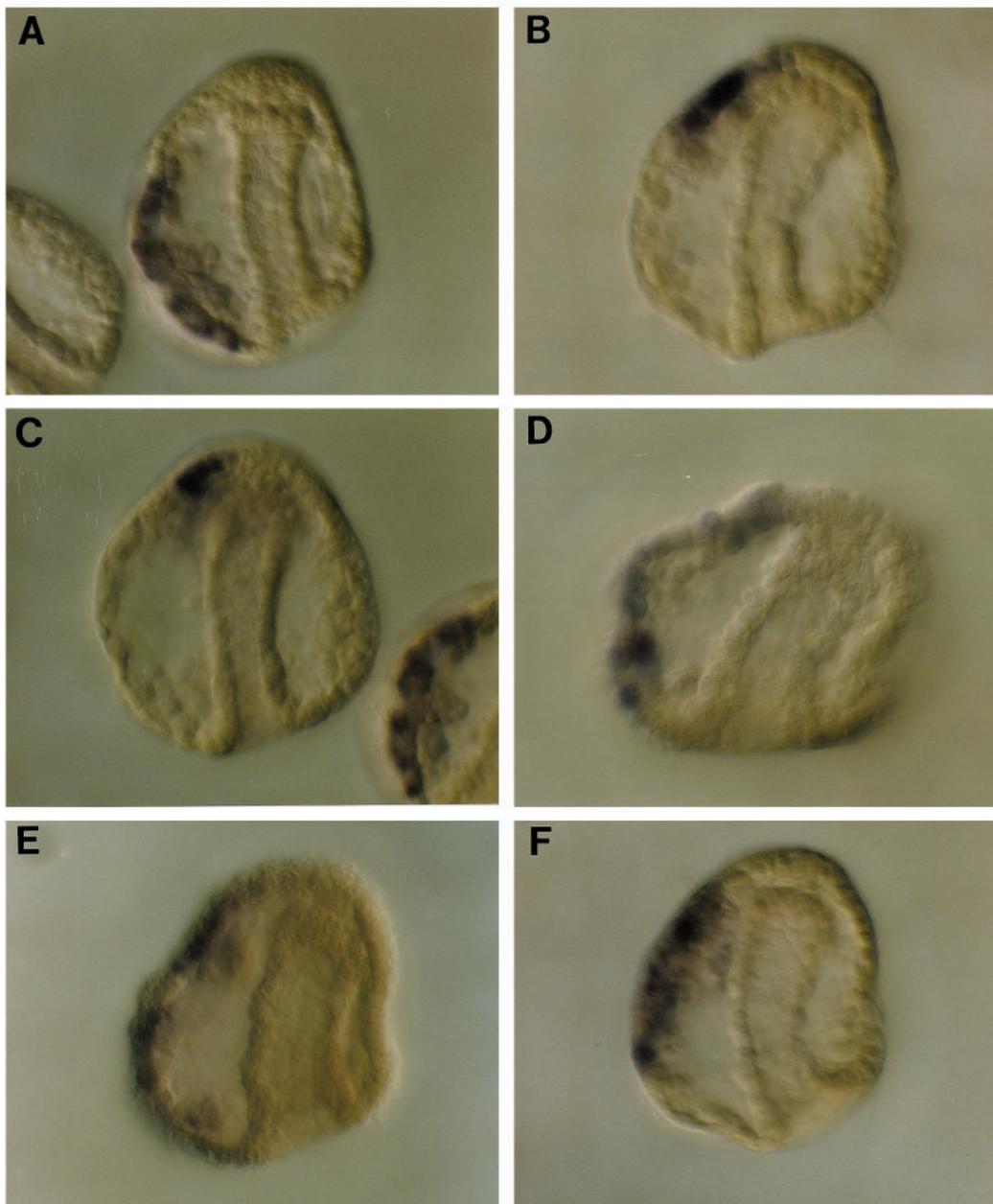


Fig. 2. Expression pattern of the *CyIIIa*•*CAT* fusion gene at late gastrula stage. The spatial distribution of *CAT* transcripts in late gastrula-stage embryos was detected by whole-mount in situ hybridization, using a digoxigenin-labeled antisense *CAT* RNA probe. Six late gastrula-stage embryos with expression restricted to the aboral ectoderm are shown, oriented with the oral surface to the right. (A) An embryo showing extensive staining along the aboral ectoderm extending to the lower aboral-oral boundary. (B,C) Two embryos displaying a patch of labeled cells toward the upper aboral-oral boundary. (D,E,F) Three embryos expressing *CAT* transcripts in large regions of the aboral ectoderm.

1993; Yuh et al., 1994). The previous experiments on *CyIIIa*•*CAT* expression cited above had relied on in situ hybridization of radioactive probes to embryo sections. The expression pattern of the complete *CyIIIa*•*CAT* fusion gene at late gastrula stage (54 hours postfertilization), as visualized by the whole-mount hybridization procedure, is shown in Fig. 2. Fig. 2A displays an embryo with aboral ectoderm expression close to the lower aboral-oral boundary; in Fig. 2B,C two embryos are shown with a patch of positive cells toward the upper aboral-oral boundary. Fig. 2D-F show three embryos with a large portion of the aboral ectoderm stained. The mosaic pattern of *CAT* expression observed in these stained embryos (Fig. 2A-F) is due to mosaic incorporation of the fusion gene during the first several cleavages (Hough-Evans et al., 1988; Livant et al., 1991). Of the hundreds of embryos expressing the *CyIIIa*•*CAT* fusion gene that were examined, approximately 95% showed expression confined to the aboral ectoderm. Approximately 70% of all the injected embryos express *CAT* transcripts. Quantitative data for four different experiments with the control *CyIIIa*•*CAT* construct are given in Tables 1 and 2.

Ectopic expression pattern of the *mZ12CyIIIa*•*CAT* fusion gene at late gastrula stage

Two adjacent SpZ12-1 binding sites were initially identified by

Calzone et al. (1988; there referred to as 'P6' sites). A preliminary experiment showed that deletion of a region of about 200 bp (−1033 to −821), which includes these sites, sharply derepresses spatial regulation of the *CyIIIa* gene, causing ectopic expression in mesenchyme cells (data not shown). To assess the contribution of the SpZ12-1 binding sites to the spatial control of *CyIIIa* gene expression, both sites within the 5' *CyIIIa* regulatory region were mutated, generating the *mZ12CyIIIa*•*CAT* fusion gene. As shown in Fig. 3A, these sites occur in reverse orientation 13 base pairs apart. They include a 9/10 nucleotide pair reverse repeat of the sequence TGTTGC(T/C)AGG. By site-directed mutagenesis we altered each of the nucleotides in this core sequence from purine to pyrimidine or vice versa. As shown in Fig. 3B, these substitutions actively abolish DNA-binding activity (Fig. 3B).

