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Spatial and temporal information processing in the sea urchin embryo: modular and intramodular organization of the *CyIIIa* gene *cis*-regulatory system

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SUMMARY

The *CyIIIa* cytoskeletal actin gene of *Strongylocentrotus purpuratus* is expressed specifically in the aboral ectoderm. In earlier work we identified a 2.3 kb *cis*-regulatory region that is necessary and sufficient for correct spatial and temporal expression of a *CyIIIa•CAT* gene. This region includes about 20 sites of specific protein-DNA interaction, at which at least nine different transcription factors may be bound. All except two of these factors have been cloned. In this work we have analyzed by deletion or mutagenesis each specific interaction. A specific function was identified for every binding site examined. These individual functions include control of amplitude and timing of expression at different phases of embryogenesis, and control of spatial expression. We show that particular negative regulatory interactions are required to repress expression of the *CyIIIa•CAT* construct in oral ectoderm and in skeletogenic mesenchyme at different stages. In further experiments we determined the overall functional organization of the *CyIIIa cis*-regulatory system, and we show that this system is modular in its regulatory structure. The 'proximal module' (with respect to the transcription start site) extends upstream for about 800 base pairs, and includes nine target sites serviced by six different transcription factors. Its major role is to establish *CyIIIa* expression in the aboral ectoderm territory as the blastomere founder cells are specified and the oral-aboral axis is determined, and to activate the *CyIIIa* gene late in cleavage. The 'middle module,' which lies upstream of the proximal module, acquires major control of *CyIIIa* function after the blastula stage. It includes six target sites, serviced by four different factors. The middle module is responsible for a sharp increase in expression occurring during gastrulation, mediated by the positively acting factors that bind within it. The middle module also includes sites at which two different negatively acting spatial control factors bind, the functions of which are required for correct spatial expression late in embryogenesis. The 'distal module' contains a number of sites at which a positively acting factor binds, but this module exercises no spatial regulatory function. Interactions within the distal module are required for the normal levels of function of both the proximal and middle modules.

Key words: *Strongylocentrotus purpuratus*, *CyIIIa*, gene regulation, spatial information processing, aboral ectoderm, embryogenesis

INTRODUCTION

Genes encoding cell differentiation products lie at the termini of the genetic networks that organize development. In Type 1 embryogenesis, of which the sea urchin provides a canonical case, some such genes begin to be expressed remarkably early in development (Davidson, 1990, 1991). The example considered in this paper, the *CyIIIa* cytoskeletal actin gene of *Strongylocentrotus purpuratus*, is transcribed exclusively in the cell lineages that give rise to the embryonic aboral ectoderm (Cox et al., 1986; Lee et al., 1986; Akhurst et al., 1987; Shott et al., 1984; Cameron et al., 1989a). The aboral ectoderm of advanced embryos and larvae consists of a single cell type, a squamous epithelium that forms the outer wall of the embryo and larva and that is largely jettisoned at metamorphosis. The *CyIIIa* gene is transcriptionally activated in mid to late cleavage, long in advance of the appearance of the squamous aboral epithelial cell type, in the eleven clones of which the aboral ectoderm is composed (Cameron et al., 1987, 1990). Its activation follows by only one or two cell cycles the segregation of aboral ectoderm founder cells from oral ectoderm and other lineages (Lee et al., 1992; Hickey et al., 1987). The early lineage-specific expression of genes such as *CyIIIa* directly reflects the outcome of the process of founder cell specification, which results in the territorial presentation of suites of active transcription factors, positive and negative.

In this paper we focus on the complex *cis*-regulatory apparatus that controls *CyIIIa* gene expression. Earlier work had demonstrated that 2.3 kb of DNA sequence extending upstream of the *CyIIIa* transcription start site suffice to confer spatially, temporally and quantitatively accurate expression on *CyIIIa•CAT* transgenes injected into unfertilized or fertilized eggs (Flytzanis et al., 1987; Hough-Evans et al., 1987, 1990; Livant et al., 1988; Franks et al., 1990). About 20 sites of high

specificity DNA-protein interaction have been mapped within the 2.3 kb *cis*-regulatory domain, and these are serviced by at least nine different transcription factors (Calzone et al., 1988; Thézé et al., 1990). These interactions are unusually well known, as all but two of the nine factors have been cloned and to some extent characterized. In Fig. 1A a map is presented showing the *CyIIIa* target sites and the nomenclature by which they are denoted, and indicating the relevant transcription factors (see legend for references). In this work we have used deletion or mutagenesis to investigate the functional significance of most of the individual sites of interaction that had not been examined earlier. Remarkably, a specific regulatory function can be attributed to every site examined. We have defined a large sub-region of the *cis*-regulatory domain, the 'proximal module,' the major role of which is to transduce the spatial information generated by the relevant early embryonic specification processes. These are the processes that determine the orientation of the oral/aboral axis, and that determine the positions of the aboral ectoderm founder cells (Davidson, 1989, 1990; Cameron and Davidson, 1991). From the gastrula stage onward, as the aboral ectoderm differentiates, the main quantitative and spatial control of *CyIIIa* gene expression is shifted to a different sub-region of the *cis*-regulatory domain, that we term the 'middle module,' and that is serviced by a new set of DNA-protein interactions. A third, 'distal module,' interacts with both of the other modules we have identified. The experiments that we describe in the following demonstrate the overall functional organization of the *CyIIIa cis*-regulatory system.

MATERIALS AND METHODS

Preparation of eggs for injection and embryo culture

Eggs and sperm of *Strongylocentrotus purpuratus* were obtained by stimulating adult animals with 25 V of direct current, which allows repeated spawning of each individual over periods of weeks or months. Embryos were cultured at 15°C, essentially as described previously (McMahon et al., 1985). One × penicillin/streptomycin (20 units/ml penicillin; 50 μg/ml streptomycin; Boehringer Mannheim Biochemicals) was added to the Millipore-filtered sea water (MFSW). Embryos were collected at the appropriate stage and processed for CAT measurements or in situ hybridizations.

Microinjection

Unfertilized eggs were prepared for injection as previously described (McMahon et al., 1985), fertilized and microinjected with approx. 2-5 pl injection solution after fertilization, using a Picospritzer II (General Valve Corporation). Each pulse was 10-30 mseconds long; 40 psi of air pressure was applied. Injection solutions contained 20% glycerol, 0.1 M KCl, approx. 1000 molecules reporter construct per pl, and a 5-fold mass excess of *Hin*dIII-digested genomic sea urchin carrier DNA.

Construction of reporter gene constructs

(1) Control constructs

The 14.7 kb *CyIIIa•CAT* construct described by Flytzanis et al. (1987), or the shorter, 9.4 kb *CyIIIa•pGEMCAT* were used as control constructs. The latter carries the *Sph*I-*Bam*HI fragment of *CyIIIa•CAT* subcloned into *pGem-3Zf(–)* (Promega). Both plasmids contain the 2.3 kb 5′ *CyIIIa* regulatory region, transcription initiation site, 5′ leader sequence of the *CyIIIa* primary transcript that is interrupted by a 2.2 kb intron (Akhurst et al., 1987), and the *CyIIIa•CAT* fusion gene. Restriction sites of *CyIIIa•CAT* or *CyIIIa•pGEMCAT* which were used for cloning the mutant constructs described below are indicated in Fig. 1C. *CyIIIa•CAT* and *CyIIIa•pGEMCAT* were linearized for microinjection at *Sph*I or *Kpn*I sites, respectively.

(2) Constructs generated by restriction fragment cloning

ProxΔP4,3A was cloned by inserting the *Bsa*HI fragment of *CyIII•CAT* into the *Cla*I site of *psp72* (Promega). The insert contains approx. 350 bp proximal to the transcription start site plus the CAT fusion gene, and is oriented in *psp72* so that the *Acc*65I-site of the polylinker is upstream of the insert. To create plasmid *Prox*, the *Bsi*WI-*Nru*I fragment of *CyIIIa•CAT* which carries the upstream P3A and the P4 site was isolated, and cloned into the *Acc*65I and *Nru*I sites of *ProxΔP4,3A*. The construct termed *ProxΔP3A* was cloned by digesting *Prox* with *Nhe*I and *Xba*I which removes only the small fragment containing the P3A-site from *Prox*, and religating the long fragment obtained after digest. For *ProxΔP4*, the *Rsa*I-*Hae*III fragment of *CyIIIa•CAT* containing the P4 and the upstream P3A site was subcloned in *pBluescript II KS (+)* (Stratagene). This construct was digested with *Nhe*I and *Xba*I, which cuts out an insert carrying only the P3A site. It was inserted into *ProxΔP4,3A*, thus creating *ProxΔP4*. All constructs described in this paragraph were linearized with *Xho*I for injection.

ProxΔP5 was cloned analogously to *Prox*, with the exception that the *Bsa*HI fragment of *ΔP5* was inserted into *psp72* in the first cloning step. *ΔP5* was generated by digesting *CyIIIa•CAT* with *Hin*dIII and religating the large product of the digest. *ProxmP1* was cloned using the same strategy applied in cloning *Prox* and *ProxΔP5*, but this time starting with the *Bsa*HI fragment of *mP1* which is described below. Plasmids *ProxΔP5*, *ΔP5* and *ProxmP1* were cut with *Sp*hI before injection.

ΔP6,7 contains the *Sph*I-*Acc*I fragment (which carries the distal P8 sites) of *CyIIIa•CAT* cloned into *Prox* after digestion with the same two restriction endonucleases. It was linearized with *Xho*I. *ΔP8* was cloned by isolating the *CyIIIa•CAT-Eag*I/*Aat*II insert and cloning it into *pGem-3Zf(–)*. It was linearized at the unique *Eag*I site. Plasmids *RTB1+1*, *RTBΔ5* and *RTBΔ5+1* are derivatives of *RTB1* which is described in detail by Makabe et al. (1995). Briefly, *RTB1* contains the proximal P8 sites, the P3B, P4, and P5 sites, the distal cluster of P8 sites, and an SV40 transcription start site. For *RTB1+1*, the *Ava*II-*Bam*HI fragment of *CyIIIa•CAT* carrying both P1 sites was subcloned into the *Sma*I site of *puc19* (Boehringer Mannheim Biochemicals) after filling in the ends. The resulting plasmid was digested with *Hin*dIII and *Eco*RI, the insert isolated and subcloned into *pBluescript II KS (+)*. It was released with *Pst*I, and cloned into *RTB1* at its *Pst*I site, producing construct *RTB1+1*. After examining *RTB1+1* for correct orientation of the inserted fragment, the plasmid was *Xho*I-linearized for injection. *RTBΔ5+1* was cloned as follows: *RTB1* was digested with *Bgl*II. The *Bgl*II fragment carries all protein binding sites, the transcription start site and the reporter gene, but only part of the polylinker described by Makabe et al., 1995. It was subcloned in *psp72*. The resulting plasmid was cut with *Hin*dIII, and the two large fragments resulting from the digest were ligated. Following confirmation of correct orientation of both fragments, the *Bgl*II fragment of the resulting plasmid was cut out and reinserted into *RTB1*, thus generating *RTBΔ5*. *RTBΔ5+1* was cloned in analogous manner to *RTB1+1*. *RTB1*, *RTB1+1*, *RTBΔ5* and *RTBΔ5+1* were linearized with *Xho*I.

