Development 124, 4717-4727 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV5131

SpMyb functions as an intramodular repressor to regulate spatial expression of *CyIIIa* in sea urchin embryos

James A. Coffman[1,2], Carmen V. Kirchhamer[2,*], Michael G. Harrington[2] and Eric H. Davidson[2]

[1]Stowers Institute for Medical Research and [2]Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA
*Present address: Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA

SUMMARY

The *CyIIIa* actin gene of *Strongylocentrotus purpuratus* is transcribed exclusively in the embryonic aboral ectoderm, under the control of 2.3 kb *cis*-regulatory domain that contains a proximal module that controls expression in early embryogenesis, and a middle module that controls expression in later embryogenesis. Previous studies demonstrated that the SpRunt-1 target site within the middle module is required for the sharp increase in *CyIIIa* transcription which accompanies differentiation of the aboral ectoderm, and that a negative regulatory region near the SpRunt-1 target site is required to prevent ectopic transcription in the oral ectoderm and skeletogenic mesenchyme. This negative regulatory region contains a consensus binding site for the myb family of transcription factors. In vitro DNA-binding experiments reveal that a protein in blastula-stage nuclei interacts specifically with the myb target site. Gene transfer experiments utilizing *CyIIIa* reporter constructs containing oligonucleotide substitutions indicate that this site is both necessary and sufficient to prevent ectopic expression of *CyIIIa*. Synthetic oligonucleotides containing the myb target site were used to purify a protein from sea urchin embryo nuclear extracts by affinity chromatography. This protein is immunoprecipitated by antibodies specific to the evolutionarily conserved myb domain, and amino acid sequences obtained from the purified protein were found to be identical to sequences within the myb domain. Sequence information was used to obtain cDNA clones of SpMyb, the *S. purpuratus* member of the myb family of transcription factors. Through interactions within the middle module, SpMyb functions to repress activation of *CyIIIa* in the oral ectoderm and skeletogenic mesenchyme.

Key words: *Strongylocentrotus purpuratus*, myb, *CyIIIa*, transcriptional regulation, embryogenesis

INTRODUCTION

The *CyIIIa* gene of *Strongylocentrotus purpuratus* encodes a cytoskeletal actin that is specifically transcribed in the aboral ectoderm of the embryo and larva, under the control of a 2.3 kb *cis*-regulatory domain. Studies of this *cis*-regulatory system have served as a paradigm for the analysis of information processing systems that regulate transcription during embryonic development (see Kirchhamer et al., 1996, for review). In vitro analyses have identified over twenty protein target sites within the *CyIIIa* regulatory domain, at which specific interactions with at least nine different transcription factors occur (Calzone et al., 1988; Thézé et al., 1990). All of these factors have been isolated by affinity chromatography (Calzone et al., 1991; Coffman et al., 1992; Char et al., 1993; Zeller et al., 1995a; Coffman et al., 1996), and eight of them have been cloned (see Fig. 1A). Concomitantly, in vivo studies utilizing gene transfer technology have identified regulatory functions for each target site (Franks et al., 1990; Hough-Evans et al., 1990; Kirchhamer and Davidson, 1996; Coffman et al., 1996).

The *CyIIIa cis*-regulatory domain consists of two functionally distinct modules, the locations of which are shown in Fig. 1A (Kirchhamer and Davidson, 1996; Kirchhamer et al., 1996). The proximal module includes both positively and negatively acting spatial control elements which together suffice to initiate expression exclusively in the aboral ectoderm early in embryogenesis. The middle module functions dominantly later in embryogenesis. It is required both for the sharp increase in *CyIIIa* transcription that occurs after blastula stage, and for spatially localizing that transcription to the aboral ectoderm (Kirchhamer and Davidson, 1996). It was recently demonstrated that the positive regulatory function of the middle module is mediated by the SpRunt-1 target site (Coffman et al., 1996). Two negative spatial regulatory functions have also been identified within the middle module. Interactions with the zinc finger repressor SpZ12-1 (see Fig. 1A) are required to prevent transcription in the skeletogenic mesenchyme (Wang et al., 1995). A second middle module repressor was inferred from in vivo competition (Hough-Evans et al., 1990), and from the results of deletion of a region of the middle module known as the P7II target site (Fig. 1A; Kirchhamer and Davidson, 1996). Interactions at this site were found to be required to prevent ectopic transcription in both the oral ectoderm and skeletogenic mesenchyme. The P7II target site had been mapped to a 63 bp sequence within the middle module which is shown in Fig. 1B. The purpose of the present investigation

was to determine what protein target sites within the sequence shown in Fig. 1B mediate its negative regulatory function, and to identify the responsible repressor protein.

Previous analysis of the P7II region revealed that it contains binding sites for SpGCF1 (Fig. 1A,B), a transcription factor that binds multiple target sites in the regulatory domains of various genes (Zeller et al., 1995a). The ability of SpGCF1 to homomultimerize and loop DNA in vitro led to the hypothesis that its function may be to facilitate direct communication between proteins bound at distant target sites (Zeller et al., 1995b). The facts that target sites for SpGCF1 are widespread, occurring in both the proximal and the middle modules, and that where analyzed, these target sites function positively in transcriptional regulation (Franks et al., 1990; Kirchhamer et al., 1996), suggested that SpGCF1 is not the factor responsible for the negative spatial function of the P7II region. However, because of the high abundance of SpGCF1 in nuclear extracts, it was initially difficult to detect any other proteins that bind in the same region. To overcome this difficulty we made use of automated sequential affinity chromatography (Coffman et al., 1992) to analyze P7II-specific binding proteins that remain in nuclear extract following passage over columns bearing specific SpGCF1 sites. We thus discovered that a sea urchin myb-domain transcription factor, SpMyb, specifically binds the P7II target site. Gene transfer experiments demonstrate that the ectopic expression mediated by the SpRunt-1 interaction within the middle module of the *CyIIIa* gene (Coffman et al., 1996; Kirchhamer et al., 1996) is repressed by the addition of a single synthetic Myb site. SpMyb is thus a key negative spatial regulator of the middle module.

MATERIALS AND METHODS

Construction of reporter genes

Oligonucleotides

Synthetic oligonucleotides used in the constructs shown in Fig. 1C are listed below. The following double-stranded oligonucleotides (corresponding to the boxed sequence in Fig. 1B) were used both as probes in DNA-binding assays and as artificial target sites in reporter constructs (Fig. 1C). Myb target sites are in larger boxes and in bold face, GCF1 sites are in smaller boxes, nucleotide base substitutions that alter the wild-type sequence are underlined, and sequences containing *Sac*II sites for the purpose of cloning are in lower case.

Oligonucleotide pair **MG**:

MG.s: 5′–ccgcggCTAA**CCGTTA**CCCTTAACCTAA–3′
MG.as: 3′-GATT**GGCAAT**GGGAATTGGATTggcgcc-5′

Oligonucleotide pair **G**:

G.s: 5′-ccgcggGTACCCGGTACCCTTACCCTAC-3′
G.as: 3′-CATGGGCCATGGGAATGGGATGggcgcc-5′

Oligonucleotide pair **M**:

M.s: 5′-ccgcggTTAA**CCGTTA**ACCTTAACCTAA-3′
M.as: 3′-AATT**GGCAAT**TGGAATTGGATTggcgcc-5′

Note that four of the five base substitutions in oligonucleotide pair G fall outside of the canonical myb target site, within sequences that in the wild-type are partial myb recognition sequences (4/6: TAAC), and also that these changes introduce an additional GCF1 site. For the purpose of cloning, the two individual oligonucleotides of each pair were annealed, concatamerized using T4 DNA ligase, and digested with *Sac*II.

Constructs

The 9.4 kb *CyIIIa.CAT* construct (line 1 of Fig. 1C) described by Kirchhamer and Davidson (1996) was used as control construct. Constructs *MG.CAT*, *M.CAT* and *G.CAT* (lines 3, 4 and 5 of Fig. 1C) were generated by opening plasmid *ΔP7II.CAT* (line 2 of Fig. 1C; Kirchhamer and Davidson, 1996) at the unique *Sac*II site and inserting the oligonucleotide pairs **MG**, **M** or **G**, respectively (see above). The resulting constructs were sequenced to verify that they contained only a single oligonucleotide insert, and that the insert was in the proper orientation with respect to the wild-type sequence.

All plasmids carry the remaining complement of regulatory sites of the *CyIIIa* 5′ regulatory region, transcription initiation site, 5′ leader sequence of the primary transcript and a *CyIIIa-CAT* fusion gene. The plasmids were linearized for microinjection at unique *Sph*I or *Kpn*I sites.

Microinjections and whole-mount in situ hybridization

Gametes of *Strongylocentrotus purpuratus* were obtained, microinjections carried out and embryos cultured as described previously (McMahon et al., 1985; Kirchhamer and Davidson, 1996). Embryos were collected at the appropriate stage and processed for whole-mount in situ hybridization (Ransick et al., 1993; Kirchhamer and Davidson, 1996). Embryos were analyzed and images stored with an imaging system using ProgRes software and a Zeiss Axioskop microscope.