Patterns of expression of the *mZ12CyIIIa*•*CAT* fusion gene at late gastrula stage were determined by whole-mount in situ hybridization as above. The mutation has a remarkable effect on spatial expression of the fusion gene, evidently resulting in its derepression in mesenchyme cells, but only in mesenchyme cells. Examples are shown in Fig. 4. Two major phenotypes occur, viz. embryos displaying expression in both mesenchyme cells and aboral ectoderm cells (Fig. 4A,B,D-F), and embryos with expression only in mesenchyme cells (Fig. 4C). A detailed analysis of about 200 stained embryos is summarized in Table

Table 1. Ectopic expression pattern of the *mZ12CyIIIa*•*CAT* fusion gene in mesenchyme cells at late gastrula stage (approx. 54 hours)

Construct	Batch	Total embryos stained*	Embryos with correct expression†	Embryos with ectopic expression in mesenchyme cells	Ectopic expression as % of stained embryos‡
<i>CyIIIa</i> • <i>CAT</i> (control)	1	41	40	1	2.4
	2	45	41	4	8.9
	Total	86	81	5	5.6
<i>mZ12CyIIIa</i> • <i>CAT</i>	1	48	26	22	46
	2	53	30	23	44
	3	95	50	45	47
	Total	196	106	90	46

*Embryos were scored if two or more cells were stained.

†Embryos with correct expression were scored if two or more cells were stained in a patch within the aboral ectoderm.

‡Ectopic expression as % of stained embryos = [\sum embryos stained ectopically/ \sum stained embryos] \times 100.

Table 2. Expression pattern of *mZ12CyIIIa*•*CAT* fusion gene at mesenchyme blastula stage (approx. 30 hours)

Construct	Batch	Total embryos stained*	Embryos with correct expression†	Embryos with ectopic expression in mesenchyme cells	Ectopic expression as % of stained embryos‡
<i>CyIIIa</i> • <i>CAT</i> (control)	1	18	18	0	0
	2	36	36	0	0
	Total	54	54	0	0
<i>mZ12CyIIIa</i> • <i>CAT</i>	1	22	22	0	0
	2	48	46	2	4
	Total	70	68	2	3

*Embryos were scored if two or more cells were stained.

†Embryos with correct expression were scored if two or more cells were stained in a patch within the ectoderm.

‡Ectopic expression as % of stained embryos = [\sum embryos stained ectopically/ \sum stained embryos] \times 100.

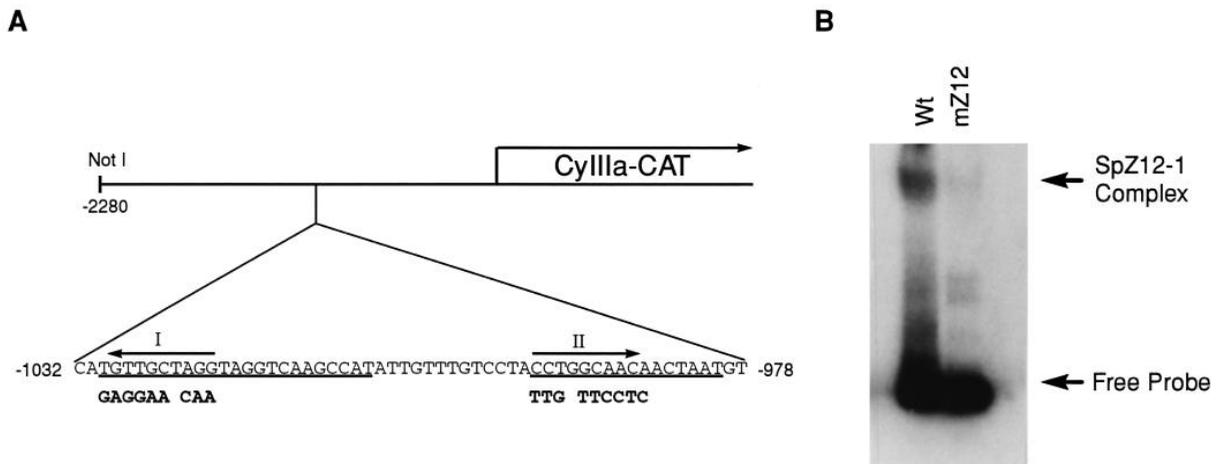


Fig. 3. *mZ12CyIIIa•CAT* fusion gene and mutated target sites. (A) A schematic representation of the *mZ12CyIIIa•CAT* fusion gene. The *mZ12CyIIIa•CAT* fusion gene includes the 5' *CyIIIa* regulatory sequence and portions of a non-translated exon, extending from -2280 to $+235$ bp relative to the transcription start site. The sequences of two adjacent SpZ12-1 binding sites, I and II, in inverse orientation are at -1030 to -1008 and -994 to -979 bp, respectively (underlined). Underlined sequences are derived from the DNase I footprinting experiment shown below. The arrows indicate the inversely repeated core target site sequences noted in text. Substituted nucleotides in the mZ12 mutant gene are indicated below, in bold type. (B) DNA binding activity of the wild-type (Wt) and the mutant (mZ12) SpZ12-1 binding sites. ^{32}P -labeled probes include the two adjacent SpZ12-1 binding sites, contained in the DNA fragment that extends from positions -1082 to -894 of the 5' *CyIIIa* regulatory region. Gel shift assays were carried out using blastula-stage nuclear extracts as previously described (Calzone et al., 1988). The nine nucleotide substitutions within each of the SpZ12-1 binding sites suffice to abolish all DNA binding activity, and only the free probe is observed.

1. About the same fraction of embryos express the *mZ12CyIIIa•CAT* fusion genes as they do the *CyIIIa•CAT* control construct. However, 46% of embryos bearing the *mZ12CyIIIa•CAT* fusion gene display ectopic mesenchyme cell expression. This is 8-fold higher than the low level of ectopic expression observed in the control *CyIIIa•CAT* embryos. Importantly, ectopic expression is confined to *skeletogenic* mesenchyme, and is not seen in secondary mesenchyme, as judged from the position of the stained cells at this stage of embryogenesis. These data indicate that a negative regulatory interaction mediated by the SpZ12-1 binding sites is required for repression of the *CyIIIa* gene in skeletogenic mesenchyme cells at the late gastrula stage.

Expression pattern of the *mZ12CyIIIa•CAT* fusion gene at mesenchyme blastula stage

We wished to determine whether the negative control of *CyIIIa* expression in mesenchyme cells that is mediated by the SpZ12-1 sites is part of the initial aboral ectoderm specification mechanism. Thus we examined the expression of the *mZ12CyIIIa•CAT* construct at earlier stages. At the mesenchyme blastula stage, the skeletogenic mesenchyme cells have just ingressed into the blastocoel and can be easily identified morphologically, though at this stage it is difficult to distinguish aboral from oral ectoderm. Surprisingly, in these experiments we observed CAT transcripts exclusively in the ectoderm in almost all of the embryos staining for CAT RNA. No ectopic expression was detected in mesenchyme cells at this stage, in contrast to the late gastrula stage, as just discussed. Three typical embryos displaying CAT expression in the ectoderm but not in the newly ingressed skeletogenic mesenchyme cells, are shown in Fig. 4G-H. As indicated in Table 2, only 3% of the stained embryos from this series of

experiments show ectopic expression in mesenchyme cells, which is probably indistinguishable from the control (*CyIIIa•CAT*) level. It should be noted that the possibility of ectopic expression in the oral ectoderm at this stage cannot be excluded, even though it is unlikely (i.e., since no oral expression is seen in gastrula stage embryos bearing the *mZ12CyIIIa•CAT* fusion). Thus the repression of mesenchyme expression mediated by the SpZ12-1 binding sites is a later control function, which is required to maintain ectodermal expression of the *CyIIIa* gene only after gastrulation.