(3) Constructs generated using PCR mutagenesis

For the cloning of *mP1*, the starting point was a construct called *IFCCP* that contains the *Pst*I-*Acc*I, *Bam*HI-*Hin*dIII and *Bam*HI-*Bam*HI fragments of *CyIIIa•CAT* in *psp72* in correct order and orientation. The first two fragments are joined by polylinker sequences containing a *Sal*I site. *IFCCP* was digested with *Sal*I and *Sac*I, the resulting 1.3 kb fragment isolated and subcloned into *pBluescript II*

KS (+). This insert carries the proximal P8 sites, the two P1 sites, and part of the reporter gene. Site-directed PCR mutagenesis of the P1 binding sites TGGTCCCCCACAGT (upstream) and TGGTGTCATCCAGT (downstream; consensus sequence underlined) was carried out by conversional mutation of bases conforming to the P1 consensus site (Thézé et al., 1990), using mutagenic, partially overlapping primers, P1→T3 and P1→T7. Their sequence (below), with the overlapping region bold and italic, and the substituted nucleotides underlined, was as follows:

P1→T3 (downstream): 5′-AAGAGAAAGAGA<u>CTGAGCTTA</u>-<u>ATTATC</u>CTGCTTGAT***TGCTTGGTCGAG***-3′

P1→T7 (upstream): 5′-TAGTACATTACT<u>ACTACGACAATA</u>-<u>GTATCATTTCACT</u>***CTCGACCAAGCA***-3′

The first round of PCR introduced base substitutions in each P1 site in two separate fragments, using a combination of mutant inside and non-mutant outside primers (commercially available T3 and T7 primers). The 25 μl PCR reactions contained 100 ng template, 0.25 μM P1->T3 or P1->T7, respectively, 2.5 μM T7 or T3 primers, 0.4 mM of each dNTP, and one unit of Vent DNA polymerase (New England Biolabs). Twenty cycles of amplification were carried out (94°C, 30 seconds; 55°C, 1 minute; 72°C, 1 minute) in a GeneAmp PCR System 9600 (Perkin Elmer). The second round of PCR was performed using only outside primers (2.5 mM each) and 100 ng of each of the PCR fragments obtained in the first round. This amplification reaction rejoined the two fragments. The final PCR product was reintroduced into *pBluescript II KS (+)*, sequenced using the dideoxy method (Sanger et al., 1977), digested with *Bsu36*I and *Sac*I and inserted into *CyIIIa•CAT* to generate *mP1*.

Plasmid *ΔP7II* was generated using a different approach: two inside primers, FC98 and FC99, flanking a approx. 60 bp fragment carrying the P7II site (TAACCTAACCTTAAAGCC, −1206 to −1223 of the sequence published by Thézé et al., 1990), and two oligos at the ends of the fragments of *CyIIIa•CAT* to be amplified (outside primers, MP1 and MP2) were synthesized. Both inside primers carry a short, new, overlapping sequence that introduced a restriction site for *Sac*II absent in both *pGem-3Zf (−)* and *CyIIIa•CAT* for ease of screening for mutant clones. The sequences of the primers used, with the overlapping nucleotides underlined, and the newly introduced *Sac*II site in bold and italics, were as follows:

FC98 (−1176 to −1163): 5′-<u>AATAT</u>***CCGCGG***<u>TCGTATGTGAC</u>-<u>TTAATAAA</u>-3′

FC99 (−1242 to −1255): 5′-<u>ATACGA</u>***CCGCGG***<u>ATATTGTTGG</u>-<u>GTCAAGGA</u>-3′

MP1 (−2360 to −2329): 5′-TAGCATGCTGGCAAATACATGTC-TGTATTCC-3′

MP2 (+1010 to +1041): 5′-AGGTCGACGACAAGAGCGGCAA-CATCATCG-3′

The first round of PCR deleting the fragment with the P7II site was carried out as described above (with the exception that 30 rounds of PCR were done) and included two separate amplification reactions, one with MP1 and FC99, and one using MP2 and FC100. The second round of PCR fused and amplified the primary products. The final product, which lacks the sequence from −1177 to −1241 of *CyIIIa•CAT*, was digested with *Sph*I and *Sal*I and inserted in *CyIIIa•pGEMCAT* to generate *ΔP7II*, which was linearized at the *Kpn*I site for injection. The plasmids *ΔP3A.F*, *ΔP3A.H*, and *ΔP3A.HF* were cloned as follows: for each P3A binding site, one mutagenic oligonucleotide (inside primer) was designed which lacks only the binding site plus several bases upstream and downstream of it, but carries complementary sequence 5′ and 3′, so that the following amplification reaction deleted the sequence containing the P3A sites. Oligonucleotide 3AD1 deleted the sequence <u>T</u>GAA<u>GCGCA</u>AA-CAAAC (downstream), 3AD2 CGG<u>CGGCG</u>C (upstream; bases corresponding to the P3A consensus site are underlined). T3 and T7 oligonucleotides were used as outside primers. The sequence of 3AD1 and 3AD2 is given below, with the arrow indicating the location of the gap.

3AD1: 5′-TTGTTTTTAAAAAGAATAAA↓ACTTTATTAAGC-AAAAAAGCAC-3′

3AD2: 5′-AGTAAGCATCTTACAAATCGTA↓TGCGGGGTTC-GCCT-3′

The starting point for the PCR reaction deleting P3A.F (downstream) was a construct containing the *Hin*dIII-*Bam*HI fragment of *CyIIIa•CAT* subcloned into *pBluescript II KS (+)*. PCR mutagenesis was carried out as described above, using 3AD1 and T7 primers. The primer pair used to delete P3A.H (upstream) after subcloning the *Pst*I-*Hin*dIII fragment of *CyIIIa•CAT* into *pBluescript II KS (+)* was 3AD2 and T3. Both mutated fragments were connected with each other and the remaining 5′ regulatory region of *CyIIIa* by insertion into *IFCCP* (described above) at the *Hin*dIII-*Bam*HI, and *Kpn*I-*Sph*I sites, respectively. This created a construct lacking both P3A2 sites, *ΔP3A.HF*. By using the same sites mentioned above to replace one fragment at a time with the respective wild-type fragment, plasmids *ΔP3A.H* and *ΔP3A.F* were generated. *ΔP3A.F*, *ΔP3A.H*, *and ΔP3A.HF* were cut at the unique *Not*I site before injection.

CAT measurements

CAT enzyme activity was determined in lysates of 50-100 embryos. Samples were collected at various stages in development and processed as reported by McMahon et al. (1984).

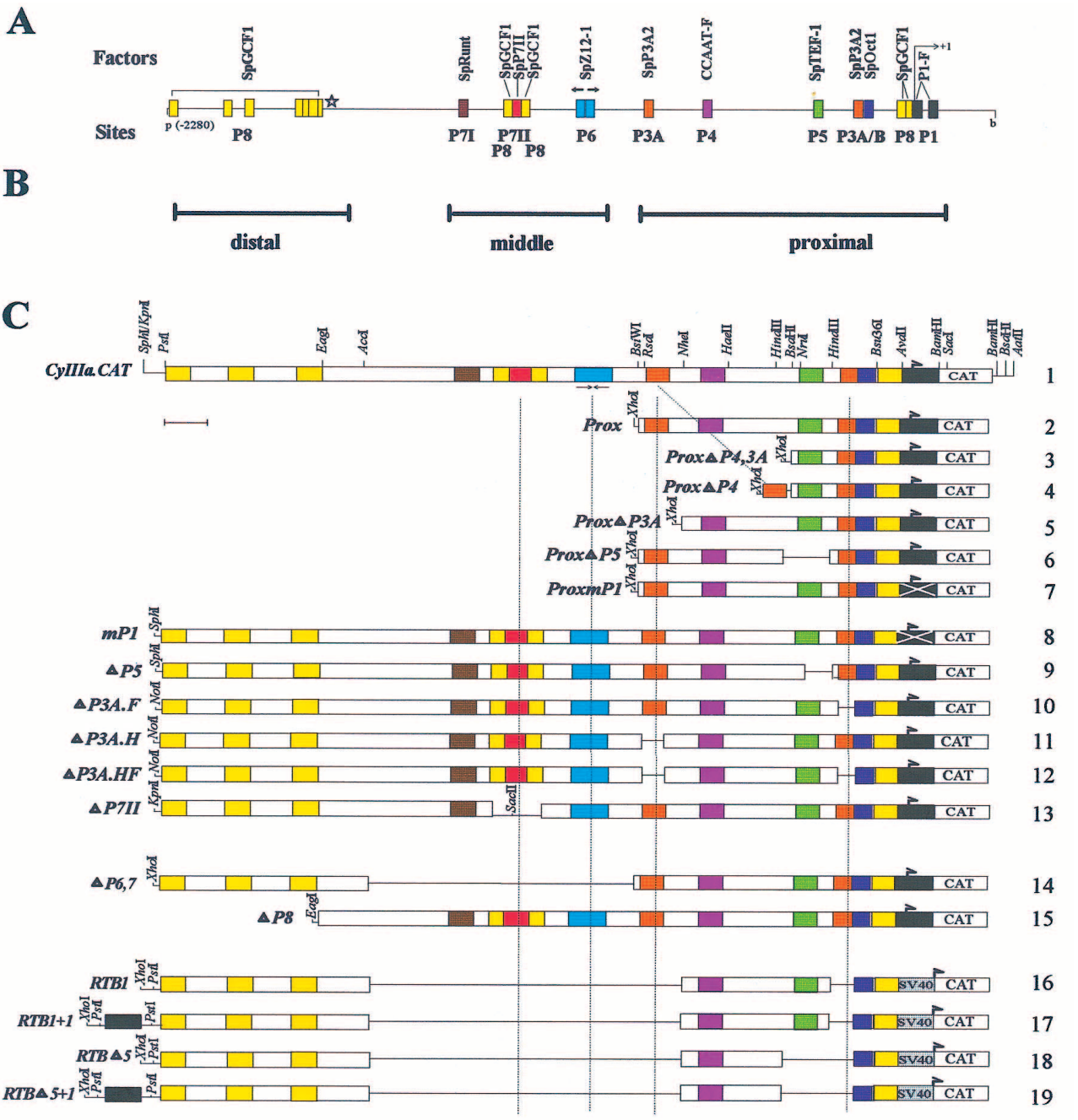
Whole-mount in situ hybridization

Whole-mount in situ hybridizations were carried out according to the method of Ransick et al. (1993), with the following modifications. (1) Embryos were fixed in Streck Tissue Fixative (Streck Laboratories) at 4°C for 1 to 5 days and briefly washed in MFSW, before transferring them into the working buffer. (2) The chromogenic reaction using NBT and BCIP (Sigma) was allowed to develop overnight at 4°C when handling embryos expressing an average of $<5\times10^5$ CAT molecules/embryo. (3) A procedure to enhance staining developed by Barth and Ivarie (1994) was adapted as follows: 5% low molecular mass polyvinyl alcohol (Sigma) was added to the staining buffer, when the amount of CAT protein/embryo was expected to be $1<2\times10^5$ CAT molecules/embryo. Additional rinses with working buffer followed, before stopping the staining reaction as described by Ransick et al. (1993). Embryos were analyzed and images stored with the help of a Roche Instruments imaging system using ProgRes software and a Zeiss Axioskop microscope. Representative examples were printed on a Nikon CP-300 Full Color Printer.