Large-scale embryo culture, protein purification, and protein microsequencing

Large-scale embryo cultures, preparation of nuclear extracts, and automated sequential affinity chromatography (ASAC) were carried out as previously described (Calzone et al., 1991; Coffman et al., 1992). For protein microsequencing, peak fractions from four separate ASAC runs of an affinity column bearing oligonucleotide pair **M** (see above) were pooled, dialyzed against Buffer 0.1 (20 mM Hepes-KOH, pH 7.9; 0.1 M KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 0.1 mM PMSF, 20% glycerol), brought to 20 ng/μl with poly(dIdC/dIdC), and reloaded onto a tandem series of four columns bearing the **M** oligonucleotide pair. The columns were loaded and eluted using a Gilson ASPEC programmed with our standard ASAC Software (Coffman et al., 1992), and the fractions analyzed by both electrophoretic mobility shift and SDS-PAGE. This second round of affinity purification in the presence of nonspecific competitor DNA resulted in a substantial enrichment of the myb-specific DNA-binding activity. Peak fractions were again pooled, dialyzed against Buffer 0.1 (without glycerol), and concentrated by ultrafiltration using Centricon 10 mini-concentrators (Amicon). The pooled, concentrated **M** oligonucleotide column eluate was brought to 1% trifluoroacetic acid, subjected to reverse phase HPLC (SMART, Pharmacia) on a C4 column (ABI), and eluted with a linear acetonitrile gradient. Individual peaks were collected and analyzed by electrophoretic mobility shift and by SDS-PAGE. Myb-specific DNA-binding activity was found in a single peak which contained two species of protein, an abundant species of approx. 30 kDa and a minor species of approx. 80 kDa. The approx. 30 kDa species corresponds in size to the predominant myb-specific DNA binding protein identified by 2-dimensional EMSA (J. A. Coffman, unpublished result), and probably represents a proteolytic fragment of the full-length protein (see Results). This fraction was subjected to digestion with a lysylendopeptidase (Achromobacter Protease 1; Wako). The resultant peptides were resolved by reverse phase HPLC (Pharmacia SMART equipped with a C18 column) and sequenced on a Porton automated sequencer.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed essentially as described by Calzone et al. (1988); Coffman et al. (1996). For detection of myb DNA-binding activity in either affinity column eluate or in vitro translation reactions, 20 ng/μl poly (dIdC/dIdC) was used as nonspecific competitor in each 10 μl binding reaction, which included 1 μl of protein extract. For detection of immunoprecipitated, renatured myb DNA-binding activity, 10 ng/μl poly (dIdC/dIdC) was used in each 15 μl binding reaction, which included 10 μl of renatured

protein eluted from the antibody complex (see below). EMSAs were visualized using a phosphorimager and ImageQuant software (Molecular Dynamics).

Immunoprecipitations

Immunoprecipitation of affinity-enriched myb DNA-binding activity was performed as follows: 100 μl of P7II affinity column eluate was brought to 1% SDS and heated to 90°C to denature the proteins, then brought to 1 ml in immunoprecipitation buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 1 mM PMSF). 250 μl aliquots of this preparation were incubated overnight at 4°C with 25 μl protein A sepharose that had been pre-coated with either anti-BP-2 antibodies (Boyle et al., 1986) or antibodies from nonimmune serum from a different rabbit. The resulting immunoprecipitates were collected by centrifugation, washed three times in 1 ml each of PBS, and eluted with 100 μl denaturation buffer (20 mM Hepes-KOH, pH 7.9; 0.1 mM EDTA, 1 mM dithiothreitol, 6 M guanidinium-HCl, 1 mM $MgCl_2$). The eluted proteins were renatured over 0.5 ml Biogel P6 (Biorad) columns that had been pre-equilibrated in renaturation buffer (10 mM Hepes-KOH, pH 7.9; 0.1 M KCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol) and subjected to EMSA as described above.

cDNA cloning

A highly conserved region of the myb DNA binding domain was amplified by polymerase chain reaction, using the degenerate primers designed by Stober-Grässer et al. (1992). Plasmid prepared from a blastula-stage λZap cDNA library was used as template. The amplified 168 bp fragment was excised, electroeluted, cloned into pGEM-T (Promega), and sequenced. This fragment was then used to screen a blastula-stage λZap cDNA library by standard procedures. A first round of screening yielded two clones, 7.1.1 and 15.1.1, which contain inserts of approx. 1.2 and approx. 1.5 kb respectively. The latter insert was sequenced and found to contain an uninterrupted open reading frame. An approx. 500 bp *Xba*I-*Eco*RI fragment from the 3′ end of the 15.1.1 insert was therefore used to rescreen the blastula-stage λZap cDNA library, yielding clones 9.1.1, 10.1.1, and 12.1.1. Clone 10.1.1 contains the longest insert (approx. 2.2 kb) and was sequenced. The SpMyb sequence presented here was compiled from the combined double stranded sequences of clones 15.1.1 and 10.1.1.

DNA sequence analysis

DNA sequencing and sequence analysis was carried out as previously described (Coffman et al., 1996), using an ABI 373A automated sequencer and IBI Pustell sequence analysis software. Nested deletions of clones 10.1.1 and 15.1.1 were constructed using a Pharmacia kit.

In vitro transcription and translation

In vitro transcription and translation were performed using Ambion T3 mMessage Machine and reticulocyte lysate kits, respectively. For in vitro transcription, clone 15.1.1 was linearized with *Bsm*I, which cuts at nucleotide position 711 of the sequence depicted in Fig. 5 (126 bp downstream from the myb domain). In vitro translation reactions were carried out using the manufacturers protocol, except that the reactions were supplemented with 0.1 mM L-methionine. The use of nonradioactive methionine allowed for unambiguous detection of the translation products by EMSA (described above).

Genomic and RNA blot hybridizations

Genomic DNA and RNA blot hybridizations were carried out as described previously (Coffman et al., 1996), using as probe either an *Xba*I-*Eco*RI fragment of SpMyb corresponding to a C-terminal portion of the coding sequence, or the cloned PCR fragment corresponding to repeat 3 of the DNA-binding domain. Probes were labeled with [^{32}P]dCTP (NEN), using the Oligolabeling kit from Pharmacia. Washes were carried out in either 0.1× SSC, 1% SDS (high stringency) or in 1× SSC, 0.2% SDS (lower stringency) at 65°C.

RESULTS

Characterization of protein target sites within the P7II region using synthetic oligonucleotides

Fig. 1B shows the 63 bp sequence, referred to here as the P7II region, which was previously shown to be required for negative spatial regulation of *CyIIIa* (Hough-Evans et al., 1990; Kirchhamer and Davidson, 1996). Earlier studies revealed that this segment of DNA interacts with one or more proteins (Calzone et al., 1988; Thézé et al., 1990). Our initial experiments were therefore designed to determine exactly what target sites lie within the P7II region. When the entire P7II region is used as a probe, the protein most commonly detected in crude nuclear extract by electrophoretic mobility shift assays (EMSA) is SpGCF1, a relatively abundant protein that functions positively in regulating *CyIIIa* (Franks et al., 1990; Zeller et al., 1995a; Kirchhamer and Davidson, 1996). Because the P7II region clearly functions *negatively* in regulating *CyIIIa*, we suspected that it might contain a target site for another protein, which would be obscured by the high abundance of SpGCF1 in nuclear extracts. We found previously that a tandem series of columns bearing oligonucleotides containing a highly specific SpGCF1 target site (P8II from Thézé et al., 1990) can be used to remove this protein efficiently from nuclear extracts, allowing downstream purification of rarer proteins in the same sequential affinity chromatography run (Coffman et al., 1992). Nuclear extract was thus passed over two columns bearing a SpGCF1 target site, and then over a column bearing an oligonucleotide fragment of the P7II region (boxed sequence in Fig. 1B). In addition to the GCF1 site (shown in blue in Fig. 1B), the P7II oligonucleotide contains a sequence (shown in red Fig. 1B) that matches perfectly the consensus binding site for the myb family of DNA-binding proteins, viz $PyAAC^{G}/_{T}G$ (in reverse orientation in Fig. 1B; see Lipsick, 1996; Graf, 1992; and Luscher and Eisenman, 1990 for reviews).