Molecular cloning of the SpZ12-1 gene

A cDNA clone, designated SpZ12-1, was isolated by screening a 4-hour sea urchin embryo λ ZAP expression library with an oligonucleotide probe containing multiple SpZ12-1 binding sites (Vinson et al., 1988; Höög et al., 1991). Nucleotide sequence analysis of a full-length SpZ12-1 cDNA clone indicated an open reading frame of 1779 bp (Fig. 5). This reading frame encodes a 593 amino acid protein, the predicated relative molecular mass of which is about 70×10^3 .

The deduced amino acid sequence of the SpZ12-1 protein reveals a potential DNA-binding domain consisting of twelve tandem Zn fingers (Fig. 5A). The finger domain is located near the carboxyl terminus and occupies more than 50% of the entire protein. All twelve fingers of the SpZ12-1 protein are variants of the TFIIIA type of Zn finger, the consensus amino acid sequence of which is Tyr/Phe-X-Cys-X_{2/4}-Cys-X₃-Phe-X₅-Leu-X₂-His-X₃-His-X₅, where X may represent a variety of different amino acid (Berg et al., 1990; Pavletich et al., 1991, 1993). In each case, the cysteines are separated by two amino acids and each finger is connected with the next by a Thr-Gly-Glu-Lys-Pro consensus linker. Each of these sequences is capable of binding a zinc ion via the cysteine and

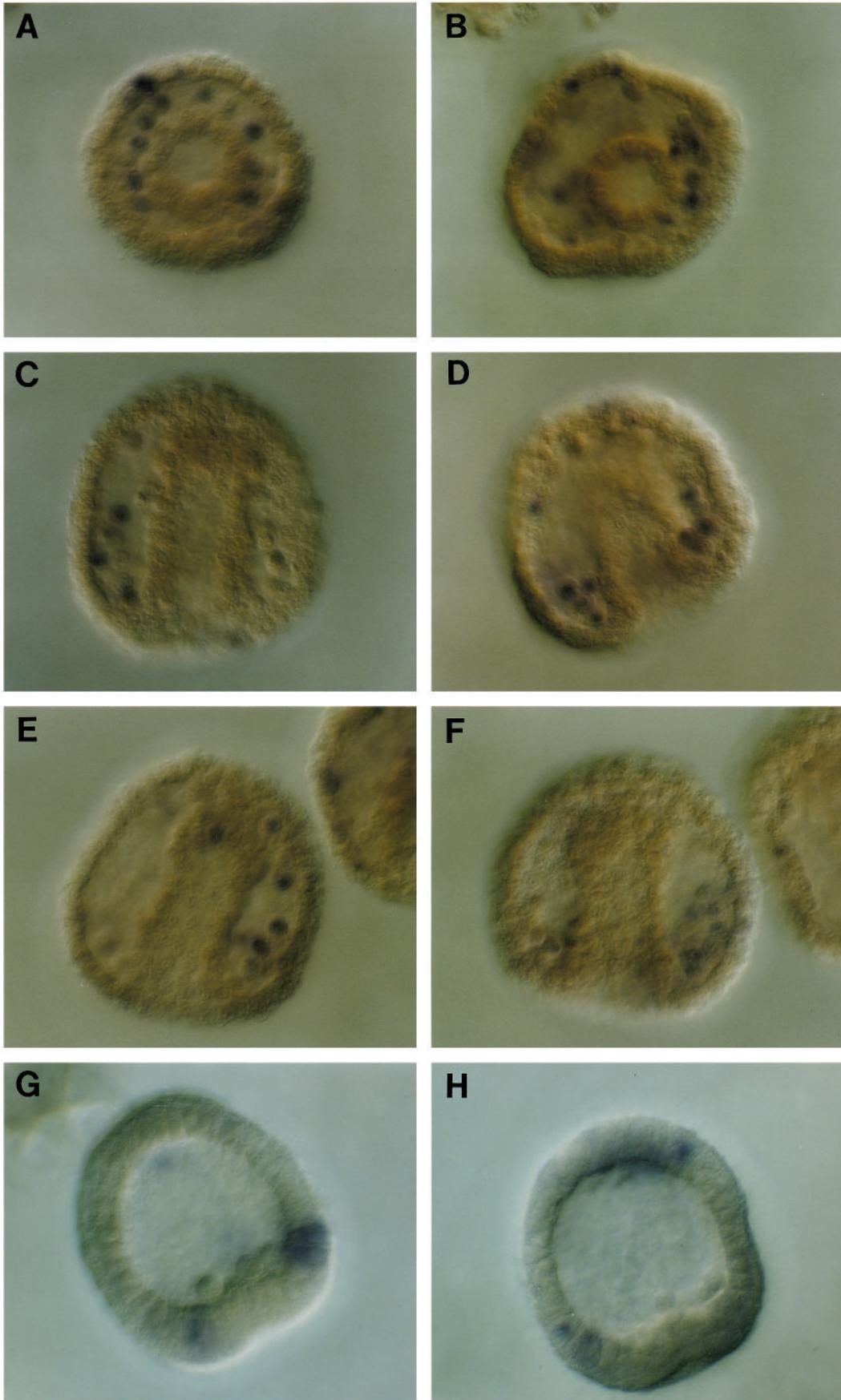


Fig. 4. Expression pattern of the *mZl2Cylla•CAT* fusion gene at gastrula stage and mesenchyme blastula stage. (A,B) Gastrula-stage embryos expressing CAT transcripts in closely spaced skeletogenic mesenchyme cells surrounding the archenteron, and along the inner ectodermal wall, vegetal view. Note that both embryos show a patch of stained cells in the aboral ectoderm toward the top left sides. (C-F) Side views, vegetal pole down. The archenteron, topped with delaminating secondary mesenchyme cells, can be seen in the optical sections shown. (C) A gastrula-stage embryo in which expression occurs only in skeletogenic mesenchyme cells. (D-F) Three other gastrula-stage embryos displaying expression in skeletogenic mesenchyme cells. In other optical sections it can be seen that these particular embryos also express CAT transcripts in the aboral ectoderm. (G) A mesenchyme blastula-stage embryo showing staining in the ectoderm close to the vegetal plate, but not in the ingressed skeletogenic mesenchyme cells. (H) A mesenchyme blastula-stage embryo with expression located in the ectoderm closer to the animal pole, but no expression in the newly ingressed skeletogenic mesenchyme.

histidine side chains to generate structural configurations that are essential for DNA binding (Berg et al., 1990). Fig. 6B shows sequence alignment of SpZ12-1 and *Drosophila Krüppel* Zn fingers (Rosenberg et al., 1986). These two finger proteins share all the consensus amino acids of the TFIIIA type of Zn finger, but also display a great deal of similarity in the region represented by the terminal 'X5' in this canonical consensus (see shading in Fig. 5B). Computer searches of GenBank with the protein sequence outside of the Zn finger domain did not reveal significant similarities to any other known proteins.