RESULTS

Spatial expression of the control *CyIIIa•CAT* construct

In this as in other recent studies from our laboratory using chloramphenicol acetyltransferase (CAT) reporter fusion genes (Yuh et al., 1994; Makabe et al., 1995; Wang et al., 1995a) we have relied on whole-mount in situ hybridization of CAT mRNA to assess spatial expression, and our initial task was to establish this method for the control *CyIIIa•CAT* construct. This construct is shown diagrammatically in Fig. 1C (line 1). It includes the entire 2.3 kb upstream region studied earlier by radioactive in situ hybridization on sections (see Introduction for references). As illustrated in Fig. 2A, *CyIIIa•CAT* is expressed in large patches of aboral ectoderm both at mesenchyme blastula stage (Fig. 2A1) and late gastrula stages (Fig. 2A2,3). Incorporation of injected



reporter genes in sea urchin embryos is mosaic, and thus only a subset of nuclei of the aboral ectoderm display staining. In about 75% of sea urchin embryos injected, exogenous DNA is incorporated, randomly with respect to cell fate, in the nucleus of one blastomere per embryo, at 2nd, 3rd or 4th cleavage, and the exogenous DNA is subsequently replicated together with the DNA of the host cell lineage. Thus, in most embryos, $\frac{1}{4}\,\frac{1}{16}$th of the cells retain the exogenous DNA (Hough-Evans et al., 1987; Franks et al., 1990; Livant et al., 1991). Table 1 summarizes the results of 13 whole-mount in situ hybridization experiments on *CyIIIa•CAT* expression, each carried out on an average of 10^2 embryos. About 60-90% of embryos developing from injected eggs display staining. Spatial expression is remarkably accurate: on the average only 3.2% of stained embryos display ectopic expression in the oral ectoderm, and 1.3% in the skeletogenic mesenchyme. No ectopic expression in vegetal plate derivatives was ever observed. As we discuss below in detail, assays of CAT enzyme activity show that the time course of *CyIIIa•CAT* expression also faithfully reflects endogenous *CyIIIa* expression (Lee et al., 1986, 1992). Thus, the fusion gene is activated toward the end of cleavage; expression peaks at the late gastrula stage; and it then decreases slowly throughout embryogenesis.

Our objective in the present work was to investigate the nature of each known protein-DNA interaction in the *CyIIIa cis*-regulatory domain, and the functional significance of their arrangement on the DNA. Reporter gene constructs derived

Fig. 1. Schematic illustration of the sea urchin *CyIIIa* cytoskeletal actin gene *cis*-regulatory region and reporter gene constructs used in this study. (A) The 5′ regulatory region of *CyIIIa*. Colored boxes in the regulatory domain of the *CyIIIa* gene, which extends from −2280 to +50 bp, represent oligonucleotide probes that form specific gel shift complexes with nuclear proteins from blastula stage embryos (Calzone et al., 1988; Thézé et al., 1990; J. Coffman and E. Davidson, unpublished data). Restriction sites that flank the whole region are *Bam*HI (b) and *Pst*I (p). The arrow indicates the transcription start site. Sequence elements that act as target sites for transcription factors in vitro are numbered P1 through P8 below the *cis*-regulatory region. The nuclear factors binding to seven of the nine different species of sites have been isolated by affinity chromatography, partially sequenced, and cloned. Their identities are indicated above the DNA, except for the factors that bind at the P1 site and at the CCAAT (P4) site, which have not yet been cloned. SpOct-1 is an octamer factor (Char et al., 1993); SpTEF-1 is an *S. purpuratus* factor homologous to the human enhancer factor TEF-1 (J. Xian and E. Davidson, unpublished data). The CCAAT-binding factor (Calzone et al., 1988; Barberis et al., 1987) could be identical to a factor cloned by Li et al. (1993). SpP3A2 is a protein of novel sequence (Calzone et al., 1991, Zeller et al., 1995c); SpZ12-1 is a zinc finger protein similar to the *Drosophila* Krüppel protein (Wang et al., 1995a);SpRunt is the *S. purpuratus* homologue of the *Drosophila* runt protein (Coffman et al., 1995); SpP7II is a factor with no homologies to other known proteins (J. Coffman and E. Davidson, unpublished data). SpGCF1 is a novel protein that, once bound, has been shown to loop DNA in vitro by multimerizing (Zeller et al., 1995a,b). Direct evidence that the factors indicated are indeed those interacting with the *CyIIIa* target sites in crude nuclear extract and in vivo, is available for SpGCF1, SpP7II, SpRunt-1, SpP3A2, and SpZ12-1, and there is only one gene encoding Octamer binding proteins of POU class in the *S. purpuratus* genome. The assignment of SpTEF-1 is provisional. (B) The *CyIIIa cis*-regulatory region is organized in three functional units, or modules, indicated by brackets. (C) Schematic diagrams of reporter gene constructs used in this study. DNA fragments of the *CyIIIa cis*-regulatory region included in the constructs are shown as boxes. The sequences carrying known protein binding sites are color-coded as in (A) and drawn larger than the actual sites for ease of illustration. Vertical dashed lines denote positions of the binding sites of the negative spatial regulators identified in this work and in previous studies (see text). Fragments that were deleted either by PCR mutagenesis, or by restriction fragment cloning, are shown as solid lines. PCR-generated point mutations of binding sites are indicated by a white X in the respective binding site. The relative positions of the *cis*-regulatory sequences are shown to scale, but the CAT fusion gene is much longer than indicated, and all vector sequences are omitted. The bar indicates 100 bp of the regulatory domain. Restriction sites used to clone reporter gene constructs are indicated in the figure.

from *CyIIIa•CAT* that were used in this work are shown in Fig. 1C. Some of these carry point mutations within, or deletions of single binding sites. Others contain fragments of the *CyIIIa* 5′ upstream region extending over several hundred base pairs. As in the control experiments with *CyIIIa•CAT*, these constructs were introduced into fertilized eggs, and transgenic embryos were collected at the appropriate stage for whole-mount in situ hybridization, and/or assays of CAT enzyme activity.

The proximal regulatory module

The smallest fragment of the *CyIIIa* regulatory domain that faithfully directs CAT reporter expression to the aboral ectoderm extends about 800 base pairs upstream of the start site. This is the construct *Prox* of Fig. 1C (line 2). An embryo expressing *Prox* in aboral ectoderm cells is illustrated in Fig. 2B. Table 1 shows that accurate expression is observed in 96% of stained embryos carrying *Prox* transgenes. The level of expression is significantly lower than in embryos bearing *CyIIIa•CAT*, however, as shown by the decrease in the fraction of embryos generating detectable quantities of CAT mRNA (to about 55%; Table 1, and see below).

In the following we term the region of the *CyIIIa* regulatory domain included in the *Prox* construct the ‘proximal module’ (see Fig. 1B). The proximal module includes target sites for interaction with at least six different transcription factors. Considered together with earlier evidence, the experiments described in the next sections determine the functional significance of each of these interactions.

Interactions at the P3A sites are required to prevent ectopic expression in the oral ectoderm

An in vivo competition study of Hough-Evans et al. (1990) had earlier indicated that interactions at the P3A sites within the proximal module exercise a negative spatial control function in *CyIIIa•CAT* expression. In their experiments, excess DNA fragments bearing P3A target sites were coinjected with the *CyIIIa•CAT* construct, and expression of the latter was observed, by section in situ hybridization, to spread to the oral ectoderm. Competitive interference with none of the other interactions occurring within the proximal module (shown in Fig. 1) caused ectopic expression. To confirm the spatial control function of interactions at the P3A sites using the more sensitive and revealing whole-mount in situ hybridization procedure, and to determine the significance of each of these sites, we constructed the fusions called *Prox*Δ*P4,3A*, *Prox*Δ*P3A*, Δ*P3A.F*, Δ*P3A.H* and Δ*P3A.HF* (Fig. 1C).

Construct *Prox*Δ*P3A* carries the proximal module but lacks the upstream of the two P3A target sites (Fig. 1C, line 5). As Table 1 shows, an average of 28% of embryos bearing *Prox*Δ*P3A* display ectopic CAT mRNA expression in the oral ectoderm, a dramatic (approx. 10×) increase over ectopic expression values for *CyIIIa•CAT* or *Prox*. Embryos bearing *Prox*Δ*P3A* continue to express the transgene in aboral ectoderm at the same rate as do embryos carrying the *Prox* construct. Similarly, when a full-length construct containing all protein binding sites but the upstream P3A site (*ΔP3A.H*; Fig. 1C, line 11) is injected, the reporter gene is expressed ectopically in the oral ectoderm in approx. 28.5% of all animals analyzed (Table 1). A representative whole-mount in situ hybridization of an embryo bearing Δ*P3A.H* and expressing CAT mRNA in oral ectoderm cells is shown in Fig. 2C. The same result is obtained when the upstream P3A site plus the adjacent P4 site are removed from the proximal module (construct *Prox*Δ*P4,P3A*; Fig. 1C, line 3). This construct is also expressed in oral ectoderm (as well as aboral ectoderm), though the level of expression was so low as to make it difficult to obtain the usual numbers of stained embryos (Table 1). The P4 site of interaction (Fig. 1A) is irrelevant for spatial expression, since the percentages of ectopic oral ectoderm expression produced by *Prox*Δ*P3A* and *Prox*Δ*P4,3A* are almost the same (28 and 24%, respectively; Table 1). It follows that removal of the P4 site alone from *Prox* should have no effect on the spatial expression of the reporter gene. To test this assumption, we injected *Prox*Δ*P4*. This construct contains both

Table 1. Whole-mount in situ hybridization of various reporter gene constructs at the mesenchyme blastula or late gastrula stage