In order to distinguish between SpGCF1 and putative myb DNA-binding activities eluted from the P7II affinity column, we constructed synthetic 22bp oligonucleotides that dissociated these sites. Three different double-stranded oligonucleotides were synthesized: (1) a fragment of the wild-type sequence with both the myb and GCF1 sites (oligo **MG**) that served as a control; (2) a mutated sequence that abolishes the myb site but adds an additional GCF1 site (oligo G); and (3) a mutated sequence that abolishes the GCF1 site but retains the myb site (oligo **M**; see Materials and Methods for oligonucleotide sequences). Each of these oligonucleotides was used as a probe in EMSA experiments in order to analyze the DNA-binding specificity of proteins eluted from the P7II affinity column. As is shown in Fig. 2, both oligonucleotides containing the myb target site (i.e., oligonucleotides **MG** and **M**) specifically bound a protein eluted from the wild-type P7II affinity column (Fig. 2A). In contrast, when the same oligonucleotides were used in EMSA to probe total nuclear extract, only SpGCF1 was detected, by probes **G** and **MG** (Fig. 2B). Probe **M** detected nothing under these conditions in total nuclear extract from blastula-stage embryos, indicating that the active myb-site-specific factor is relatively rare in these

extracts. Nonetheless, an estimated 100-fold enrichment of this factor achieved by a single round of affinity chromatography allowed its detection. These results demonstrate that while the wild-type oligonucleotide **MG** interacts with both SpGCF1 and a myb site-specific factor, oligonucleotide **M** interacts exclusively with the myb-site-specific factor, and oligonucleotide **G** interacts exclusively with SpGCF1.

Interactions at the myb target site function negatively to regulate spatial expression of *CyIIIa*

Gene transfer experiments were performed to determine the function of each of the target sites contained within the P7II region. Toward this end, each of the synthetic oligonucleotides used for the DNA/protein binding experiments described above was separately cloned into the deleted region of the *ΔP7II.CAT* construct (Fig. 1C, lines 3-5). The resulting constructs were microinjected into eggs, which were then allowed to develop to gastrula stage and analyzed by whole-mount in situ hybridization (WMISH). The results of this experiment are summarized quantitatively in Table 1, and representative examples are shown in Fig. 3. Remarkably, each of the constructs that contained a 'wild-type' myb site, i.e., *MG.CAT* and *M.CAT* gave rise to spatially normal patterns of expression (Fig. 3A,B). This result is quantitated in lines 3 and 5 of Table 1. Note that the small fractions of embryos displaying ectopic expression of these constructs are comparable to the fractions obtained with the wild-type *CyIIIa.CAT* construct (Table 1, line 1; see also Kirchhamer and Davidson, 1996). In contrast, *G.CAT*, the construct containing the mutated myb target site, gave rise to significant ectopic expression in both the oral ectoderm and the skeletogenic mesenchyme, as illustrated in Fig. 3C1, C2. This result is shown in line 4 of Table 1. It is important to note that the amount of ectopic expression obtained with this construct is similar to that observed when *ΔP7II.CAT* is injected (Table 1, line 2; Kirchhamer and Davidson, 1996). Since *G.CAT* includes a perfectly good GCF1 site, we conclude that the latter site plays no role in the negative regulatory function of the P7II region. In contrast, *M.CAT*, the construct lacking GCF1 sites in the oligonucleotide insert, exhibited somewhat lower levels of expression (Table 1, line 3), a result that is consistent with the earlier evidence cited above that SpGCF1 facilitates transcriptional activation. These results demonstrate that, within the context of the rest of the *CyIIIa* regulatory system, the myb target site contained within the P7II region is necessary and sufficient to prevent ectopic transcription in the oral ectoderm and skeletogenic mesenchyme. Furthermore, the five point mutations that eliminate interactions with the myb site binding protein in vitro produce quantitatively the same effect on spatial expression in vivo as does the entire 63 bp

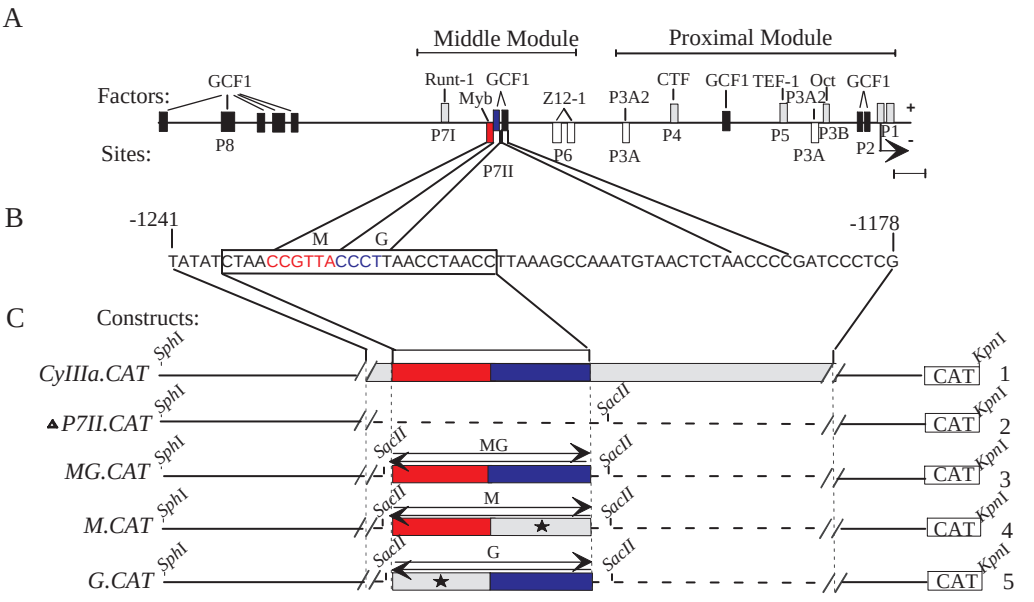

Fig. 1. Schematic representation of the sea urchin *CyIIIa* cytoskeletal actin gene regulatory region and reporter constructs used in this study. (A) Linear representation of the 5′ regulatory region of *CyIIIa*. Specific target sites, designated P1-P8 (as in Calzone et al., 1988; Thézé et al., 1990) are listed below the line. Cloned *trans*-acting factors known to interact with these elements are listed above the line (see Kirchhamer and Davidson, 1996 and Coffman et al., 1996, for references]. For simplicity, the prefix Sp has been omitted from the transcription factor designations. Boxes (not to scale) above the line represent target sites that interact with proteins that have a positive effect on transcription (+; Franks et al., 1990; Kirchhamer and Davidson, 1996; Coffman et al., 1996), while negatively acting factors bind to sites shown as boxes below the line (–; Hough-Evans et al., 1990; Wang et al.; Kirchhamer and Davidson, 1996). Sites for the ubiquitous transcription factor SpGCF1 (Zeller et al., 1995a,b) are shown as black boxes. The *CyIIIa* regulatory region is organized as two functional units, or modules, indicated by brackets (Kirchhamer and Davidson, 1996; Kirchhamer et al., 1996). The myb site which is the subject of this study (red) is located within the middle module, which also includes sites for the transcription factors SpRunt-1 (Coffman et al., 1996) SpZ12-1 (Wang et al., 1995) and SpGCF1 (blue and black). The bent arrow indicates the transcription start site. The scale bar represents 100 bp. (B) Sequence of the P7II region that was deleted in the study of Kirchhamer and Davidson (1996) (see construct 2 in C). Only the strand corresponding to the transcribed strand of *CyIIIa* is shown. Target sites are color coded as in A, with the myb site (**M**) in red, and the GCF1 site (**G**) in blue. Note that this region also contains a second GCF1 site (black box in A). The oligonucleotides used as target sites in this study were permutations of the boxed sequence. The numbering is with respect to the *CyIIIa* transcription start site, as in Thézé et al. (1990). (C) Schematic diagram of reporter constructs used in this study. DNA fragments of the *CyIIIa cis*-regulatory region which were not modified are depicted as solid horizontal lines. The SpMyb and SpGCF1 sites of the middle module are color-coded as in A. Dashed horizontal lines denote deletions, stars denote multiple point mutations that destroy individual sites. Restriction sites used for the cloning of experimental constructs are indicated in the figure, as are oligonucleotides used to introduce artificial target sites (arrows). The entire regulatory domain is drawn to scale (as in A), but not the protein-binding sites, deletions, oligonucleotides or *CAT* fusion gene.