Characterization of the SpZ12-1 protein

Since the sequence analysis of the SpZ12-1 protein revealed twelve Zn fingers near the carboxyl terminus, it is likely that a potential DNA-binding domain is located within this region. To test this possibility, gel shift assays were performed to compare the DNA-binding activity of the wild-type and two deletion mutants of the SpZ12-1 protein. DNA fragments encoding the full-length sequence (SpZ12-1), the amino-terminal region (SpZ12-1ΔZF), and the Zn finger domain (SpZ12-1ΔN) of the SpZ12-1 protein were cloned into pET expression vectors (Studier et al., 1986), as shown in Fig. 6A. The encoded proteins were expressed and purified from *Escherichia coli*,

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1 AATTTCGGCCGATTCGGGCCGTGCACATCCAGGAGAGATCCAGCACAGCAGACAAAATGACACAGGCGATGCTCTCTAAGAAA
1 M D L S K K
91 CGAAAAAGAGATGCACCTATACCGGTTCACAGTCCCGGTTATAGCACCGGTTACAGCACAGGTAACCATTAACGGGAGCCAGTGGAGGAA
7 R K R D A P I P V T V P V I A P V T A Q V T I K R E P V E E
181 ACTAGTGATGATGGCTACTTTACTAGTCTCGGGAGGGGAGCGAGAAACATTATGCAAAGTCGTCAGCCATGCTTTTCAGTACGCTACG
37 T S D D G Y F T S L L G R G S E K H Y A K S S S H V F Q Y A T
271 CAACATCAGGGTATTCCTTTTTCAGTCCAAGCAGCCCTGTCTATCACCATCCAGTACCAAAACAATAATCGAGATCAAAACAGAGACGGTG
67 Q H Q G I P F S P S S P V Y H H P V P N N N I E I K Q E T V
361 ACGGACGAAGATAGTGGCAGGTGTGCTTCAAACCTTACGATAATGCAGGCACATGCGAGGACTACTTTCCGTTATCACTGCATGATCTG
97 T D E D R C E V C F K P Y D N A A H A R T T F R Y Q L H D L
451 GAGTGTGGTGTGTGTATGAAATCCTGCGCGAGCATCGAGTCGAGGCAGCAGGAGGACACTTGGATTCTCGGAGCGATGACATGTCCAGC
127 E L V L C M K S C A S I E S R Q Q E E H L D S R S D D M S T
541 GCTGAAGATGAGACGACCGGGTTCGCGAGTACAGGGAGCTCGGAACGACAAGAGAGGTATGAGCAGTTCGCTGAAGAGTATCAGGGA
157 A E D E D R C E V C F K P Y D N A A H A R T T F R Y Q L H D L
631 ATTGGGAATCTAGATGGTGTGCGGATGGTACGGATGAGGAGAACGAGGGGGCGTGAGCATCGAGGTGACAGTGAAGAGCAGCCAGTCG
187 I G N L D G D A D G T D E E N E G A L S I E A D S E S S Q S
721 GAGCCATTCACAGCCATACATTCGCGGTTCGCGAGTACATTGACGGGATGACCAACCTTGGGCCAGATCCAGAGTGTCTTCATGG
197 E P F T R P Y I A G K P Y I D G I A T I W A R S R V S W
811 AGTGAGGCCCTACGAATGAAAACCTGCGGAAACGGTTCGCCAGAGAGCTGTCTACCGCTCAGACCAAGATCCACACCGGAGAGAGA
247 S E A L R M Q N C G K R F A Q K S C L T R H T K I H T G E R
901 CTCATCAATCGCAGGATGACCAAGTCTTCCCGGTCGCGCAATTCGCGGTCCCATCGGAGCGCACACCGGGGAGAAAGTGTCTTC
277 L H Q C D E C T K S F T R A S N L R S H M R T H T G E K C F
991 CAGTGCCCATCTGCGAGAAGCTCTTCGGTCAGAGTTCCTGGTGAAGCGTCACATCCGCACCCAGCCGCGGAGAAAGAGGTCAAGTGC
307 Q C P I C Q K L F G Q C S W L K R H I R T H T G E K E V K C
1081 CCGCTTCGACGACGGTTCAGCTCCACGACCTCTTCAGTCCCATATGAGAACACACACGCGGGGAGAAACCTTCGCTGCCCTCCATA
337 P L C S K W V S S T T S L Q S H M R T H T G E R P F A C C L I
1171 TGTGCAAGAGCTTTCAGTGTCAAGTGCACCTGACGGTTCATATGCGTATCCACAGGCAACAAGCCATACGTTGTGAGGTGTGTGGT
367 C D K S F S V K C T L T V H M R I H T G N K P Y V C E V C G
1261 GCTGCATTCATACAGGGGACCCAGTGTCCACTCAGATGCGGGTGCATACGGGTGAGAGCCATACATGTTGAGATCTGTATCGGCAG
397 A A F I O G T O L S T H M R V H T G E K P Y M C E I C H R O
1351 TTCGGCAAGAAAGACAACCTCCTCACCATGTCAAGATTCACACTGAAGGGCAGAATTTTGTACTGCAAGTCTGCAACCTACGTTTCAAC
427 F G K K D N L L T H V K I H T E G O N F D C K F C N L R K
1441 AACTCTGAGGAGCTCAAGACCCATACCCTGATCCATATAGAAGAGAGACCATTCAGTGTGCAACAAGCCCTCAAGAGAAAG
457 N S E E L K T H T L I H I E E R P F O Q C E V C N K A F K R K
1531 GACAACCTTAAAGAACACATTAAGGTTACACCAAAAGAGAAGCCTTTTGTCTGTGAGTTTGGCAAGAGGCTTCTCCAACCGCAGCCAT
487 D N L K E H I K V H T K E K P F S C E V C K E A F S N G S H
1621 CTTAAGTCCACATGCGACGTCACAGGGGGACAAGCCCTACTCTGTGAGCTCTCGGGGAAGTGTCTCACCCAGAGCAGTAGTCTGAAG
517 L K V H M R R H T G A D D K P Y S C E L C K C F T G S S L K
1711 ACCCATACCTGATCCACCGGTGAGAACTTACTCTGTACTATCTGCCATGCACCTTACCAGGCAATGCCAGCGTCAAGAAGCAC
547 T H Y L I H T G E K P Y S C T I C H A L F T R N A A S V K K H
1801 ATGAAGAGAGCCCAATGCGGACATGCGGGACAGAGGCGCTCTGAATGAGTGGGCTCAGAGCAGTAGCTTAAAGACCCACTACC
577 M K R A H N A D M P G P E E A S E *
1891 TGATCCGACCGGTGAGAAACCTTATCTCGGGGCGGTTTACAAAACCTGTGATTGAACAAAGGCTTTGTAAACGGGTCCTGTACT
1981 ATCTGCCAGCGCTCTTACCCGCAACCCACTCTTCAACACTATGCCACCATGCCGGGACCGGAGTGGCTGTGAGTGAAGCCCTC
2071 GAGGAACCATGAGGAGGAGTGAAGTACAGTCAAGCTGCTTTAGTACCCTGTCTACAAGACCGCTGTCTA
2161 TAAAGACCACATGTTTGTTCCTTGAATAATGTTTCTCATTGAACATGACTATTAAGAACCTGTCTACAAGACAATTTTGTG
2251 TATCCCTGGGTGGTCCGTTTACAGAGGTTGACTGAGTGTGTTGGTGCACCATCAACTTTTGTGTTTGTATTTTCTGATATGCACAC
2341 GGGCCTTTGATGTAGATATGATGAGGTGATTTTATACGTATTTATGCTTTTCTAAATGATTAATAAATGATTCAGATGTTTT
2431 CTCTGTGAACGATTGACTGATTGAACTTAAATCAACCAGTAAATTTGTTTGTAAATAGATAAACCCACAATATGAGGGAATTTAT
2521 GTATACCTTTTGAACAATATGAAAATAGTAGTGTCTGTTTTCATGTAGTTTCCATCTTATAAGTGTATAGTTAAATCTATTTGAAAATAT
2611 ATACATTTTAAACTTTTCTAGCTGGTTTTGGGAAATTTGAAAAAATAAAATTAATAAAAAAAAAAACCGAATT
    