Construct	Batch	Stage*	Labeled: not labeled (interpretable)†	% expr.‡	% correct expr.§	% aboral ectoderm expr.¶	% ectopic oral ecto-derm expr.**		% ectopic skeletogenic mesenchyme expr.**		% other ectopic expr.
CyIIIa.CAT	A	G	39:30 (24)	56.5	100	–	0		0		0
	B	G	34:24 (34)	58.6	91.2	–	5.9		2.9		0
	C	G	50:40 (34)	55.6	91.2	–	5.9		2.9		0
	D	G	48:23 (36)	67.6	97.2	–	2.8		0		0
	E	G	98:13 (72)	88.3	97.2	–	1.4		1.4		0
	F	G	55:24 (34)	69.6	91.2	–	5.9	**3.2**	2.9	**1.3**	0
	G	G	72:45 (38)	61.5	97.4	–	2.6		0		0
	H	G	70:28 (67)	71.4	97.0	–	3.0		0		0
	I	G	52:33 (36)	61.2	97.2	–	0		2.8		0
	J	G	55:17 (26)	76.4	96.2	–	3.8		0		0
	I	B	32:10 (30)	76.2	100	–	N/A		0		0
	J	B	34:8 (28)	96.4	N/A	N/A	N/A		3.6		
	K	G	83:28 (72)	74.8	97.2	–	2.8		0		0
Prox	D	G	92:74 (59)	55.4	98.3	–	1.7		0		0
	E	G	106:81 (69)	56.7	94.2	–	2.9	**2.3**	2.9	**1.5**	0
ProxΔP3A	C	G	85:17 (60)	83.3	63.3	95.0	35		1.7		0
	D	G	97:22 (23)	44.3	69.6	91.3	30.4	**28**	0	**3**	0
	E	G	45:18 (27)	71.4	74.1	96.3	18.5		7.4		0
ProxΔP4	C	G	9:71 (9)	11.3	100	–	0		0		0
	D	G	5:96 (5)	5.0	[100]	–	0	**0**	0	**0**	0
	E	G	7:216 (5)	3.1	[100]	–	0		0		0
ProxΔP4,3A	A	G	2:110 (2)	1.8	[50]	[100]	[50]		0		0
	B	G	21:70 (20)	23.1	80	95.0	20		0		0
	D	G	5:83 (5)	5.7	[80]	[80]	[20]	**24.3**	0	**0**	0
	E	G	7:179 (7)	3.8	71.4	100	28.6		0		0
ProxΔP5	D	G	2:282 (2)	0.7	[100]	–	0		0		0
	E	G	0:153 (0)	0	–	–	–		–		–
ΔP3A.F	F	G	57:22 (36)	72.2	80.6	97.2	19.4		2.8		0
	G	G	87:50 (52)	63.5	65.4	96.2	30.8	**25.1**	3.8	**3.3**	0
ΔP3A.H	F	G	74:13 (31)	85.1	83.9	90.3	16.1		3.2		0
	G	G	92:13 (49)	75.4	57.1	98.0	40.8	**28.5**	2.1	**1.1**	0
ΔP3A.HF	F	G	79:18 (36)	81.4	66.7	91.2	30.6		2.2		0
	G	G	106:24 (59)	81.5	67.8	96.6	32.2	**34**	0	**0.5**	0
	H	G	121:75 (97)	61.7	59.8	97.9	39.2		1		0
ΔP5	C	G	100:79 (96)	55.9	92.2	–	2.6		3.9		1.3
	K	G	103:92 (58)	52.8	93.1	–	5.2	**3.9**	1.7	**2.8**	0
ΔP7II	A	G	59:28 (38)	67.8	71.1	94.7	15.7		15.7		0
	I	G	72:22 (41)	76.6	73.2	92.7	17.1	**16.2**	12.2	**12.6**	0
	J	G	100:28 (51)	78.1	78.4	98.0	15.7		9.8		0
	I	B	121:63 (82)	65.8	100	95.1	N/A		0		0
	J	B	83:23 (57)	78.3	98.2	100	N/A	**N/A**	1.8	**0.9**	0
ΔP6,7	J	G	40:82 (27)	32.7	92.6	–	3.7		3.7		0
	K	G	23:22 (20)	51.1	100	–	0	**1.9**	0	**1.9**	0
ΔP8	H	G	71:29 (36)	71.0	94.3	–	3.8		1.9		0
	I	G	42:19 (36)	68.9	97.2	–	2.8	**3.3**	0	**1.0**	0

*Blastulae were collected at 20 to 24 hours, gastrulae at 50 to 54 hours postfertilization.
†Embryos with more than two labeled cells were scored as positive.
‡% of stained embryos = [Σ scored embryos/Σ stained embryos] × 100.
§% of correctly stained embryos = [Σ embryos stained in the aboral ectoderm only/Σ stained and interpretable embryos] × 100.
¶Dashes indicate that the percentage of correct expression and the percentage of aboral-ectoderm staining are identical.
**% of ectopically stained embryos = [Σ embryos stained ectopically/Σ stained and interpretable embryos] × 100.
Abbreviations: N/A, not applicable; expr., expression; G, late gastrula; B, mesenchyme blastula.
Bracketed data denote experiments in which the total number of stained animals is too small to obtain statistically reliable percentages.

P3A sites, but the upstream site is now in a different position, adjacent to the P5 site (see Fig. 1C, line 4). As expected, *ProxΔP4* directs expression exclusively to the aboral ectoderm in all cases analyzed (Table 1). This experiment shows that the exact location of the upstream P3A site of interaction is inconsequential for its function in negative spatial control (compare *Prox*, *ProxΔP3A*, and *ProxΔP4* of Fig. 1C). In construct Δ*P3A.F* (Fig. 1C, line 10), the downstream P3A site is deleted instead. Table 1 shows that this deletion also causes ectopic expression in the oral ectoderm, in 25% of embryos.

Are interactions at both P3A sites required? Experiments with a fusion construct lacking both P3A sites of the full-length construct (Δ*P3A.HF*; Fig. 1C, line 12) show that ectopic expression in the oral ectoderm occurs in only slightly more

Table 2. The role of P1: CAT enzyme activity in blastula-stage embryos and WMISH in gastrula stage embryos*

Experiment	Construct	CAT molecules/ embryo	Enhancement of expression by P1 site	Stained: not stained (interpretable)§	% expression¶	% ectoderm expression**	% aboral:oral expression
A			*Prox* / *ProxmP1*				
1	*Prox*	1.6×10^6		not done	–	–	–
	ProxmP1	N/D	N/A	not done	–	–	–
2	*CyIIIa•CAT*	1.3×10^7		25:12 (22)	67.6	100	95.5:4.5
	Prox	1.33×10^6		27:41 (22)	39.7	100	100:0.0
	ProxmP1	1.07×10^5	12.4	0:87 (0)	0	–	–
	mP1	9×10^6		122:67 (101)	64.6	99	98.0:2.0
3	*CyIIIa•CAT*	9.65×10^6		80:53 (73)	60.2	98.6	98.6:0
	Prox	2.63×10^5		22:51 (20)	30.1	100	100:0.0
	ProxmP1	N/D	N/A	0.65 (0)	0	–	–
	mP1	6.6×10^6		87:71 (73)	55.1	100	98.6:1.4
4	*CyIIIa•CAT*	1.6×10^6		not done	–	–	–
	Prox	1.81×10^5		not done	–	–	–
	ProxmP1	1.92×10^4	9.4	not done	–	–	–
	mP1	1.3×10^6		not done	–	–	–
B			*RTB construct+1* / *RTB construct*				
1	*RTB1*	1.7×10^5†		6:104 (6)	5.5	[50]	–
	RTB1+1	6.5×10^6	3.8	98:108 (70)	47.8	95.5	85.7:41.0
2	*RTB1*	4.5×10^5‡		5.51 (5)	8.8	[60]	–
	RTB1+1	8.3×10^5	1.8	51:80 (41)	38.9	95.1	92.7:48.8
3	*RTB1*	1.68×10^5		5:23 (5)	17.9	[80.0]	–
	RTB1+1	1.76×10^6	10.5	18:21 (17)	46.2	100	88.2:58.8
	RTBΔ5	N/D		0:41 (0)	0	N/A	N/A
	RTBΔ5+1	N/D	–	0:37 (0)	0	N/A	N/A
4	*RTB1+1*	2.48×10^6	–	52:27 (35)	65.8	97.1	91.4:42.9
	RTBΔ5	N/D		0:98	0.0	N/A	N/A
	RTB5+1	2.44×10^5	N/A	22:37 (20)	37.3	95.0	90.0:50.0
5	*RTB1*	7.1×10^5		7:28 (6)	17.1	[67]	–
	RTB1+1	1.8×10^6	2.5	25:23 (22)	52.1	95.0	72.7:36.4
	RTBΔ5	N/D		1:52 (1)	1.9	0	0
	RTBΔ5+1	4.5×10^5	N/A	7:23 (7)	23.3	100	100:42.9

*Blastulae were collected at 20-24 hours, gastrulae at 50 to 54 hours postfertilization.

†1.7×10^6 is 3.9% of the *CyIIIa•CAT* control.

‡4.5×10^4 is 0.1% of the *CyIIIa•CAT* control.

§Embryos with more than two labeled cells were scored as positive.

¶% of stained embryos = [Σ scored embryos/Σ stained embryos] × 100.

**% of embryos stained in the ectoderm = [Σ embryos stained in one or both ectodermal territories/Σ stained and interpretable embryos] × 100; % of embryos stained in the aboral ectoderm = [Σ embryos stained in the aboral ectoderm only/Σ stained and interpretable embryos] × 100; % of embryos stained in the oral ectoderm = [Σ embryos stained in the oral ectoderm only/Σ stained and interpretable embryos] × 100.

Abbreviations: N/A, not applicable; N/D, not detectable.

Bracketed data denote experiments in which the total number of stained animals is too small to obtain statistically reliable percentages.

embryos, 34%, than in the single P3A site deletions. An embryo expressing Δ*P3A.HF* in the oral ectoderm is shown in Fig. 2D. We conclude that interactions at the two P3A sites of the proximal module are required to confine expression to the aboral ectoderm. The factor which binds the P3A sites in embryos at the stages when these observations were made is SpP3A2 (Calzone et al., 1991; Zeller et al., 1995c). This factor is therefore a repressor of *CyIIIa* expression in oral ectoderm, and spatial control of proximal module function across the oral/aboral axis in the ectoderm depends on its negative regulatory function. These interactions, however, do not account for the absence of expression in vegetal plate or skeletogenic mesenchyme lineages, since no ectopic expression in these territories was observed with any of the P3A site deletion constructs.

The positively acting P4 and P5 sites of the proximal module

We noted above that *ProxΔP4* (Fig. 1C, line 4) confers accurate but only low level expression in embryos bearing this transgene, as summarized in Table 1. These results indicate directly that the P4 site functions positively in the proximal module. The in vivo competition experiments of Franks et al. (1990) confirm this positive function in the context of the whole *CyIIIa•CAT* construct. The P4 site is bound by a CCAAT factor, almost certainly the same factor studied by Barberis et al. (1987) since the site is identical to that recognized by their factor at 13/14 base pairs. CCAAT binding factors usually act positively and thus the role of the P4 site in the proximal module of the *CyIIIa* gene is not surprising. Its effect is very strong, however: Table 1 shows that deletion of