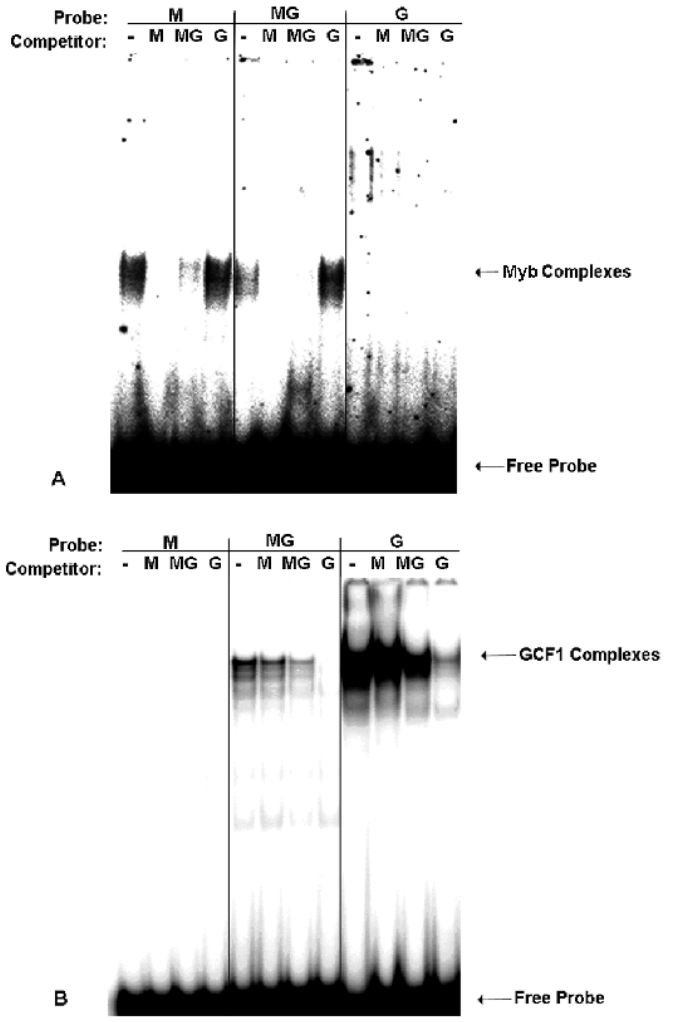

Fig. 2. Specific protein-DNA complexes formed with double stranded oligonucleotides used in this study. Each of the oligonucleotides (described in Materials and Methods) was used both as labeled probe and unlabeled competitor in EMSA with either (A) nuclear proteins eluted from an affinity column bearing a P7II target site oligonucleotide, or (B) crude nuclear extract. Probes and competitors used in each reaction are shown above each panel. M designates an oligonucleotide containing only the myb-target site; MG designates an oligonucleotide containing both myb and GCF1 target sites; and G designates an oligonucleotide containing only GCF1 target sites. In reactions that included unlabeled oligonucleotides as competitor, approximately 1,000-fold molar excess was used with respect to the probe DNA. (A) Affinity-enriched P7II-binding protein interacts with oligonucleotides **M** and **MG**, but not with the oligonucleotide **G**. (B) SpGCF1 in crude nuclear extract interacts with oligonucleotides **G** and **MG**, but not with oligonucleotide **M**. The higher degree of binding to probe **G** reflects that this oligonucleotide contains two core GCF1 sites (see Materials and Methods).

P7II deletion studied earlier by Kirchhamer and Davidson (1996).

Isolation of a cDNA clone encoding SpMyb, a sea urchin transcription factor that contains a myb DNA binding domain

To determine whether the affinity purified myb-binding protein

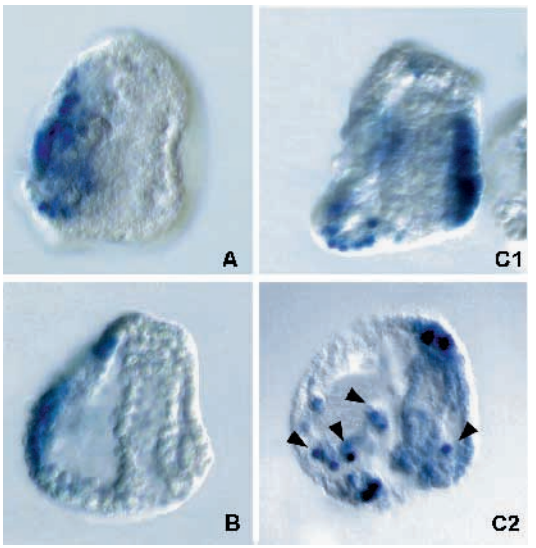

Fig. 3. Spatial expression patterns of reporter constructs. The spatial distribution of *CAT* transcripts was detected by WMISH, using a digoxigenin-labeled antisense *CAT* RNA probe. Expression domains were analyzed in late gastrula-stage embryos (50-54 hours old), shown with the oral ectoderm oriented to the right. (A) Embryo expressing the *MG.CAT* fusion gene (line 3 of Fig. 1B) exclusively in the aboral ectoderm territory. (B) Gastrula displaying *M.CAT* (line 4 of Fig. 1B) expression exclusively in the aboral ectoderm. (C1) Gastrula showing ectopic expression of *G.CAT* in the oral ectoderm (line 5 of Fig. 1B). (C2) Another embryo expressing *G.CAT* in both oral and aboral ectoderm territories and in skeletogenic mesenchyme cells (black arrowheads).

in fact contains a myb related DNA-binding domain, we first made use of a polyclonal antibody (anti-BP-2) generated against the vertebrate myb DNA-binding domain (Boyle et al., 1986). Anti-BP-2 has been shown to immunoprecipitate myb-related proteins from a wide variety of metazoan species. The EMSA depicted in Fig. 4 shows that the protein eluted from the P7II affinity column is indeed immunoprecipitated with anti-BP-2, but not with non-immune antibodies.

Affinity purified myb-binding proteins were further purified to near homogeneity by reverse-phase HPLC (data not shown), and subjected to amino acid sequence analysis. Two amino acid sequences were recovered that fall within the highly conserved first and third repeats of the myb DNA-binding domain (see Fig. 5, highlighted sequence). This result directly demonstrates the existence in the nuclei of sea urchin embryos of a myb domain protein that can be purified by virtue of interaction with its *CyIIIa* target site.

To obtain cDNA clones encoding this protein, degenerate primers (Stober-Grässer et al., 1992) were used in a polymerase chain reaction to amplify a blastula-stage cDNA specifying a highly conserved fragment of the myb DNA-binding domain. The amplified fragment was cloned, sequenced, and used as a probe to screen a blastula-stage cDNA library. With this probe we isolated clones for SpMyb (see Materials and Methods), the combined sequences of which are shown in Fig. 5. Note that the open reading frame begins at the 5′ end of the sequence. The first methionine residue, which aligns approximately with the N-termini of vertebrate myb proteins (see Fig. 6), occurs at amino acid position 4 in the open reading frame. However, we

Table 1. Late gastrula-stage expression of constructs containing either a wild-type or mutated Myb target site*

Construct	Total scored	Labeled:not labeled (interpretable)†	% expr‡	% Aboral ectoderm expression§	% Oral ectoderm expression§	% Skeletogenic mesenchyme expression§
CyIIIa.CAT	188	121:67 (113)	64.4	93.8	7.0	5.4
ΔP7II.CAT	328	206:122 (165)	62.8	95.2	17.6	36.4
MG.CAT	239	136:103 (122)	56.9	96.7	4.9	7.4
G.CAT	241	168:73 (145)	69.7	97.2	15.2	20.0
M.CAT	213	96:117 (91)	45.1	95.6	3.3	6.6

*Gastrulae were collected at 48-54 hours postfertilization; for constructs see Fig. 1C.
†Embryos with more than two labeled cells were scored as labeled.
‡% expression is [Σ labeled embryos/Σ labeled and unlabeled embryos]×100.
§% aboral ectoderm, oral ectoderm, or skeletogenic mesenchyme expression is [Σ embryos labeled in particular territory/Σ labeled, interpretable embryos]×100.

cannot be certain that this methionine represents the actual N terminus of SpMyb. The open reading frame depicted in Fig. 5 encodes an approximately 77 kDa protein, which is in the size range of known myb proteins (Boyle et al., 1986). The size also corresponds to the minor approx. 80 kDa species found in the purified preparation of SpMyb from which the amino acid sequence was obtained (see Materials and Methods).

Fig. 6 depicts an alignment of SpMyb with various other members of the myb family from vertebrates and *Drosophila*. Consistent with their common deuterostome relationship, the highest degree of similarity occurs between SpMyb and vertebrate A-Myb (39% identity), while slightly less correspondence occurs between SpMyb and vertebrate B-Myb (36% identity), and c-Myb (33% identity). The similarity between SpMyb and the *Drosophila* Myb is lowest (31% identity); moreover, DmMyb contains a long stretch of amino acids at the N terminus that are not found in any of the other aligned sequences, and is lacking certain regions that are found in both SpMyb and in all the vertebrate homologues. As can be seen in Fig. 6, the myb DNA-binding domain is the highest region of conservation, containing the three tandem repeats that form the multiple helix-turn-helix motifs of this domain. More C-terminal regions are also conserved. Some of these contain potential sites of phosphorylation by proline-directed serine/threonine kinases, including S528 of murine c-Myb (S533 in chicken c-Myb). This site (boxed in Fig. 5) is S549 of SpMyb. Phosphorylation of this serine has been shown to contribute to negative functions of vertebrate c-Myb, possibly by modulating specific protein-protein interactions (Aziz et al., 1995; Miglarese et al., 1996).