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Fig. 5. Sequences of SpZ12-1 cDNA and protein, and sequence similarities of the Zn finger domain between the SpZ12-1 and *Krüppel* proteins (Rosenberg et al., 1986). (A) The complete nucleotide sequence and the deduced amino acid sequence of the SpZ12-1 cDNA are shown. The Zn finger region is underlined. The numbered bent arrows below the amino acid sequence mark the positions of the twelve zinc fingers. The GenBank accession number is U19831. (B) Alignment of Zn finger sequences of the SpZ12-1 and *Krüppel* protein. The fingers of both proteins are numbered on the left side. Positions and amino acids that are identical among the Zn fingers are shaded.

B

SpZ12-1	1	LRMQN	C	GKR	F	AQKSC	L	TR	H	TKI	-	HTGER	L
	2	HQ	C	DE	C	TKS	F	TRASN	L	RS	HMRT	-	HTGEKC
	3	FQ	C	PI	C	QKL	F	GQSSW	L	KR	HIRT	-	HTGEKE
	4	VK	C	PL	C	SKW	V	SSTS	L	QS	HMRT	-	HTGEKP
	5	FA	C	LI	C	DKS	F	SVKLT	L	TV	HMRI	-	HTGNKP
	6	YV	C	EV	C	GAA	F	IQGTQ	L	ST	HMRV	-	HTGEKP
	7	YM	C	EI	C	HRQ	F	GKDN	L	LT	HVKI	-	HTEGQN
	8	FD	C	KF	C	NLR	F	NNSEE	L	KT	HTLI	-	HTEERP
	9	FQ	C	EV	C	NKA	F	KRKN	L	KE	HIKV	-	HTKEKP
	10	FS	C	EV	C	KEA	F	SNGSH	L	KV	HMRR	-	HTGDKP
	11	YS	C	EL	C	GKC	F	TQSS	L	KT	HYLI	-	HTGEKP
	12	YS	C	TI	C	HAL	F	TRNAS	V	KK	HMKRA	-	HNA DMP
<i>Krüppel</i>	1	FT	C	KI	C	SRS	F	GYKHV	L	QN	HERT	-	HSRDKS
	2	FE	C	PE	C	DKR	F	TRDHL	L	KT	HMRL	-	HTGEKP
	3	YH	C	SH	C	DRQ	F	VQVAN	L	RR	HRLV	-	HTGEKP
	4	YT	C	EI	C	DGK	F	SDSNQ	L	KS	HMLV	-	HTGEKP