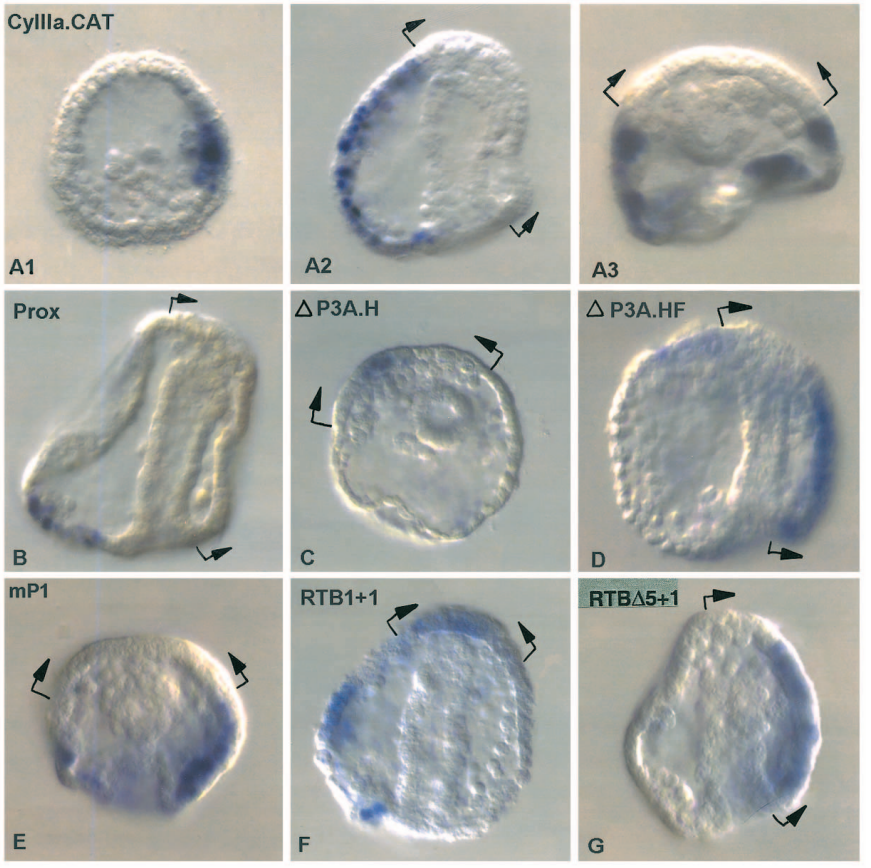


Fig. 2. Spatial expression patterns of various expression constructs, indicated in each panel. The spatial distribution of *CAT* transcripts was detected by whole-mount in situ hybridization, using a digoxigenin-labeled antisense *CAT* RNA probe. Expression domains were analyzed in mesenchyme blastula (approx. 20 hours of age; A1) or late gastrula stage embryos (50-54 hours of age; A2 through G) by rolling each mounted specimen gently under the microscope. At the late gastrula stage, oral ectoderm cells can be distinguished from aboral ectoderm cells in that they are typically thicker and more closely packed than the cells of the squamous aboral ectoderm territory (Cameron et al., 1994), and by their position relative to that of the archenteron, which at this stage makes contact with the oral ectoderm. The approximate extent of the oral ectoderm is indicated in each panel as the region within the bent arrows. The individual gastrulae are shown in one of three orientations, all of which allow distinction between the oral and aboral territories: A2, B, D, G are displayed in lateral view, with the oral side oriented to the right of the tube-shaped gut; A3, C are shown as optical sections through the animal/vegetal axis, with the oral ectoderm above the circular gut, and the aboral ectoderm below. The embryo in (F) is shown in frontal view, with the gut in the center and the oral ectoderm at the tip of the archenteron. (A1,2,3) Embryos expressing the *CyIIIa•CAT* fusion gene (Fig 1C) carrying the entire *CyIIIa cis*-regulatory region. (A1) Lateral view of a mesenchyme blastula stage embryo showing normal ectodermal expression. (A2) A gastrula stage embryo in lateral view, displaying CAT mRNA staining that extends from the upper to the lower oral-aboral ectoderm boundary. (A3) An optical section through a gastrula stage embryo showing normal expression of *CyIIIa•CAT* in patches throughout the aboral ectoderm, which is facing down. The thicker, more compact cells of the oral ectoderm (facing up) are not stained; neither are primary mesenchyme cells, which are oriented in a half-circle lining the oral ectodermal wall, nor the gut, which is the circle in the center of the embryo. (B) An embryo carrying *Prox* reporter constructs, which only contain the proximal module of the *CyIIIa* 5′ upstream region. *Prox* expression can be observed exclusively in the aboral ectoderm. (C) A gastrula expressing *ΔP3A.H*, a construct missing one of the two P3A binding sites. In this embryo CAT transcripts can be seen in a patch of the oral ectoderm. (D) An embryo expressing *ΔP3A.HF*, which carries deletions of both functional P3A binding sites. Both aboral ectoderm and ectopic oral ectoderm expression can be observed. (E) An embryo carrying the *mP1* reporter genes, which lacks functional P1 sites expressing CAT transcripts in large regions of the aboral ectoderm. (F) An embryo expressing the *RTB1+1* reporter gene. CAT transcripts are distributed throughout the ectoderm, both oral and aboral. (G) An embryo expressing *RTBΔ5+1*, in which CAT mRNA is expressed in the oral ectoderm.

the P4 site from the *Prox* construct decreases detectable expression to only about 6% of all gastrula stage embryos analyzed, compared to 56% for the *Prox* construct.

An equally or even more powerful positive function is mediated by the P5 site, which is recognized by a factor of the TEF family, SpTEF-1 (J. Xian and E. Davidson, unpublished data). Almost no expression at all is detectable by whole-mount in situ hybridization in embryos bearing *ProxΔP5* (Fig. 1C, line 6), out of >400 embryos observed (Table 1). However, the effect of the P5 site deletion is less severe in the context of the whole regulatory system. Thus, as can be seen in Table 1, construct *ΔP5* reduces the fraction of embryos expressing detectable CAT mRNA by only about 15% compared to *CyIIIa•CAT*. However, CAT enzyme measurements (data not shown) indicate that the *ΔP5* construct is actually not more than 10-20% as active as *CyIIIa•CAT*; even this lower level of activity, however, falls largely above the threshold for whole-mount in situ hybridization detection. Franks et al. (1990) also concluded by in vivo competition that interactions at the P5 site exercise a strong positive function with respect to *CyIIIa•CAT* expression.

Other data obtained previously, and summarized in the Discussion show that the P3B site, at which an octamer factor (Char et al., 1993) interacts, and the P8 site, at which the SpGCF1 factor (Zeller et al., 1995a,b) interacts, also function positively. We discuss the role of the P1 site in the following section.

Interactions at the P1 site locate expression driven by the proximal module to the ectoderm

In Table 2 we present a series of experiments that illuminate the key role played by the P1 interactions. The two P1 sites exist as direct repeats (Calzone et al., 1988; Thézé et al., 1990), one of which is in the immediate vicinity of the transcription start site, while the other is just downstream, in the transcribed leader sequences of exon 1 of the *CyIIIa* gene (see Fig. 1A). For reasons that become clear in the following, Franks et al. (1990) obtained no significant result by in vivo competition against *CyIIIa•CAT*

using excess DNA fragments bearing the P1 target sites. We found, however, that mutation of these sites almost obliterates the activity of the proximal regulatory module. This is shown in the four experiments of Table 2A: compare the CAT enzyme activity generated by *Prox* to that generated by the construct bearing mutated P1 sites, *ProxmP1* (Fig. 1C, line 7). Unfortunately, *ProxmP1* activity is too low to permit whole-mount in situ hybridization observations. Table 2A also confirms, as expected from the foregoing results, that the *Prox* construct is expressed exclusively in the aboral ectoderm.

Paradoxically, when the same mutation of the P1 target sites is inserted in place of the natural P1 sites in *CyIIIa•CAT*, only trivial effects are observed. This construct, *mP1* (Fig. 1C, line 8), produces only slightly less CAT enzyme than does *CyIIIa•CAT*, and by whole-mount in situ hybridization it is seen to be expressed detectably in almost the same fraction of embryos, exclusively in the aboral ectoderm. An embryo bearing *mP1* and displaying accurate aboral ectoderm expression is illustrated in Fig. 2E, and detailed results are shown in Table 2A (experiments 2, 3 and 4). Since the CAT enzyme comparisons of Table 2 refer to a single developmental time point, the mesenchyme blastula stage (~20 hours), we carried out time course studies of CAT expression driven by *CyIIIa•CAT*, *mP1*, *Prox*, and *ProxmP1*. Results are shown in Fig. 3A. Here it can be seen that *Prox* expression achieves a level of only a few percent of *CyIIIa•CAT* expression after the first few hours following activation of the gene, when *Prox* accounts for a larger fraction of the total activity (~20% at 18 hours). The *ProxmP1* construct is almost inactive throughout development (see normalized data in inset of Fig. 3A). On the other hand, *mP1* is about 70% as active as *CyIIIa•CAT* from the blastula stage onward. This result accounts entirely for the failure of Franks et al. (1990) to observe a significant effect in their in vivo P1 competition experiment. It follows, furthermore, that additional regulatory elements missing from the *Prox* construct are responsible for most (i.e., approx. 70%) of the activity of *CyIIIa•CAT*, and for these regulatory elements, the P1 interactions are irrelevant.

To determine the spatial regulatory significance of the P1 interactions, we utilized the *RTB1* vector described earlier by Makabe et al. (1995). This vector contains the distal and proximal P8 sites and the P4, P5 and P3B sites of the *CyIIIa* gene, linked to an SV40 promoter, and the CAT reporter (see Fig. 1C, line 16). Makabe et al. (1995) showed that *RTB1* acts as a powerful, but spatially neutral enhancer, since when a locator element of a gene expressed exclusively in skeletogenic mesenchyme, *SM50*, is inserted in it, active skeletogenic mesenchyme-specific expression is obtained. Therefore, some or all of the factors interacting at the *RTB1* target sites are present in embryonic territories outside the aboral ectoderm (as well as within this territory); other evidence (see Discussion) in fact indicates that the factors interacting at the P8 site, the P4 site and the P3B site are globally active in the embryo. *RTB1* itself is expressed at a very low level, a few percent or less of *CyIIIa•CAT*, and entirely randomly with respect to embryonic territory, as can be seen in Table 2B. Here five experiments are summarized which demonstrate that when the natural P1 sites of the *CyIIIa* gene are inserted into *RTB1* (construct *RTB1+1*, Fig. 1C, line 17), the level of expression increases 2.5-10 fold, and it thus becomes