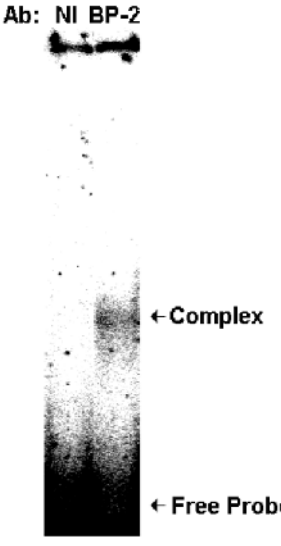

Fig. 4. The Myb-site oligonucleotide (M) binds a myb-related DNA-binding protein. The anti-BP-2 polyclonal antibody, generated against the conserved myb DNA-binding domain (Boyle et al., 1986), was used to immunoprecipitate proteins eluted from the **M** oligonucleotide affinity column as described in the Materials and Methods. Nonimmune serum was used as a control. Renatured proteins recovered from each immunoprecipitate were subjected to EMSA. The lane labeled NI depicts an EMSA performed using proteins recovered from beads coated with antibodies from nonimmune serum; the lane labeled BP-2 depicts an EMSA performed using proteins recovered from beads coated with anti-BP-2 antibodies.

In order to demonstrate that the cloned SpMyb is able to bind specifically to the myb target site from the *CyIIIa* regulatory domain, a fragment of SpMyb corresponding to the DNA-binding domain was transcribed and translated in vitro, and subjected to EMSA with the **M** oligonucleotide as probe. As can be seen in Fig. 7, the SpMyb protein fragment specifically interacts with the functional myb target site (oligonucleotide **M**), but not with the mutated target site (oligonucleotide **G**).

SpMyb is encoded by a single-copy gene which gives rise to a single embryonic transcript

The results illustrated in Fig. 8 indicate that SpMyb is the only myb-family member in sea urchin embryos. Fig. 8A shows a genome blot of *Eco*RI-digested DNA from three different individuals that was hybridized with a fragment encoding the C-terminal region of SpMyb. The single bands in lanes 1 and 2 indicate that these individuals are each homozygous for two different restriction fragment variants. The two bands in lane 3 indicate that this individual is either homozygous for a third variant containing an internal *Eco*RI site, or is heterozygous, carrying two different alleles. The degree of polymorphism apparent on this blot is consistent with that found in natural populations (Britten et al., 1978). When the genome blot was stripped and rehybridized at lower stringency with a probe representing the myb DNA-binding domain, essentially the same result was obtained (data not shown), suggesting that there is only one canonical myb-domain protein encoded in the genome of *S. purpuratus.*

To determine the size of the SpMyb transcript, poly(A)$^+$ RNA isolated from blastula-stage embryos was transferred to nylon and hybridized with the same fragment as used to probe the genome blot. The results are illustrated in Fig. 8B, which shows that SpMyb is encoded by a single transcript at this stage, of approximately 6 kb. No additional transcripts were detected when the conserved region of the myb domain was used to probe this same blot (data not shown). We therefore

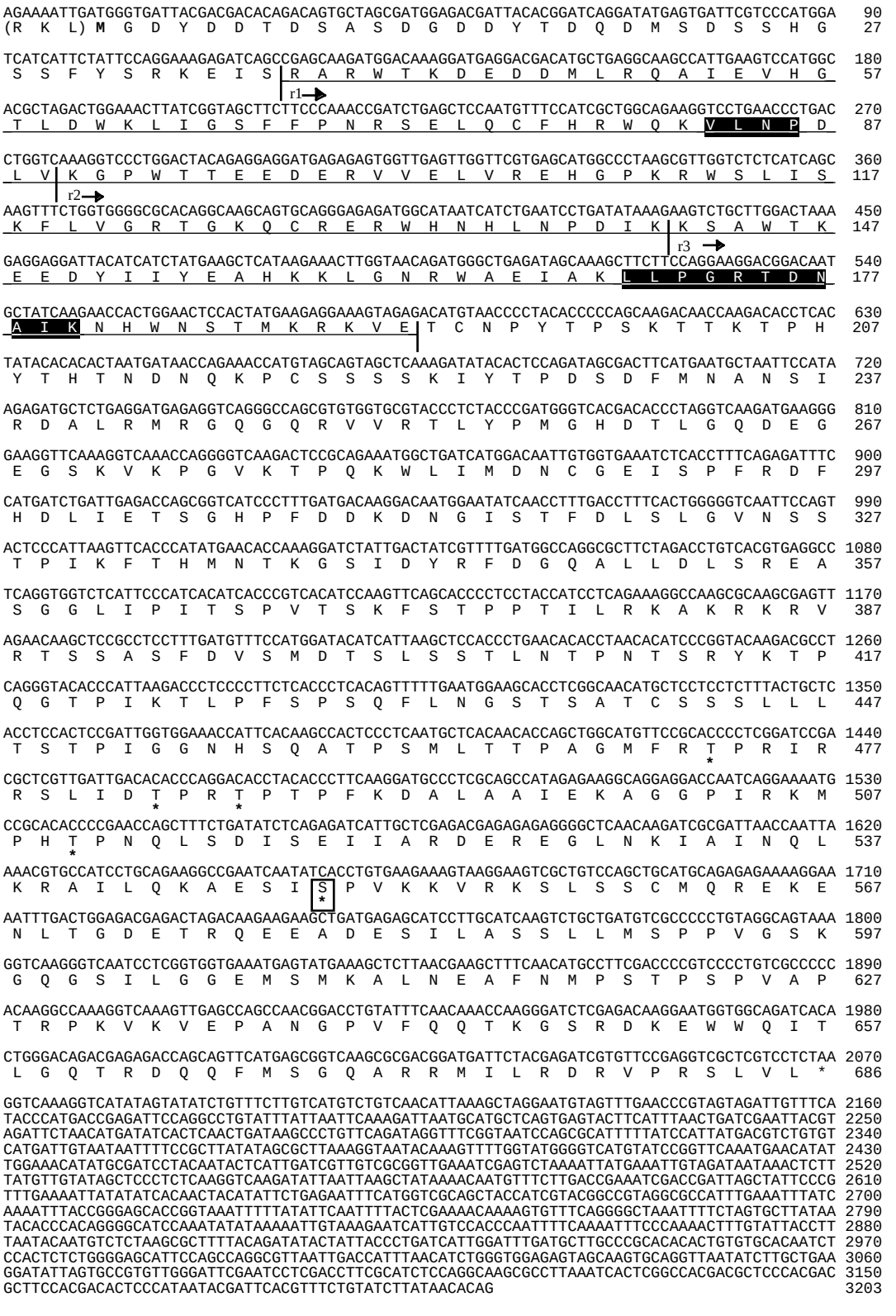

Fig. 5. The cDNA sequence and conceptual translation of SpMyb (GenBank accession no. U96090). While the open reading frame begins at the 5′ end of the sequence, a methionine that approximately aligns with the N-termini of vertebrate myb proteins (see Fig. 6) is found at position 4 (in boldface; this is position 1 in the numeration of the amino acid sequence). It is not known whether this is the actual start codon of SpMyb; the parentheses around the first three amino acid residues of the ORF indicate that they may or may not be translated. The myb DNA-binding domain is underlined, with the beginning of each of its three imperfect tandem amino acid repeats (r1, r2, r3) indicated by arrows. Amino acid sequences obtained from purified protein are highlighted. Asterisks in bold-face underneath amino acids mark potential phosphorylation sites; the boxed Serine (S) represents a potentially phosphorylated residue with known regulatory function in vertebrate c-Myb (Aziz et al., 1995; Miglarese et al., 1996). The termination codon is also marked by an asterisk.

conclude that, within the limits of detection employed here, SpMyb encodes the only myb-domain protein expressed in blastula-stage embryos of *S. purpuratus*.

Distribution of SpMyb transcripts in prism-stage embryos

Whole mount in situ hybridization was employed to visualize the distribution of *SpMyb* transcripts in prism-stage embryos. Examples of the staining pattern obtained with an antisense, digoxigenin-labeled SpMyb probe are shown in Fig. 9B,D. As a control, embryos were stained with *Endo16*, which is specifically localized to the midgut and hindgut at this stage (Fig. 9A,C; Ransick et al., 1993). In contrast, SpMyb transcripts appear to be largely localized to the oral ectoderm, mesenchyme and endoderm at this stage (Fig. 9B,D). SpMyb is therefore present in regions where it is required to repress *CyIIIa* transcription (oral ectoderm and mesenchyme), and in other regions as well (endoderm). The temporal expression pattern, as well as a more detailed analysis of the spatial pattern of both *SpMyb* transcripts and protein will be the subject of a separate report.