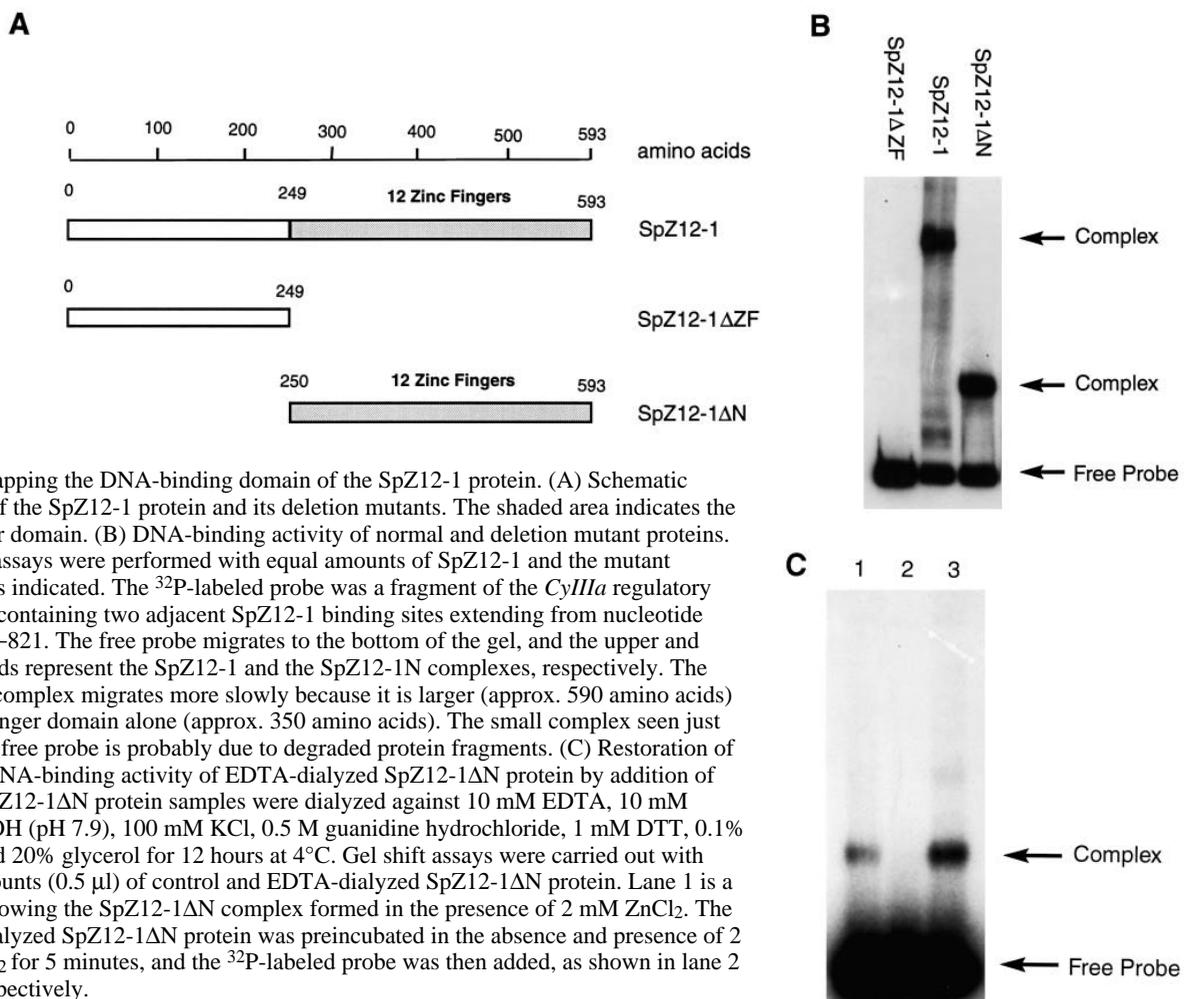


Fig. 6. Mapping the DNA-binding domain of the SpZ12-1 protein. (A) Schematic diagram of the SpZ12-1 protein and its deletion mutants. The shaded area indicates the zinc finger domain. (B) DNA-binding activity of normal and deletion mutant proteins. Gel shift assays were performed with equal amounts of SpZ12-1 and the mutant proteins as indicated. The ^{32}P -labeled probe was a fragment of the *CyIIIa* regulatory sequence containing two adjacent SpZ12-1 binding sites extending from nucleotide -1033 to -821. The free probe migrates to the bottom of the gel, and the upper and lower bands represent the SpZ12-1 and the SpZ12-1N complexes, respectively. The SpZ12-1 complex migrates more slowly because it is larger (approx. 590 amino acids) than the finger domain alone (approx. 350 amino acids). The small complex seen just above the free probe is probably due to degraded protein fragments. (C) Restoration of specific DNA-binding activity of EDTA-dialyzed SpZ12-1ΔN protein by addition of ZnCl_2 . SpZ12-1ΔN protein samples were dialyzed against 10 mM EDTA, 10 mM HEPES-KOH (pH 7.9), 100 mM KCl, 0.5 M guanidine hydrochloride, 1 mM DTT, 0.1% NP-40 and 20% glycerol for 12 hours at 4°C. Gel shift assays were carried out with equal amounts (0.5 μl) of control and EDTA-dialyzed SpZ12-1ΔN protein. Lane 1 is a control showing the SpZ12-1ΔN complex formed in the presence of 2 mM ZnCl_2 . The EDTA-dialyzed SpZ12-1ΔN protein was preincubated in the absence and presence of 2 mM ZnCl_2 for 5 minutes, and the ^{32}P -labeled probe was then added, as shown in lane 2 and 3, respectively.

and after renaturation (see Materials and methods) the proteins were tested for their ability to bind to the SpZ12-1 binding sites of the 5' *CyIIIa* regulatory region by gel shift assay. Results are shown in Fig. 6B. The SpZ12-1 and SpZ12-1ΔN proteins bound to the ^{32}P -labeled DNA probe equally well. However, no binding whatsoever was observed when the SpZ12-1ΔZF protein was tested. These results clearly demonstrate that the DNA-binding domain of the SpZ12-1 protein is located within the Zn finger region. Fig. 6C shows the results of an experiment demonstrating that Zn ions are required for the DNA-binding activity of the SpZ12-1 protein. DNA binding is abolished by dialyzing purified SpZ12-1ΔN protein with a low concentration of EDTA, and restored when ZnCl_2 is added back to the reaction mixture.

Using bacterially expressed, full-length SpZ12-1 protein, we further defined the SpZ12-1 binding sites within the 5' *CyIIIa* cis-regulatory region by DNase I footprinting experiments. As shown in Fig. 7A, the SpZ12-1 protein protects the two adjacent regions indicated as SpZ12-1 binding sites I and II. Alignment of the protected sequences of the sites in Fig. 7B shows that they include the inversely repeated consensus motif TGTTGC(T/C)AGG detected earlier (Thézé et al., 1990).

Identification of the SpZ12-1 protein in the early embryo

Since the SpZ12-1 clone was originally obtained by direct

ligand screening, it was essential to determine whether the protein it encodes was present in the early embryo. A polyclonal antibody was raised against the recombinant SpZ12-1ΔZF protein, which lacks the Zn finger domain (Fig. 6). The specificity of the antibody was confirmed by reaction with both blastula-stage nuclear extracts and the full-length bacterially expressed SpZ12-1 protein (Fig. 8A). Only one immunoreactive band was detected with blastula-stage nuclear extracts, which is identical in size to the SpZ12-1 protein that is encoded by the cloned cDNA. Gel shift assays were then performed, using a blastula-stage nuclear extract and a ^{32}P -labeled SpZ12-1 binding site probe. The specific SpZ12-1 protein-DNA complex was isolated from the gel; the complexed protein was released by treatment with SDS; and it was then displayed by SDS gel electrophoresis. This gel was blotted, and the filter reacted with the anti-SpZ12-1 antibody, as just described above. Fig. 8B shows that the authentic immunoreactive component of the DNA-binding complex that derived from the embryo extract is identical in size to the full-length bacterially expressed SpZ12-1 protein. Furthermore, no other proteins binding to the specific sites could be recovered from the embryo extracts. Fig. 8C shows a supershift experiment using the same polyclonal antibody. Here it can be seen that essentially all the complex is removed to either a higher molecular mass band or to an aggregate that remained bound in the well. It follows that SpZ12-1 is in all probability the