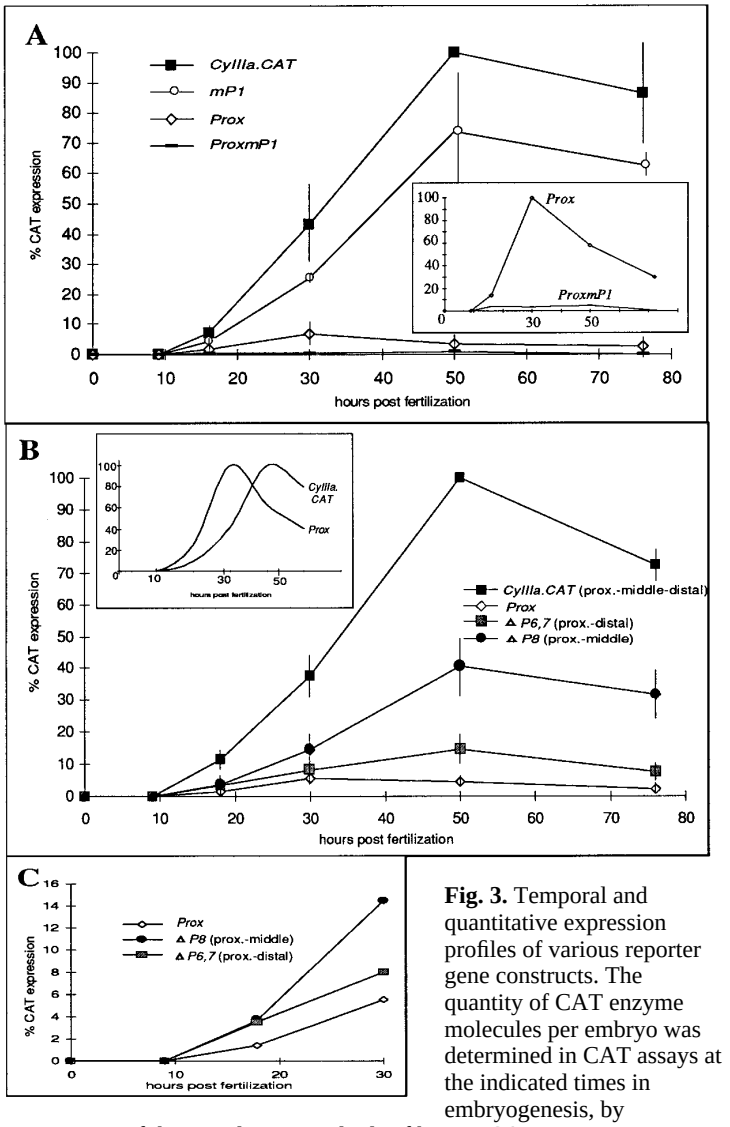


Fig. 3. Temporal and quantitative expression profiles of various reporter gene constructs. The quantity of CAT enzyme molecules per embryo was determined in CAT assays at the indicated times in embryogenesis, by comparison of the samples to standards of known CAT concentration. Data points of each panel were normalized to the time point of highest expression of one construct, as indicated below, and this value was set to 100%. Vertical lines through the data points represent standard deviations, and were calculated after normalization (*n* varies from 3 to 11). Symbols assigned to each construct are indicated in the panels. (A), Time course of *CyIIIa•CAT* expression, compared to expression of *Prox*, *mP1*, and *ProxmP*1 (see Fig. 1C). Data points for each separate experiment were normalized to the peak of expression of *CyIIIa•CAT* at 50 hours in that experiment. Inset: normalized for *ProxmP1* and *Prox* expression, taking the point of maximal activity of *Prox*, i.e., the 30 hour value, as 100%. (B) Time course of *CyIIIa•CAT* expression compared to that of *Prox*, Δ*P6,7* (lacking the middle module), and Δ*P8* (lacking the distal module). Data points from each experiment were normalized to the peak of expression of *CyIIIa•CAT*, i.e., the 50 hour point for that experiment. Inset: Normalized comparison of time course of *CyIIIa•CAT* expression to that of *Prox*. Values obtained in CAT assays were normalized to the respective points of maximal expression of each construct. The lines demonstrating the temporal patterns of the two are a least squares spline fit to the normalized data. (C) Magnification of the early results shown in (B), for constructs *Prox*, Δ*P8*, and Δ*P6,7*.

possible to detect the locus of expression by whole-mount in situ hybridization. Table 2B shows that addition of the P1 sites specifically directs expression to the ectoderm, but to *both* oral and aboral ectoderm. An embryo bearing *RTB1+1* and expressing CAT mRNA in both oral and aboral ectoderm is shown in Fig. 2F. Only background levels of vegetal plate and skeletogenic mesenchyme expression are observed in these experiments. Since it remained possible in the absence of further information that ectoderm specificity could be due in part to interactions at the P5 site, we removed this site, creating the *RTBΔ5* vector (Fig. 1C, line 18). This alteration essentially obliterates background expression of the vector. Weak but detectable expression is obtained when the P1 sites are added back (construct *RTBΔ5+1*, Fig. 1C, line 19), and experiments 4 and 5 of Table 2B show again that the P1 sites direct expression to the oral and aboral ectoderm. Fig. 2G illustrates an embryo bearing *RTBΔ5+1*, and expressing CAT mRNA in the oral ectoderm. The *RTBΔ5+1* experiment eliminates the P5 site as the ectoderm-specific regulatory element. We conclude that the P1 interactions are responsible for the ectoderm specificity of the proximal module; the P1 sites apparently function as an ectoderm locator element in the sense of Makabe et al. (1995).

The spatial control function of the proximal module can now be seen to be determined by both positive and negative interactions. On the animal/vegetal axis, ectodermal expression is specified by the positive P1 interactions, and on the oral/aboral axis it is turned off in oral ectoderm by interaction at the two P3A sites. The remaining interactions in the proximal module are required to enhance expression, and to set the timing of gene expression, a point that we return to in the following section.

Temporal and quantitative control of expression

We have seen that the proximal module accounts for only a small fraction of the total expression profile of *CyIIIa•CAT*. The general location of other regulatory sequences that control timing and amplitude of expression is indicated in the experiments shown in Fig. 3B. This figure summarizes sets of time course experiments comparing the expression of Δ*P6,7*, a construct lacking the whole middle module of the regulatory domain; Δ*P8*, a construct lacking the whole distal module (Fig. 1B and C; lines 14 and 15); *CyIIIa•CAT* and *Prox*. Each experiment was carried out on a single batch of eggs. The following conclusions can be drawn from these experiments, and from the magnified early time course data as replotted in Fig. 3C.

(i) The proximal module suffices to activate the *CyIIIa* constructs at the normal time of endogenous *CyIIIa* activation, though at a lower level than if *either* the middle or the distal modules are present in the construct (see Fig. 3C). We note that constructs lacking the proximal module, and containing only the distal module, the middle module, or both, are entirely inactive (data not shown). This is not due, as we saw earlier, to a requirement for either P1 or P5 interactions, since deletions of these sites in the whole *CyIIIa•CAT* construct decrease but do not obliterate expression. Thus we identify two additional functions of the proximal module: temporal activation of the *CyIIIa* gene early in development; and mediation of the positive functions of the middle module.

(ii) The proximal module responds to regulators that promote peak activity earlier than the peak of expression displayed by *CyIIIa•CAT*. The inset of Fig. 3B shows the relative amplitude of activation, normalized to the maximum of CAT expression of each construct. *Prox* expression reaches a peak at approx. 30 hours postfertilization and then drops dramatically in activity, while *CyIIIa•CAT* reaches a maximum about 20 hours later, at 50 hours postfertilization, and still expresses the reporter gene at 80% of its highest activity by the end of embryogenesis.

(iii) The middle module includes target sites at which the regulators responsible for most of the late rise in activity interact (compare the expression profile of Δ*P6,7* with that of *CyIIIa•CAT* in Fig. 3B), as well as for some of the early activity (Fig. 3C). We show elsewhere that interactions of a runt domain factor, SpRunt-1, at the P7I site of the middle module are required for this function (Coffman et al., 1995).

A positive function for the region carrying the most distal cluster of binding sites in the *CyIIIa cis*-regulatory region was demonstrated earlier (Flytzanis et al., 1987). Zeller et al. (1995a,b) showed that this cluster contains several binding sites for SpGCF1, a protein which can loop *CyIIIa cis*-regulatory DNA in vitro. Fig. 3B and C shows that deletion of the distal module of the *CyIIIa* regulatory system causes an approximately two-fold decrease in overall activity, throughout development. Thus we confirm that the distal module functions positively. Since Δ*P6,7*, i.e., a construct including only the distal and proximal modules, is expressed about two-fold better than is *Prox* alone (Fig. 3B,C), the distal module is able to function synergistically with the proximal module; since *CyIIIa•CAT* is expressed 2- to 3-fold better than Δ*P8* (Fig. 3B), the distal module must also function synergistically with the middle module, because the middle module is responsible for most of the late peak of *CyIIIa•CAT* expression. We could not test the effect of the distal module on the middle module in isolation, since as noted above, some element(s) of the proximal module are required to mediate middle module function.

Fig. 1A shows that SpGCF1 sites are present in both middle and proximal modules as well as in the distal module. The synergistic, positive function of the distal module demonstrated here may in some way depend on establishment of direct contacts between factors bound at the middle and proximal modules, due to multimerization of SpGCF1 bound in these three regions of the *CyIIIa* regulatory domain (Zeller et al., 1995b).

The middle module governs spatial expression after the blastula stage

All of the constructs utilized in the experiments of Fig. 3 are expressed accurately in the aboral ectoderm (for *CyIIIa•CAT*, *Prox*, Δ*P6,7*, Δ*P8*, Table 1; *Prox*, Tables 1 and 2). The distal module has no spatial control function, as shown by the normal expression of Δ*P8*, and by the fact that it simply amplifies whatever spatial pattern of expression is mediated by the downstream regulatory element present (e.g., Δ*P3A.H* and Δ*P3A.HF*, which contain the distal module, and *Prox*Δ*P3A*, which does not). Since *Prox* and Δ*P6,7* are expressed accurately, the middle module appears not to be required for correct spatial expression either. This produces an apparent paradox, however, for earlier work had shown convincingly that if certain interactions in the middle module are disrupted, ectopic spatial expression ensues. Thus, Wang et al. (1995a) demonstrated that deletion or mutation of the P6 site of *CyIIIa•CAT*,

which lie within the middle module (*cf.* Fig. 1A), causes a striking ectopic expression, specifically in the skeletogenic mesenchyme. This effect is only observed in gastrula stage and later embryos, and at mesenchyme blastula stage, expression is strictly confined to the ectoderm. In vivo competition using DNA fragments carrying the P7II target site of the middle module had also been reported to cause ectopic expression of *CyIIIa•CAT* (Hough-Evans et al., 1990).

Five whole-mount in situ hybridization experiments carried out with a deletion at the late gastrula stage of the P7II site are summarized in Table 1. This construct, Δ*P7II* (Fig. 1C, line 13), is indeed expressed ectopically, in both oral ectoderm and skeletogenic mesenchyme (Table 1). Representative stained embryos are shown in Fig. 4 (B1-B4). However, when mesenchyme blastula stage embryos bearing Δ*P7II* were examined, only ectoderm-specific expression was observed (Table 1). At this stage the newly ingressed skeletogenic mesenchyme cells are clearly visible, as illustrated in the stained Δ*P7II* embryos shown in Fig. 4 (A1, A2). Thus, both the P7II and P6 interactions exercise negative spatial control functions that are required for correct *CyIIIa•CAT* expression from the gastrula stage onward, but apparently not earlier.

Removal of these sites by deletion of the whole middle module thus does not cause ectopic expression, while their removal or mutation in the otherwise complete construct does. It follows that the interactions occurring at the P6 and P7II sites negatively control the expression pattern produced by the positive regulators that bind *within* the middle module. We conclude that the middle module has two essential roles that are functionally interrelated: it is responsible for the sharp increase in *CyIIIa* expression occurring in the gastrula stage which is mediated by SpRunt-1; but to confine this late embryonic step-up of expression to the aboral ectoderm, expression must be turned off in the skeletogenic mesenchyme and the oral ectoderm. The functions of the P6 and P7II interactions, in other words, are necessary because SpRunt-1 is apparently active in skeletogenic mesenchyme and oral ectoderm, as well as in the aboral ectoderm. This follows directly from the fact that P6 and P7II deletion or mutation results in expression in all three territories.

DISCUSSION

The *CyIIIa cis*-regulatory system considered as a genetic information processing device

A priori the pattern of expression of the *CyIIIa* cytoskeletal actin gene of *S. purpuratus* would seem rather simple. The gene is activated following segregation of aboral ectoderm lineage founder cells, and its expression is confined to the progeny of these cells throughout embryogenesis. But the complexity of the functional interactions in the *cis*-regulatory domain of this gene belies any notions of simplicity at the level of the mechanism of control. We are aware from earlier work of at least 20 individual sites of high specificity DNA-protein interaction within the *CyIIIa cis*-regulatory sequence, serviced by nine different transcription factors. In the experiments of this paper, and some prior studies, we have tested the functional significance of every target site in the regulatory domain, except for the individual copies of the multiple SpGCF1 sites in the distal and middle regions of the sequence (Zeller et al., 1995a,b). We show that a specific function can be attributed to every interaction; they are *all* biologically meaningful.