DISCUSSION

SpMyb, a myb-domain transcription factor in sea urchin embryos

We report here the cloning and initial characterization of SpMyb, a sea urchin member of the myb family of transcription factors. SpMyb was identified as a nuclear protein in sea urchin embryos that specifically interacts with a functional target site in the *cis*-regulatory domain of *CyIIIa*. This target site, which lies within the region referred to as P7II in earlier work (Hough-Evans et al., 1990; Kirchhamer and Davidson, 1996), is required to prevent ectopic expression of *CyIIIa* in the oral ectoderm and skeletogenic mesenchyme. Six lines of evidence indicate that SpMyb is the protein that mediates the negative regulatory function of this target site in vivo: (i) when

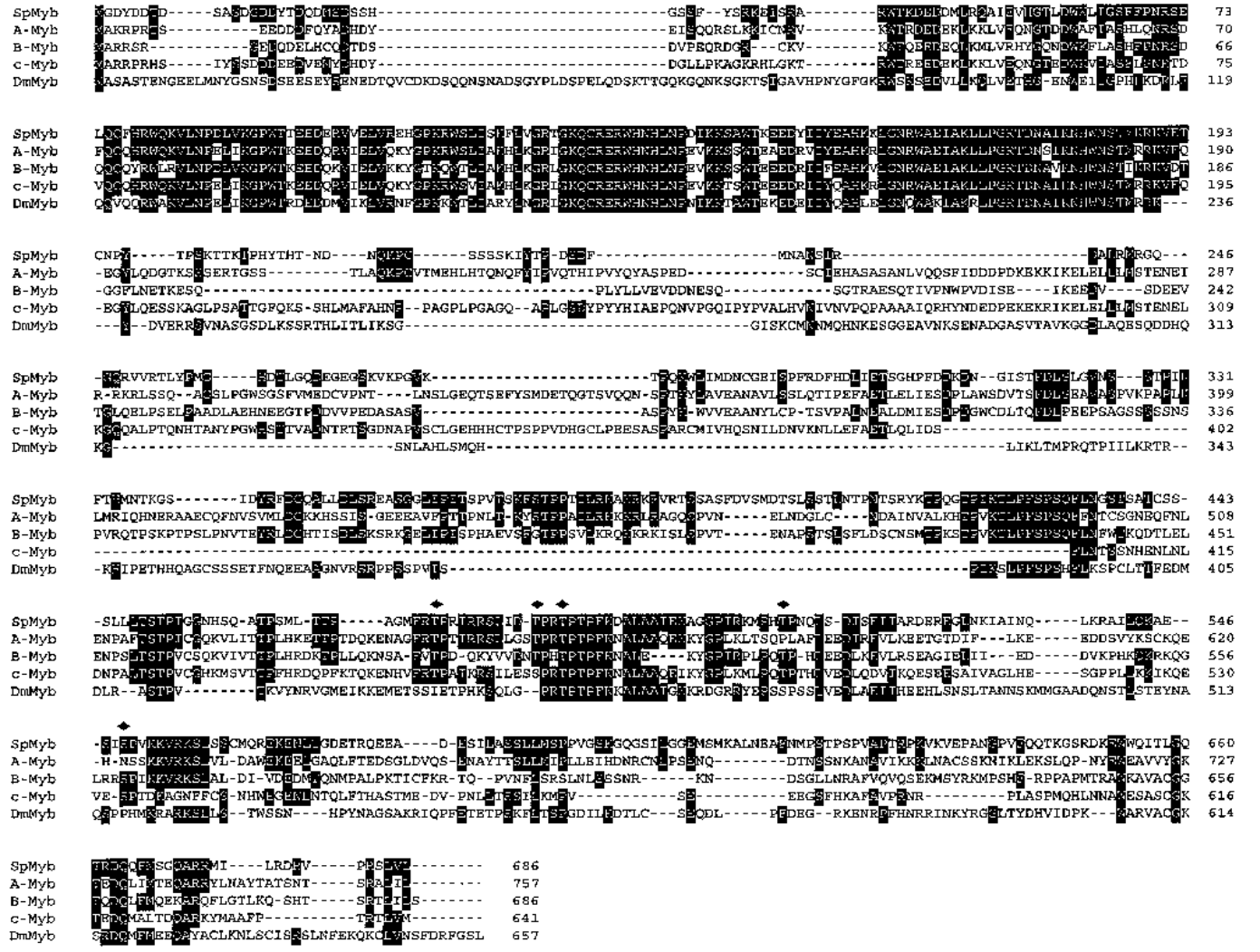

Fig. 6. Multiple sequence alignment of SpMyb with three vertebrate myb proteins (A-Myb, B-Myb, and c-Myb; each from *Gallus gallus*) and a myb protein from *Drosophila melanogaster* (DmMyb). The alignment was performed using the Clustal V program (Higgins et al., 1992). For SpMyb, the first methionine residue in the sequence shown in Fig. 5 was used as the N terminus in this alignment. Highlighted residues indicate positions that are found in common between SpMyb and at least one of the other proteins. The diamonds above the aligned sequences indicate sites of phosphorylation previously defined in the murine homologue of c-Myb (Aziz et al., 1995; Miglarese et al., 1996).

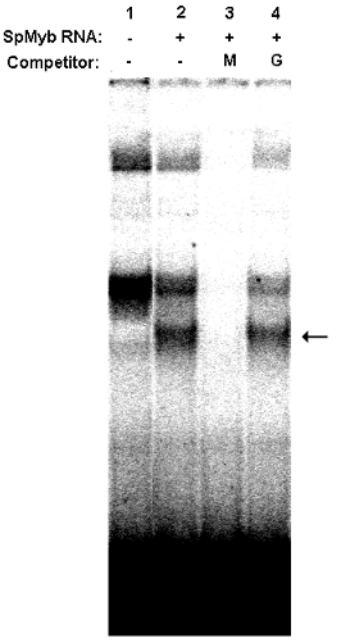

Fig. 7. DNA-binding activity of SpMyb translated in vitro from RNA encoded by the SpMyb cDNA clone. A fragment of the SpMyb cDNA corresponding to the DNA-binding domain (nucleotides 1-720 in Fig. 5) was transcribed and translated in vitro, and subjected to EMSA with the myb-site probe (oligonucleotide **M**). Lane 1, unprogrammed reticulocyte lysate; note that there are proteins in the lysate that bind the myb-site probe. Lanes 2-4, reticulocyte lysate programmed with the SpMyb DNA-binding domain. In lane 3, 1,000-fold molar excess of unlabeled oligonucleotide **M** was included as competitor; in lane 4, 1,000-fold molar excess of unlabeled oligonucleotide **G** was included as competitor. Note that the reticulocyte myb binding proteins are also competed by the **M** oligonucleotide. Use of the smaller myb DNA binding domain as template for in vitro translation allowed the reticulocyte and the sea urchin proteins (indicated by arrow) to be distinguished by complex size.

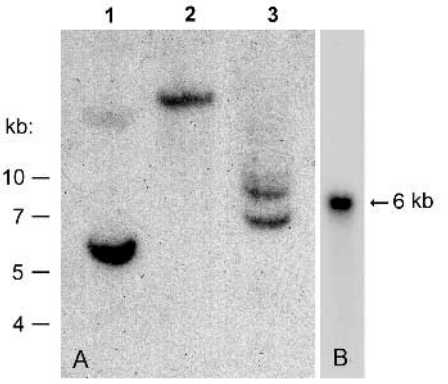

Fig. 8. Genomic and RNA blots probed with SpMyb. (A) Blot of *Eco*RI-digested genomic DNA from three different individuals, hybridized with SpMyb at high stringency. The pattern of bands shows that SpMyb is a single copy gene (see text). (B) Blot of poly(A)+ RNA from 24 hour blastulae, hybridized with SpMyb at high stringency. A single band of approximately 6 kb is detected.

cloned into the region of *CyIIIa.CAT* constructs from which the P7II region had been deleted, short oligonucleotides bearing the *CyIIIa* myb target site reconstitute the spatial regulatory function of that region in vivo, whereas oligonucleotides containing point mutations that alter bases known to be critical for myb target-site recognition do not (Table 1 and Fig. 3); (ii) a protein isolated from blastula-stage nuclear extracts interacts specifically with oligonucleotides containing the myb target site, as is shown by in vitro competition experiments (Fig. 2); (iii) this protein is immunoprecipitated by an antibody specific to the myb domain (Fig. 4); (iv) amino acid sequences obtained from the affinity-purified protein are identical to sequences in the conserved DNA binding domain of SpMyb; (v) DNA and RNA blot hybridization experiments employing different parts of cloned SpMyb as probe indicate that there is only one canonical myb domain protein encoded in the sea urchin genome, and that only a single species of *SpMyb* transcript is expressed in the blastula-stage embryo (Fig. 8); and (vi) SpMyb is expressed in the territories where the P7II target site is required to prevent ectopic expression of *CyIIIa* (Fig. 9). While it remains formally possible that a protein other than SpMyb is actually responsible for the negative spatial regulatory function of the P7II target site, this alternative is now improbable, since no other proteins were found to interact specifically with the P7II region, either by EMSA or by the highly sensitive technique of affinity chromatography combined with EMSA.