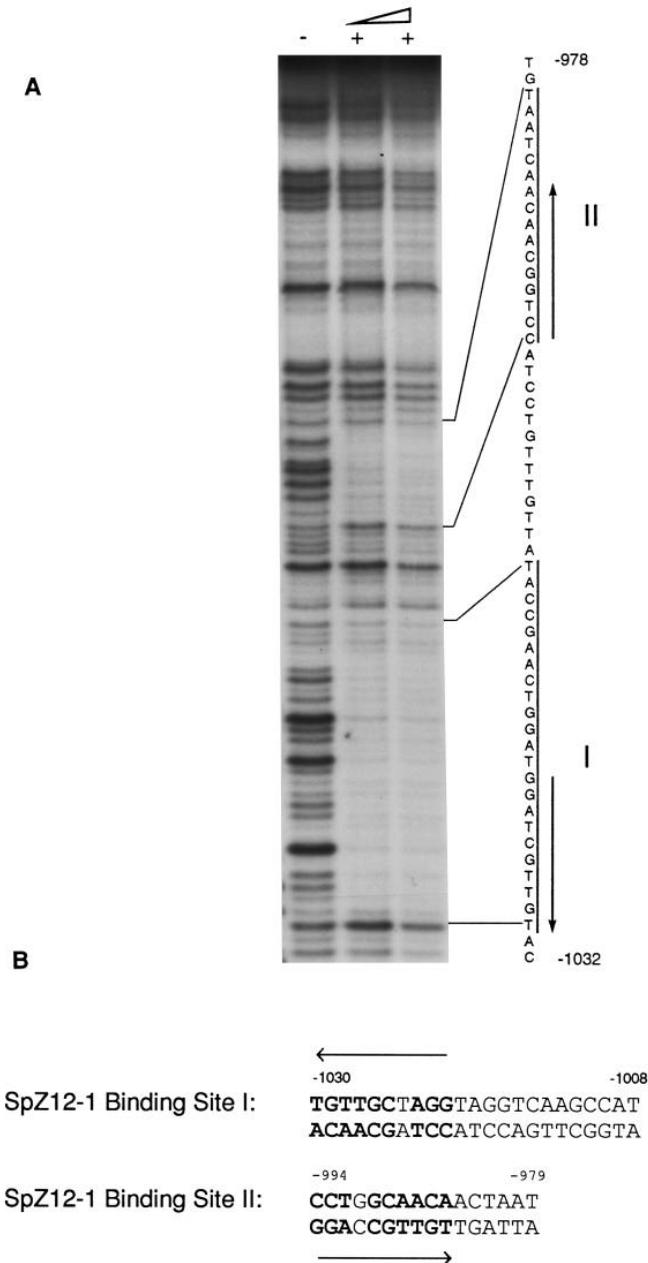


Fig. 7. DNase I footprinting using the bacterially expressed, full-length SpZ12-1 protein. (A) Footprints. DNase I footprint assays were carried out with increasing amounts of bacterially expressed SpZ12-1 protein as indicated (Calzone et al., 1991). Lane (-) shows DNase I digestion pattern without added protein. The probe is the coding strand of the *CyIIIa* cis-regulatory region containing two SpZ12-1 binding sites (-1033 to -821). Protected regions of sites I and II are bracketed and their sequences are underlined. The inverted repeat sequence element in each site, and their relative orientations, are indicated by the arrows. (B) Protected sequences. The nucleotides constituting the inverted repeat present in binding sites I and II are indicated in bold type.

DNA-binding factor that interacts with the target sites in the *CyIIIa* gene that we studied, and that is responsible for the spatial repression function we identified.

DISCUSSION

Identification of a negative *cis*-element within the 5' *CyIIIa* regulatory region

Previous studies suggest that the *CyIIIa* gene is under negative spatial control in the early embryo (Franks et al., 1988, 1990; Hough-Evans et al., 1990). The first such evidence came from an interspecific experiment (Franks et al., 1988) in which ectopic spatial expression was seen when a *CyIIIa*•*CAT* fusion gene was injected into eggs of a distantly related second species, *L. variegatus*. This phenomenon implies that the normal spatial regulation of the *CyIIIa* gene is at least in part negative. Ectopic spatial expression can also be induced in *S. purpuratus* by an in vivo competition. If excess molar amounts of either the P3A2 or P7II sites (see Fig. 1) are coinjected with the *CyIIIa*•*CAT* construct, ectopic expression of *CAT* transcripts is also detected, in oral ectoderm, and oral ectoderm plus skeletogenic mesenchyme, respectively (Hough-Evans et al., 1990). As expected if the interactions at these sites are of negative import, the competition for the factors binding these two target sites did not significantly decrease the overall level of expression (Franks et al., 1990). In contrast, competition for the target sites of a number of other positively acting factors depresses the level of expression stoichiometrically, while the normal spatial pattern of expression remains unchanged (Franks et al., 1990; Hough-Evans et al., 1990). Recent studies have confirmed that the P3A2 and P7II binding sites are in fact required for negative spatial control of the *CyIIIa* gene. However, when the SpZ12-1 binding sites were coinjected in molar excess with the *CyIIIa*•*CAT* construct in these in vivo competition experiments, neither ectopic expression nor decrease in *CAT* activity was observed (Franks et al., 1990; Hough-Evans et al., 1990). This could have been due to the relatively high prevalence of SpZ12-1 protein (Calzone et al., 1988), which could have made it difficult to obtain an effective excess of competitor sites in vivo. In the present study, when the SpZ12-1 binding sites of the *CyIIIa*•*CAT* construct were altered by site-directed mutagenesis, approximately 46% of embryos bearing this fusion gene showed ectopic expression, specifically in mesenchyme cells, at the late gastrula stage (Table 1). This percentage directly reflects the probability of incorporation of the fusion gene into mesenchyme cell lineages during the first several cleavages (Hough-Evans et al., 1988; Sucov et al., 1988). Thus, other recent experiments carried out with a skeletogenic mesenchyme-specific construct (*SM50*•*CAT*) demonstrate that the maximal percentage of embryos with stained mesenchyme cells from a single injection is about 30-40% (unpublished data). Our results imply that all embryos that have incorporated the mutant fusion gene in skeletogenic mesenchyme cell precursors will express *CAT* transcripts in these cells. We conclude that the SpZ12-1 binding sites are a third negative spatial control element, interactions at which are required to repress mesenchyme cell expression of the *CyIIIa* gene.

Late effect: repression of the *CyIIIa* gene in skeletogenic mesenchyme cells

The results from embryos bearing the *mZ12CyIIIa*•*CAT* fusion gene at the mesenchyme blastula stage (Fig. 4 and Table 2), together with the data from the late gastrula stage (Fig. 4 and

Table 1) clearly show that ectopic mesenchyme cell expression of this fusion gene occurs long after mesenchyme cell specification. It is important to note that there are several skeletogenic mesenchyme marker genes, which are activated at late cleavage stage, transcripts of which rapidly accumulate in these mesenchyme cells as ingression occurs (Benson et al., 1987; Killian et al., 1989). The skeletogenic mesenchyme is probably autonomously specified during cleavage (Davidson, 1989). Ectopic expression of CAT transcripts was not detected in skeletogenic mesenchyme cells in embryos bearing the *mZ12CyIIIa•CAT* fusion gene at the blastula stage, when the cells ingress into the blastocoel. Therefore, the negative function of the SpZ12-1 sites for the *CyIIIa* gene is not part of the initial autonomous specification program. This spatial control function appears to be required only after gastrulation. A plausible interpretation is that during the postgastrular period the *CyIIIa* gene begins to respond to a positive regulator that is active in both aboral ectoderm and skeletogenic mesenchyme, so the role of the repressive interaction we have studied would be to confine the effect of this positive regulator to the aboral ectoderm.