Each interaction in the regulatory domain transduces an item of regulatory information presented to the gene in terms of the concentration and activity of the transcription factors which recognize that site. The factors are presented differentially in the various nuclei of the embryo, and at different times in development. We think of the *cis*-regulatory domain as a 'hardwired' information processing *system*, that integrates the functional significance of the individual interactions occurring within it. The 'wiring' of this metaphor denotes the sequence and arrangement of the transcription factor target sites in the *cis*-regulatory DNA. We show how certain of the interactions specified by these target sites bring lineage-specific spatial information to the gene, the initial outcome of the early blastomere specification processes. Other interactions reflect the cell differentiation processes occurring later in development. Still other interactions convey temporal information, and control the amplitude of expression. The detailed biochemical mechanisms by which these factors affect transcription, positively or negatively, are largely unknown, and our focus here is on the regulatory significance of the DNA target sites at which these interactions occur.

We might ask why a terminal downstream gene such as *CyIIIa* carries its own complex information processor, rather than, for instance, to answer to a few positive transcriptional signals the presentation of which is controlled at a higher level of zygotic regulatory hierarchy. One answer is that at least early in development many and perhaps all of the key factors controlling *CyIIIa* expression are maternal, so that zygotic control of their transcription is not an issue. This is known to be true of SpP3A2 (Zeller et al., 1995c); SpGCF1 (Zeller et al., 1995a); SpZ12-1 (Wang et al., 1995b); SpRunt-1 (Coffman et al., 1995); and is probably true of the P1 factor, SpTEF-1, the CCAAT binding factor and SpOct1 (Calzone et al., 1988; F. Calzone and E. Davidson, unpublished data). A more general answer, as suggested earlier (Davidson, 1990) might be that information processing at the gene is a basic and integral feature of developmental genetic programming, irrespective of the maternal or zygotic provenance of the transcription factors.

How the *CyIIIa cis*-regulatory system works

In Fig. 5 we present, in diagrammatic form, a representation of the role(s) of interactions at each target site. These diagrams also specify the interrelations that constitute the integrative functions of the *cis*-regulatory system. The specific experimental results upon which each element of the diagrams rests are indicated in the legend. We here consider only the major features of the system.

Modular organization

The *CyIIIa* regulatory system is modular in the sense that the three clusters of individual target sites that we define as proximal, middle, and distal modules (Fig. 5) display separable regulatory functions, though important interactions also occur between elements of the different modules. The *proximal module* is the smallest subregion of the *CyIIIa* regulatory domain that is capable of autonomously conferring aboral ectoderm specific expression on the reporter gene (Table 1). This module also suffices to cause transcriptional activation at the late cleavage/early blastula stage (Fig. 3C). The *middle*

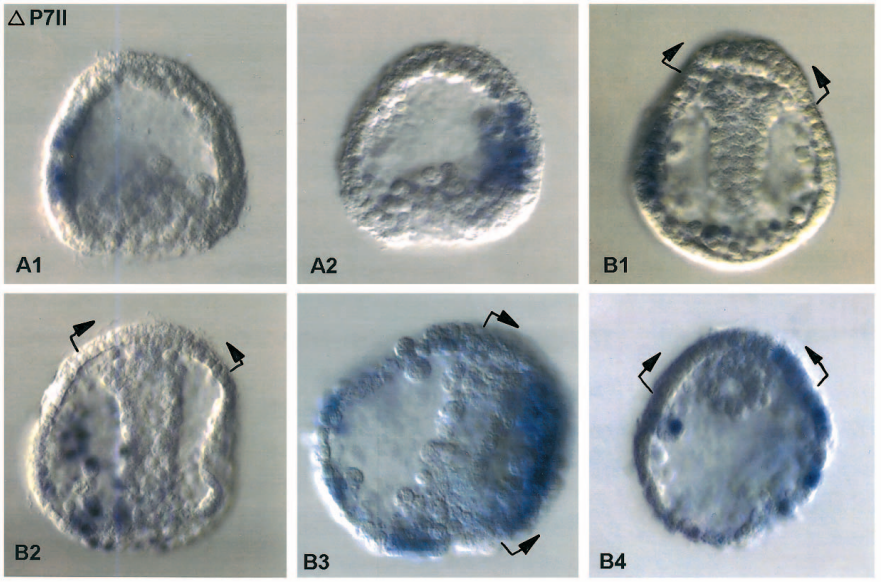


Fig. 4. Expression pattern of the Δ*P7II* reporter construct. The spatial distribution of *CAT* transcripts in mesenchyme blastula (A1,2) or late gastrula stage embryos (B) was determined in whole-mount in situ hybridization experiments, using a digoxigenin-labeled antisense *CAT* RNA probe. Late gastrulae were oriented in frontal view (B1,2), which allows identification of the skeletogenic mesenchyme cells based on their positioning at or around the base of the tube-shaped gut. Other gastrulae are shown in lateral view (B3), or as a transverse optical section (B4). The approximate extent of the oral ectoderm is indicated in each panel as the area inside the bent arrows. (A1,2). Mesenchyme blastula stage embryos displaying large patches of staining in the ectoderm. The skeletogenic mesenchyme cells remain unstained. (B1,2). Gastrula stage embryos showing expression in both ectoderm and skeletogenic mesenchyme cells. (B3) Lateral view of an embryo with a large period expression domain in the oral ectoderm, which is oriented to the right. (B4), Optical section through an embryo with expression in skeletogenic mesenchyme cells (only one of which is seen in the focal plane shown) and in the oral ectoderm.

module is responsible for the sharp increase in expression after the early gastrula stage (Fig. 3B), and it operates an entirely distinct spatial control system than is utilized in the proximal module. The *distal module* is a separable regulatory element, that has no spatial control function of its own. It can be added to proximal or middle module constructs, the expression of which it enhances to a modest degree, irrespective of their spatial domain of expression.

The proximal and middle modules of the *CyIIIa* regulatory system utilize separate positive regulatory interactions, that function only in certain spatial domains of the embryo, in addition to their separate negative spatial control elements. The linchpin of the proximal module is the P1 interaction, and that of the middle module is the SpRunt-1 interaction occurring at the P7I site (see Fig. 5). Without P1 sites the proximal module is almost totally inactive, while this mutation scarcely affects the function of the middle module (Fig. 3, Table 2). On the other hand, mutation of a few base pairs defining the runt domain target within the P7I site obliterates the gastrula stage transcriptional activation function of the middle module (Coffman et al., 1995). The separate negative control mechanisms of these two modules function independently in that they control different positive functions, as indicated in Fig. 5A,B. This explains why interference with any one of these negative interactions in the context of the whole system (i.e., deletion or mutation of the P3A, P6, or P7II target sites of *CyIIIa•CAT*) results in ectopic expression (Table 1; Wang et al., 1995a).

Early embryonic functions of the proximal module

We regard the proximal module as the *cis*-regulatory interpreter of the initial specification process that sets up the spatial territories of the embryo, for the following reasons. (i) The proximal module suffices to initiate transcription in late cleavage when transcription of the *CyIIIa* gene is activated (Fig. 3C). (ii) Its activity peaks earlier than that of the middle module, the whole *CyIIIa•CAT* construct, or the *CyIIIa* gene itself (insert, Fig. 3B; *cf*. Lee et al., 1986, 1992). (iii) The SpP3A2 factor, which controls aboral *vs* oral ectoderm expression by interactions at two sites within the proximal module, is the only factor binding the P3A site that is present at functional nuclear concentrations beyond the cleavage stage (Zeller et al., 1995c); (iv) Neither of the negative spatial regulatory functions of the middle module appear to operate before the end of the mesenchyme blastula stage. Nonetheless, we note that there remains an uncertainty, in that we cannot *directly* show that the P3A sites control oral/aboral ectoderm expression prior to the gastrula stage, because the oral and aboral portions of the ectoderm cannot be distinguished until that stage. Nor can we directly exclude an early repressive role in the oral ectoderm for the P7II interaction, in addition to that of the SpP3A2 interactions.

Temporal control of activation within the proximal module is likely to be due to the appearance, in the embryo nuclei, of a factor binding at the P5 site (probably SpTEF-1; J. Xian and E. Davidson, unpublished data) after mid-cleavage, and/or to the appearance of the P1 factor. Calzone et al. (1989) showed that DNA binding activity at the P5 site increases >50 fold between 7 and 24 hours. We show here that deletion of the P5 site from the proximal module obliterates its function (Table 1), so the nuclear advent of this factor could indeed provide a key switch for gene activation. Exactly the same arguments hold for the P1 factor, which increases in concentration in embryo nuclei >10× between 7 and 24 hours (Calzone et al., 1988), and which is also obligatory for proximal module function. The nuclear levels of factors binding at all the other sites within the proximal module change little over the period when the *CyIIIa* gene is activated, if at all (Calzone et al., 1988).

Later embryonic functions of the middle module

The negative spatial control functions of the middle module are mediated by interactions at the P6 and P7II sites. The factors binding at these sites (SpZ12-1 and SpP7II) act by

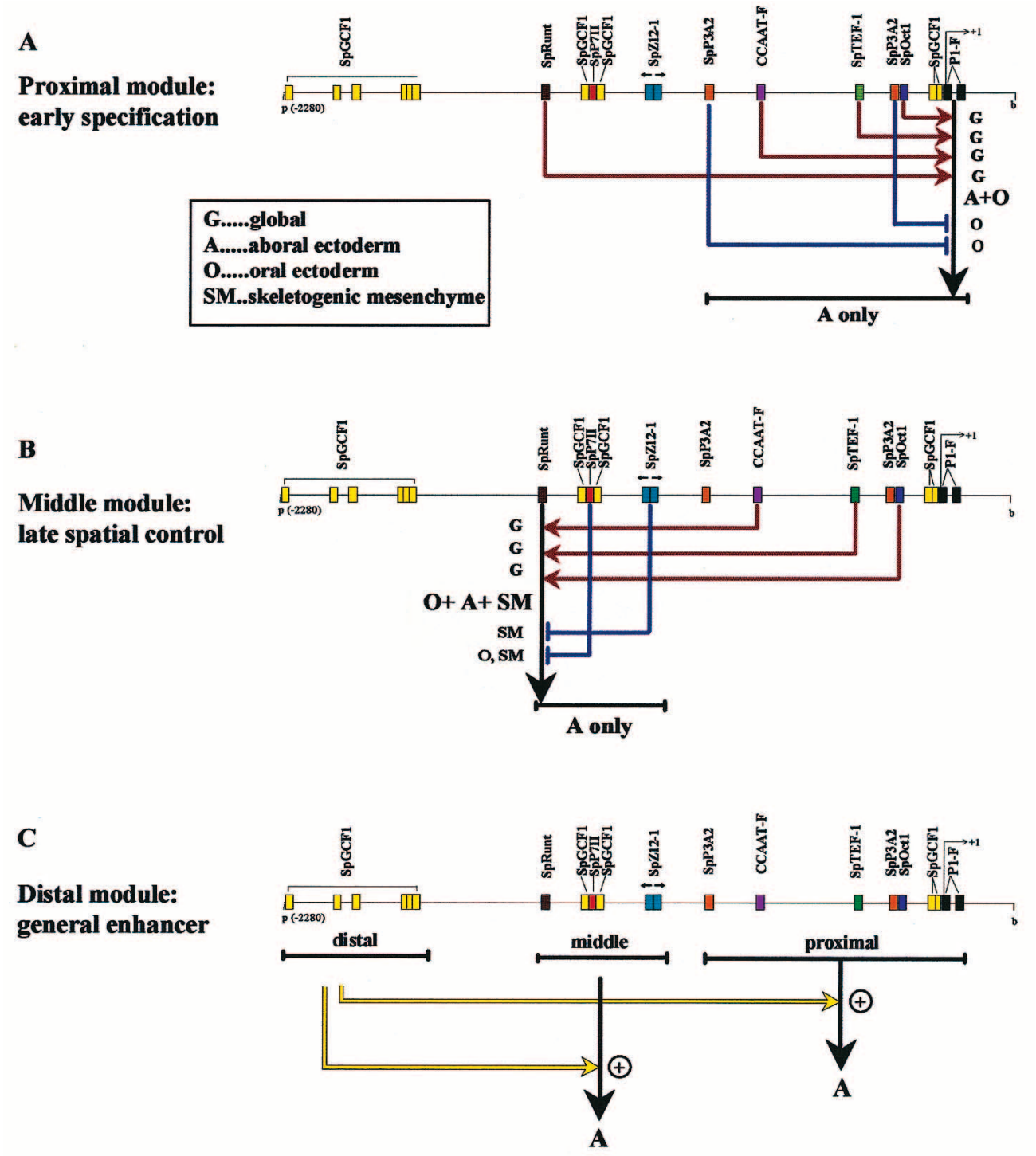


Fig. 5. For legend see p. 346.