Several regions of the sequence in SpMyb are also conserved in various other myb proteins. As expected, the highest conservation occurs within the myb DNA-binding domain, the primary structure of which is highly conserved in organisms as distant as *Dictyostelium discoideum* (Stober-Grässer et al., 1992; Lipsick, 1996). A C-terminal region previously shown to be conserved in both *Drosophila* and vertebrate myb proteins (reviewed by Lipsick, 1996) is also found in SpMyb. In addition, SpMyb contains blocks of residues that are also found in the 'negative regulatory domain' of vertebrate c-Myb (Dubendorff et al., 1992). In vertebrates, c-Myb serves as an

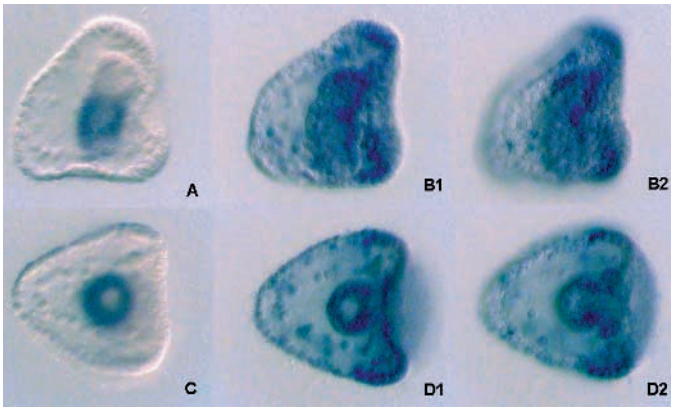

Fig. 9. Visualization of SpMyb transcripts in prism-stage embryos by WMISH. (A) Lateral view of a control embryo, hybridized with Endo16, which is at this stage localized to the mid- and hindgut (Ransick et al., 1993). (B1) Lateral view of an embryo hybridized with SpMyb. (B2) Same embryo as in B1, focusing on the surface of the ectoderm. (C) Anal view of a control embryo hybridized with Endo16, as in A. (D1) Anal view of an embryo hybridized with SpMyb. (D2) Same embryo as in D1, from a different focal plane. Note that SpMyb transcripts appear to be more sparse or possibly absent in the aboral ectoderm.

activator of some genes and as a repressor for others (see, e.g. Reddy et al., 1994; Vandenbark et al., 1996; reviewed by Lipsick, 1996). Vertebrate A-Myb has been shown to be an activator where analyzed (see, e.g. Golay et al., 1994; Takahashi et al., 1995). B-Myb has been shown to function both as a repressor (Foos et al., 1992), and as a cell-type-specific activator (Tashiro et al., 1995). We have shown that in the sea urchin embryo, SpMyb functions as a repressor of *CyIIIa* transcription. In the following we discuss evidence that the specific target of that repression is the SpRunt-1 activator.

SpMyb functions as an intramodular repressor to regulate spatial expression of *CyIIIa*

Previous analyses demonstrated that the middle module of the *CyIIIa* regulatory domain becomes the dominant regulatory module during differentiation of the aboral ectoderm after the blastula stage of embryonic development, and that the activation function of the middle module is mediated by the SpRunt-1 target site (Kirchhamer and Davidson, 1996; Coffman et al., 1996). In addition, it was shown that constructs lacking the entire middle module and retaining only the proximal module produce normal patterns of expression even though they lack the P7II regions, whereas constructs lacking the P7II region of the middle module but in which the remainder of the middle module is present are expressed ectopically (Kirchhamer and Davidson, 1996). These results suggested that the P7II region mediates repression by interfering with the transactivation function of SpRunt-1 in the oral ectoderm and skeletogenic mesenchyme. Such regulation could either be direct, by means of protein-protein interactions between SpMyb and SpRunt-1, or indirect, occurring by means of interactions of SpRunt-1 and SpMyb with a common intermediate protein or proteins. Intramodular or local repression, referred to as 'quenching' by Gray and Levine (1996), endows the developmental *cis*-regulatory module with the independent capability of processing spatial regulatory information in given regions or cell types of the embryo (for discussion, see Arnone and Davidson, 1997; Kirchhamer et al., 1996; Gray and Levine, 1996).

It is likely that SpRunt-1 is active not only in the aboral ectoderm, wherein it functions to activate *CyIII*a, but also in the oral ectoderm and skeletogenic mesenchyme, where SpMyb appears to be required to quench SpRunt-1-mediated activation. Late in development SpMyb is itself expressed strongly in the oral ectoderm, mesenchyme and endoderm. The repressive functions of the middle module are evident only after gastrulation at least in mesenchyme cells (Wang et al., 1995; Kirchhamer et al., 1996), and the localized expression of SpMyb occurs at this time. Thus its function as a repressor appears to be transcriptionally regulated in the postgastrular embryo, the terminal period of differentiation for many embryonic cell types.

The *CyIIIa* middle module encodes information that promotes evolutionarily conserved interactions between transcriptional regulatory proteins

The putative functional interaction between SpMyb and SpRunt-1 is interesting in light of the known interactions between vertebrate homologues of these two transcription factors. Vertebrate myb-domain and runt-domain factors have been shown to interact synergistically in the activation of the T-cell receptor δ and γ enhancers (Hernandez-Munain and Krangel, 1994; Hsiang et al., 1995) and in the activation of a murine leukemia retroviral enhancer (Zaiman and Lenz, 1996). In the case of the former, the interaction does not involve cooperative DNA binding, suggesting that it may require intermediary proteins (Hernandez-Munain and Krangel, 1995). In vertebrate systems, both runt-domain and myb-domain transcription factors are also known to interact with Ets-domain transcription factors in the regulation of various genes (e.g., Reddy et al., 1994; Wotton et al., 1994; Zaiman and Lenz, 1996; Dudek et al., 1992). We note that a perfect target site for an Ets-domain factor occurs 20 bp upstream of the myb target site in the *CyIIIa* middle regulatory module (–1252 to –1257 in the sequence published by Thézé et al., 1990), and a close match (5/6) for the Ets target site occurs immediately adjacent to the SpRunt-1 target site. There is no evidence at present that either of these sites bind Ets-domain proteins or function in regulating *CyIIIa* transcription. However, an Ets factor is expressed in sea urchin embryos, and its mRNA is prevalent in early embryos (Chen et al., 1988).

The interaction between SpMyb and SpRunt-1, and perhaps an Ets factor as well, are specified by the target sites encoded in the *cis*-regulatory DNA of the *CyIIIa* middle module, which can thus be thought of as a 'hard-wired' genomic regulatory subsystem (Arnone and Davidson, 1997). The comparative evidence indicates the remarkable conservation of the main working component of this subsystem, a sequence element that includes target sites for runt, myb, and Ets transcription factors. Here this element functions as a spatial on-or-off switch, a role that is likely to be useful in many different developmental regulatory contexts, and in many different genes.

The authors are indebted to Dr Joseph Lipsick (Stanford University Medical Center) for the kind gift of BP-2 antiserum. We thank Nicolette Halloran and Jina Yun for technical assistance, and Drs Ellen Rothenberg, Maria Arnone, and Leonard Bogarad for critical reviews of the manuscript. This work was supported by the Stowers Institute for Medical Research, by a grant from the NICHHD (HD-05753), and from NSF subcontract (STC DIR 8809719).

REFERENCES

Arnone, M. I. and Davidson, E. H. (1997). The hardwiring of development: organization and function of genomic regulatory systems. *Development* **124**, 1851-1864.

Aziz, N., Miglarese, M. R., Hendrickson, R. C., Shabanowitz, J., Sturgill, T. W., Hunt, D. F. and Bender, T. P. (1995). Modulation of c-Myb-induced transcription activation by a phosphorylation site near the negative regulatory domain. *Proc. Natl. Acad. Sci. USA* **92,** 6429-6433.

Boyle, W. J., Lipsick, J. S. and Baluda, M. A. (1986). Antibodies to the evolutionarily conserved amino-terminal region of the v-*myb*-encoded protein detect the *c-myb* protein in widely divergent metazoan species. *Proc. Natl. Acad. Sci. USA* **83**, 4685-4689.

Britten, R. J., Cetta, A. and Davidson, E. H. (1978). The single-copy DNA sequence polymorphism of the sea urchin Strongylocentrotus purpuratus. *Cell* **15**, 1175-1186.

Calzone, F. J., Thézé, N., Thiebaud, P., Hill, R. L., Britten, R. J. and Davidson, E. H. (1988). Developmental appearance of factors that bind specifically to *cis*-regulatory sequences of a gene expressed in the sea urchin embryo. *Genes Dev*. **2**, 1074-1088.

Calzone, F. J., Höög, C., Teplow, D. B., Cutting, A. F., Zeller, R. W., Britten, R. J. and Davidson, E. H. (1991). Gene regulatory factors of the sea urchin embryo. I. Purification by affinity chromatography and cloning of P3A2, a novel DNA binding protein. *Development* **112**, 335-350.