SpZ12-1 is probably the particular transcription factor that exercises the negative control function we have assigned to its target site

The argument that SpZ12-1 is in fact the sea urchin protein that binds to the *CyIIIa* target site we studied relies on four separate items of evidence: (i) The recombinant protein against which the antibody was raised is exactly the same size as the single immunoreactive polypeptide present in sea urchin embryo nuclear extract (Fig. 8A). (ii) A nuclear protein that reacts with this antibody is released from complexes formed with the SpZ12-1 site probe, and this protein is also identical in size to the recombinant SpZ12-1 encoded by our cDNA clone (Fig. 8B). This observation proves that SpZ12-1 is present in the sole gel shift complex formed by sea urchin embryo nuclear protein with the SpZ12-1 probe. It does not exclude the possibility that some other DNA binding protein (that the antibody does not detect) could also be present on some of the DNA probe molecules, if this other protein fortuitously produces the same size complex as does SpZ12-1. (iii) However, all of the complex present is abolished by chelation, and is restored by readdition of Zn (Fig. 7C), so if another DNA binding protein is present it must also be a Zn finger protein. (iv) Finally, we show in a supershift experiment that all of the complex is removed to higher molecular mass by the antibody against cloned SpZ12-1 (Fig. 8C). This excludes any DNA-

binding complex other than those formed by SpZ12-1 molecules. A remaining possibility that we cannot formally exclude is that different proteins react with the SpZ12-1 site in vivo, if these proteins are for some reason excluded from our nuclear extracts. However, even crude egg lysates display only the same immunologically reactive $70 \times 10^3 M_r$ proteins that produce the same characteristic SpZ12-1 gel shift complex (D. G.-W. Wang et al., unpublished data). Thus it is likely that SpZ12-1 is indeed the factor that services the *CyIIIa* target sites, the function of which we demonstrated in vivo. The binding activity of the SpZ12-1 protein is highly specific, as shown in the DNase I footprinting experiments (Fig. 7). Since SpZ12-1 is of maternal origin, this factor no doubt has other functions in oogenesis or early development (D. G.-W. Wang et al., unpublished data; Anderson et al., 1994).

The SpZ12-1 protein

Examination of the SpZ12-1 protein sequence reveals twelve tandem Zn fingers of TFIIIA type near the carboxyl terminus (Berg et al., 1990). This type of Zn finger is one of the most common DNA binding motifs among eukaryotic transcription factors, and can recognize a diverse set of DNA sequences (Pellegrino et al., 1991; Pavletich et al., 1993). The similarities between the finger motif of SpZ12-1 and *Drosophila* segmentation gene *Krüppel* is striking (Fig. 5B). *Krüppel* represents a subclass of Zn finger genes of the TFIIIA type that is characterized by multiple Zn fingers in which the canonical sequence motif $CX_2CX_3FX_5LX_2HX_3H$ is separated by highly conserved seven amino acid linkers of the form TGEKP(Y/F)X (Chowdhury et al., 1987). Recent studies of two crystal struc-

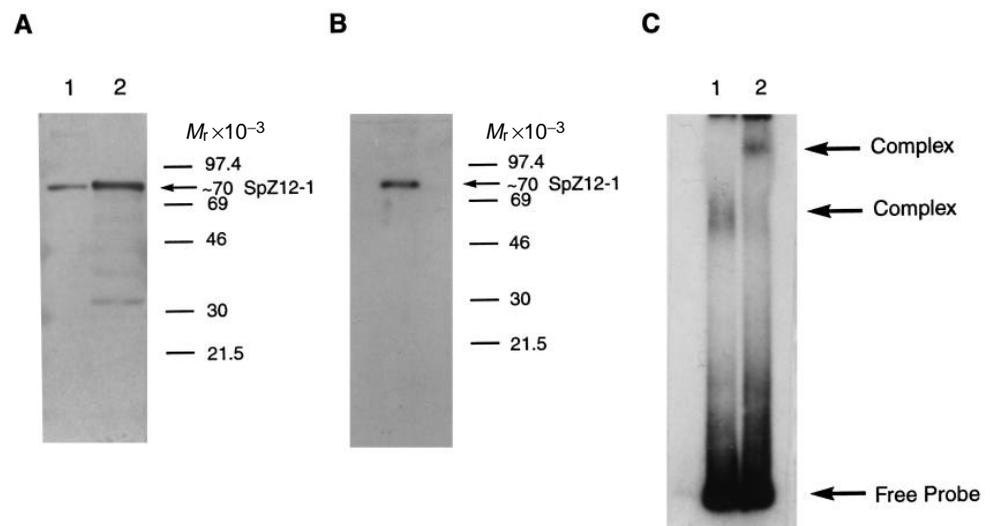


Fig. 8. Identification of the SpZ12-1 protein in blastula-stage nuclear extracts. Protein was electrophoresed on a 7% SDS polyacrylamide gel, transferred to a nitrocellulose filter and reacted with the antibody. The positions of size markers and immunoreactive bands are indicated. (A) Lane 1 and 2 were loaded with blastula stage nuclear extract and full-length bacterially expressed SpZ12-1 protein, respectively. (B) The sample was prepared as follows: the SpZ12-1 binding site probe was mixed with late blastula-stage nuclear extracts and the complex displayed by native gel electrophoresis. A gel slice containing the SpZ12-1 protein-DNA complex was excised, minced and incubated in an SDS extraction buffer. The whole mixture was then loaded. (C) The anti-SpZ12-1 antibody supershifts the entire gel shift complex. Gel shift assays were performed using blastula-stage nuclear extracts as previously described (Calzone et al., 1988). After the binding reaction, 5 μ l of the antibody was added to the reaction mixture for 10 minutes at 4°C.

tures of the Zn finger proteins, Egr 1 and GLI-1 with their cognate DNA-binding sites, show that each Zn finger domain can independently bind DNA and that each recognizes a three-nucleotide sequence; in the case of GLI-1, not all of the five Zn fingers contact the DNA; i.e., not all Zn fingers contribute equally to DNA recognition (Pavletich et al., 1991, 1993). Consistent with this observation, our data demonstrate that the twelve-Zn finger region of SpZ12-1 recognizes only a 23-nucleotide target site (Fig. 7B), implying that probably four to five of the Zn fingers are not involved in the interaction displayed by the footprint at each site. The SpZ12-1 protein sequence does not have significant similarities to any other proteins outside the Zn finger domain. For instance, despite the fact that the fingers of SpZ12-1 and *Krüppel* are similar, the amino-terminal region of SpZ12-1 has no similarity with the highly conserved *Krüppel*-associated box (KRAB). This is a 75-amino acid domain present in the amino-terminal region of more than one-third of all *Krüppel* subclass Zn finger proteins, which is probably responsible for transcriptional repression mediated by these factors (Margolin et al., 1994; Witzgall et al., 1994).

SpZ12-1 contains many potential sites for phosphorylation, but the significance of this is hard to evaluate due to the preponderance of serine, threonine and proline residues in the sequence. Our recent studies have revealed that a number of SpZ12-1 variants are present in the early embryo which can be resolved by two-dimensional gel electrophoresis. These variants appear to be the consequence of posttranslational modifications (D. G.-W. Wang and M. G. Harrington, unpublished observations). Since SpZ12-1 is maternal and is present in both cytoplasm and nucleus of the early embryo (D. G.-W. Wang et al., unpublished data) such posttranslational modifications could be the means of controlling the nuclear localization of SpZ12-1, or its regulatory function in the nucleus.

In conclusion, we have identified a specific negative spatial *cis*-regulatory function that controls *Cy13a* expression in the skeletogenic mesenchyme of late embryos. The *trans*-factor which exercises this function is probably SpZ12-1. Since we know the cell type and stage in which this function of SpZ12-1 is required, we are now in a position to determine the modifications that determine its temporal and spatial activity.

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