Char, B. R., Bell, J. R., Dovola, J., Coffman, J. A., Harrington, M. G., Becerra, J. C., Davidson, E. H., Calzone, F. J. and Maxson, R. (1993).

SpOct, a gene encoding the major octamer-binding protein in sea urchin embryos. Expression profile, evolutionary relationships, and DNA binding of the expressed protein. *Dev. Biol*. **158**, 350-363.

Chen, Z.-Q., Kan, N. C., Pribyl, L., Lautenberger, J. A., Moudrianakis, E. and Papas, T. S. (1988). Molecular cloning of the Ets proto-oncogene of the sea urchin and analysis of its developmental expression. *Dev. Biol*. **125,** 432-440.

Coffman, J. A., Moore, J. G., Calzone, F. J., Britten, R. J., Hood, L. E. and Davidson, E. H. (1992). Automated sequential affinity chromatography of sea urchin embryo DNA binding proteins. *Mol. Mar. Biol. Biotech.* **1**, 136-146.

Coffman, J. A., Kirchhamer, C. V., Harrington, M. G. and Davidson, E. H. (1996). SpRunt-1, a new member of the runt-domain family of transcription factors, is a positive regulator of the aboral ectoderm-specific *CyIIIa* gene in sea urchin embryos. *Dev. Biol*. **174**, 43-54.

Dubendorff, J. W., Whittaker, L. J., Eltman, J. T. and Lipsick, J. S. (1992). Carboxy-terminal elements of c-Myb negatively regulate transcriptional activation in *cis* and in *trans*. *Genes Dev*. **6**, 2524-2535.

Dudek, H., Tantravahi, R. V., Rao, V. N., Reddy, E. S. and Reddy, E. P. (1992). Myb and Ets proteins cooperate in transcriptional activation of the *mim-1* promoter. *Proc. Natl. Acad. Sci. USA* **89**, 1291-1295.

Franks, R. R., Anderson, R., Moore, J. G., Hough-Evans, B. R., Britten, R. J. and Davidson, E. H. (1990). Competitive titration in living sea urchin embryos of regulatory factors required for expression of the *CyIIIa* actin gene. *Development* **110**, 31-40.

Foos, G., Grimm, S. and Klempnauer, K.-H. (1992). Functional antagonism between members of the *myb* family: B-*myb* inhibits v-*myb*-induced gene activation. *EMBO J.* **11**, 4619-4629.

Golay, J., Loffarelli, L., Luppi, M., Castellano, M. and Introna, M. (1994). The human A-myb protein is a strong activator of transcription. *Oncogene* **9**, 2469-2479.

Graf, T. (1992). Myb: A transcriptional activator linking proliferation and differentiation in hematopoietic cells. *Curr. Opinion Genet. Dev.* **2**, 249-255.

Gray, S. and Levine, M. (1996). Transcriptional repression in development. *Curr. Opin. Cell Biol.* **8**, 358-364.

Hernandez-Munain, C. and Krangel, M. S. (1994). Regulation of the T-cell receptor δ enhancer by functional cooperation between c-Myb and core-binding factors. *Mol. Cell. Biol.* **14**, 473-483.

Hernandez-Munain, C. and Krangel, M. S. (1995). c-Myb and core-binding factor/PEBP2 display functional synergy but bind independently to adjacent sites in the T-cell receptor δ enhancer. *Mol. Cell. Biol.* **15**, 3090-3099.

Higgins, D. G., Bleasby, A J. and Fuchs, R. (1992). Clustal V: Improved software for multiple sequence alignment. *Computer Applications in the Biosciences (CABIOS)* **8**, 189-191.

Hough-Evans, B. R., Franks, R. R., Zeller, R. W., Britten, R. J. and Davidson, E. H. (1990). Negative spatial regulation of the lineage-specific *CyIIIa* actin gene in the sea urchin embryo. *Development* **110**, 41-50.

Hsiang, H. H., Goldman, J. P. and Raulet, D. H. (1995). The role of c-Myb or a related factor in regulating the T-cell receptor γ gene enhancer. *J. Immunol.* **154**, 5195-5204.

Kirchhamer, C. V. and Davidson, E. H. (1996). Spatial and temporal information processing in the sea urchin embryo: Modular and intramodular organization of the *CyIIIa* gene *cis*-regulatory system. *Development* **122**, 333-348.

Kirchhamer, C. V., Yuh, C. -H. and Davidson, E. H. (1996). Modular *cis*-regulatory organization of developmentally expressed genes: Two genes transcribed territorially in the sea urchin embryo, and additional examples. *Proc. Natl. Acad. Sci. USA* **93**, 9322-9328.

Lipsick, J. S. (1996). One billion years of Myb. *Oncogene* **13**, 223-235.

Luscher, B. and Eisenman, R. N. (1990). New light on Myc and Myb. Part II. Myb. *Genes Dev*. **4**, 2235-2241.

McMahon, A. P., Flytzanis, C. N., Hough-Evans, B. R., Katula, K. S., Britten, R. J. and Davidson, E. H. (1985). Introduction of cloned DNA into sea urchin egg cytoplasm: replication and persistence during embryogenesis. *Dev. Biol.* **108**, 420-430.

Miglarese, M. R., Richardson, A. F., Aziz, N. and Bender, T. P. (1996). Differential regulation of c-Myb-induced transcription activation by a phosphorylation site in the negative regulatory domain. *J. Biol. Chem.* **271**, 22697-22705.

Ransick, R., Ernst, S. G., Britten, R. J. and Davidson, E. H. (1993). Whole mount in situ hybridization shows *Endo16* to be a marker for the vegetal plate territory in sea urchin embryos. *Mech. Dev.* **42**, 117-124.

Reddy, M. A., Yang, B. -S., Yue, X., Barnett, C. J. K., Ross, I. L., Sweet, M. J., Hume, D. A. and Ostrowski, M. C. (1994). Opposing actions of *c-ets*/PU. 1 and *c-myb* protooncogene products in regulating the macrophage -specific promotors of the human and mouse colony-stimulating factor-1 receptor (*c-fms*) genes. *J. Exp. Med*. **180,** 2309-2319.

Stober-Grässer, U., Brydolf, B., Bin, X., Grässer, F., Firtel, R. A. and Lipsick, J. S. (1992). The Myb DNA-binding domain is highly conserved in *Dictyostelium discoideum*. *Oncogene* **7**, 589-596.

Takahashi, T., Nakagoshi, H., Sarai, A., Nomura, N., Yamamoto, T. and Ishii, S. (1995). Human A-*myb* gene encodes a transcriptional activator containing the negative regulatory domains. *FEBS Letters* **358**, 89-96.

Tashiro, S., Takemoto, Y., Handa, H. and Ishii, S. (1995). Cell type-specific *trans*-activation by the B-*myb* gene product: requirement of the putative cofactor binding to the C-terminal conserved domain. *Oncogene* **10**, 1699-1707.

Thézé, N., Calzone, F. J., Thiebaud, P., Hill, R. L., Britten, R. J. and Davidson, E. H. (1990). Sequences of the *CyIIIa* actin gene regulatory domain bound specifically by sea urchin embryo nuclear proteins. *Mol. Reprod. Dev*. **25**, 110-122.

Vandenbark, G. R., Chen, Y., Friday, E., Pavlik, K., Anthony, B., deCastro, C. and Kaufman, R. E. (1996). Complex regulation of human *c-kit* transcription by promoter repressors, activators, and specific myb elements. *Cell Growth Differ*. **7**, 1383-1392.

Wang, D. G. -W., Kirchhamer, C. V., Britten, R. J. and Davidson, E. H. (1995). SpZ12-1, a negative regulator required for spatial control of the territory-specific *CyIIIa* gene in the sea urchin embryo. *Development* **121**, 1111-1122.

Wotton, D., Ghysdael, J., Wang, S., Speck, N. A. and Owen, M. J. (1994). Cooperative binding of Ets-1 and core binding factor to DNA. *Mol. Cell Biol.* **14**, 840-850.

Zaiman, A. L. and Lenz, J. (1996). Transcriptional activation of a retrovirus enchancer by CBF (AML1) requires a second factor. Evidence for cooperatively with c-Myb. *J. Virol*. **70**, 5618-5629.

Zeller, R. W., Coffman, J. A., Harrington, M. G., Britten, R. J. and Davidson, E. H. (1995a). SpGCF1, a sea urchin embryo DNA binding protein, exists as five nested variants encoded by a single mRNA. *Dev. Biol.* **169**, 713-727.

Zeller, R. W., Griffith, J. D., Moore, J. G., Kirchhamer, C. V., Britten, R. J. and Davidson, E. H. (1995b). A multimerizing transcription factor of sea urchin embryos capable of looping DNA. *Proc. Natl. Acad. Sci. USA* **92**, 2989-2993.

(Accepted 5 September 1997)