repressing the positive functions of the SpRunt-1 regulators binding at the P7I site. Deletion of these sites from *CyIIIa•CAT* causes ectopic expression, in skeletogenic mesenchyme (Wang et al., 1995a) and skeletogenic mesenchyme + oral ectoderm (Table 1), respectively, but if the P7I site is removed as well, as in the Δ*P6,7* deletion (Table 1), only aboral ectoderm specific expression is observed. These negative interactions are examples of repression by 'quenching' (Gray et al., 1994). This term denotes transcriptional repression that functions by interference with the positive effects of activators that are bound at nearby loci in the upstream regulatory sequence.

From the gastrula stage onwards, the middle module is responsible for a much increased level of *CyIIIa•CAT* expression (Fig. 3B), and at the same time the level of SpRunt-1 mRNA rises sharply (J. Coffman, C. Kirchhamer and E. Davidson, unpublished data). It follows that the postgastrular peak of *CyIIIa* transcription depends on the zygotic control of SpRunt-1 transcript levels. The negative spatial regulatory functions of

Fig. 5. Functional interactions amongst individual elements of the *CyIIIa cis*-regulatory region demonstrated in this work. The target sites necessary and sufficient for correct spatial and temporal regulation of the *CyIIIa* gene are indicated as in Fig. 1, in the *cis*-regulatory control region of the gene, which provides the template for interactions with nine different transcription factors, shown over the line representing the DNA in each portion of the figure. Different subsets of these interactions occur at different stages of embryogenesis and in different embryonic blastomeres and cell types, depending on the concentrations of active transcription factors presented in the individual nuclei. In this diagram, all of the interactions of which we have knowledge are shown at once, their functional significance is indicated, and the inferred locations of the respective transcription factor activities in the embryo are denoted (G, A, O, SM; see box). In A and B three kinds of interactions are shown: *black downward arrow*s represent the key regulatory functions in the proximal module mediated by the P1 factor (A), and in the middle module mediated by the SpRunt-1 factor (B), respectively. The black downward arrows indicate functions that if prevented by deletion or mutation of the target sites obliterate the major regulatory activity of the module; and that are mediated by factors spatially confined in the embryo, the activities of which are positive. Red bent arrows indicate ancillary positive functions; i.e., if the target sites for these fractions are deleted there is no change in spatial expression, but a decrease in amplitude of expression in the module indicated by the direction of the arrowheads. Blue bent lines indicate negative functions. These are shown as blocking the activity of the key positive regulators in the indicated domains of the embryo. These functions are revealed by the appearance of ectopic expression in specific domains of the embryo when the respective target sites are deleted or mutated. (A) Functional interrelationships in the proximal module of the CyIIIa regulatory system. As discussed in text, we believe this module to have the general function of interpreting the outcome of the territorial specification process and to set off expression of the *CyIIIa* gene late in cleavage in the aboral ectoderm lineages, and it continues to control expression in these lineages throughout the blastula stage. Evidence utilized in constructing this diagram is as follows: *P1 interaction*: The experiments documented in Table 2 (see text) show that this interaction locates expression to the oral + aboral ectoderm. *SpOct 1 interaction* (P3B site): Franks et al. (1990) showed by in vivo competition that this interaction is a positive one and that it is required for *CyIIIa•CAT* expression. In addition, unpublished data of Hough-Evans and Davidson show that inclusion of the P3B target site is essential for function of any proximal module constructs that include other sites of this module. *SpP3A2 interaction*: Δ*P3A* experiments documented in Table 1 (see text) show that both P3A2 sites are required specifically to repress expression in the oral ectoderm. This interaction occurs in the proximal module since deletion of P3A2 sites in constructs containing only the proximal module give the same results as in constructs containing the whole regulatory domain. *SpTEF1 interaction* (P5 site): *Prox*Δ*P5* experiments (Table 1) show that this site functions positively, and within the proximal module. *CCAAT-F* (P4 site): the *Prox*Δ*P4* experiment in Table 1 shows that this interaction is essential for proximal module functions, and that it acts positively. *SpRunt-1 interaction* (P7I site): this interaction is shown by the experiments of Fig. 3B and of Coffman et al. (1995), to be required for the normal level of expression from the time of *CyIIIa* activation. The latter study localizes the effect shown in Fig. 3B (Δ*P6,7*) to the P7I site. We have no evidence as to how many of the interactions shown in this diagram are mediated by direct contact with the basal transcription apparatus or by interaction with intermediary effectors that do not bind DNA. At one extreme all the factors shown might interact directly, or via effectors, with the basal apparatus; at the other they could all act *via* P1, sites for which lie directly apposed to the start site. (B) *Functional interactions in the middle module. SpRunt-1 interactions* (P7I site): as discussed in the text, the middle module is responsible for the increase in transcriptional activity after gastrulation begins, and this is due to the positive function of its key regulator *SpRunt-1* (Coffman et al., 1995; Δ*P6,7*; Fig. 3B and Table 1). The spatial domain of SpRunt-1 activity is inferred from the regions to which ectopic expression spreads when the target sites for interaction with the negative regulators of the middle module are deleted or mutated. *SpZ12-1 interaction* (P6 site): this interaction was studied by Wang et al. (1995) who showed that it is required to repress expression in post-gastrula embryos, specifically in the skeletogenic mesenchyme. *SpP7II interaction* (P7I site): Δ*P7II* experiments (Table 1), and in vivo competition experiments of Hough-Evans et al. (1990) show that this interaction is required to repress expression in late embryos in oral ectoderm and skeletogenic mesenchyme (however, in the present study we were unable to confirm the report of Hough-Evans et al. of ectopic expression in any vegetal plate derivatives). The ancillary functions of the *CCAAT-F, SpTEF1*, and *SpOct1* interactions shown here are based on the in vivo competitions of Franks et al. (1990) and Hough-Evans et al. (1990), which show that these interactions are required for expression of mesenchyme blastula stage and later, but that they affect only the level of expression and not the spatial domain of expression; and on the Δ*P5* experiment (Table 1), which demonstrates the same thing for the *SpTEF-1* interaction in gastrula stage embryos. In addition, by process of elimination, either the *CCAAT* factor or *SpOct1* interaction (more probably the latter) are *required* for middle module function, in that, in the absence of the proximal module the middle module is inactive. This cannot be due to P1, SpP3A, SpTEF1 or SpGCF1 interactions (see text). (C) Functional interrelations of the distal module. The results of experiments shown in Fig. 3B,C show that the distal module is responsible for increasing the level of activity, both early and late in development, as implied by prior evidence as well (Flytzanis et al., 1987; Franks et al., 1990). The distal module carries out no spatial control function (Table 1, ΔP8 experiment; Hough-Evans et al., 1990; see text). Zeller et al. (1995b) showed that the factor binding to the multiple sites of the distal module, SpGCF1, multimerizes and loops *CyIIIa* regulatory DNA in vitro. They proposed that its function is to promote direct communication between the modules, accounting for the functions of the SpGCF1 (P8) target sites within the proximal and middle modules.

the middle module are evidently required to prevent the *CyIIIa* gene from responding to this newly produced SpRunt-1 factor in oral ectoderm and skeletogenic mesenchyme cells. Thus, the switch from proximal to middle module also represents a switch from control by positive factors of maternal origin to control by a positive factor of zygotic origin.

Specification of the oral/aboral axis and the spatial control of *CyIIIa* expression

In regularly developing sea urchin embryos the term ‘axis’ serves merely as a semantic device, for there is no process of ‘axis formation’ other than those that lead to specification of the territorial lineage founder cells. In the future ectodermal territories the first of these to arise are the **No** and **Na** oral and aboral founder cells, which appear on opposite sides of the embryo, at third cleavage. Any position on the circumference of the egg may be included in either of these cells, so far as is known, though this is difficult to establish unequivocally. In *S. purpuratus* the oral/aboral axis, i.e., the line extending from the center of the **Na** to the center of the **No** cell, is established

with reference to the plane of first cleavage (Cameron et al., 1989b) and its position is indicated by a cytoskeletal polarization that can be recognized early in cleavage by redox stains (Czihak, 1963; Cameron et al., 1987, 1989b; Davidson, 1989). The first two cleavages occur orthogonally to one another, intersecting in the animal/vegetal axis, which is prefixed in the cytoarchitecture of the egg before fertilization, and cannot be reversed experimentally. In contrast, the polarity and position of the oral/aboral axis is highly plastic, at least during the cleavage cycles that precede *CyIIIa* gene activation. A variety of experimental operations have been described that result in respecification of specific oral/aboral progenitor cells (see Hörstadius, 1973; Davidson, 1989 for review). Furthermore, the specification of the remaining oral and aboral ectoderm founder cells which segregate at 5th and 6th cleavages, is clearly dependent on intercell signaling (Davidson, 1989; see Cameron et al., 1987, 1990, for lineages). Somehow the regulatory system of the *CyIIIa* gene must recognize and respond to the conditional specification processes that determine the quadrants of egg cytoplasm inherited by the oral and aboral lineages, for shortly after the establishment of these lineages, the gene is activated exclusively in the aboral lineages.

In Fig. 6 we summarize our understanding of the mechanism by which expression of the *CyIIIa* gene is confined to progeny of the aboral ectoderm founder cells. We have concluded that interactions at the P3A sites of the proximal module are required for discrimination against expression in the oral ectoderm territory, and that the factor responsible is probably the maternal SpP3A2 repressor. Since the oral pole of the early embryo can be anywhere on the circumference, SpP3A2 must be everywhere (at least in the ectodermal territory) to start with, and become functional as a repressor in the oral lineages during specification. Physical prelocalization of the factor could not account for the localization of SpP3A2 repressive activity, since oral/aboral assignments can be reversed. Activation of the repressor could depend on its covalent modification in response to the conditional specification processes that must establish the boundaries of the ectodermal territories (Davidson, 1989). In fact, we have observed at least five variants of SpP3A2, probably different phosphorylation derivatives, but their localization in the egg is not yet known (Harrington et al., 1992). In Fig. 6 we symbolize active SpP3A2 repressor by closed circles, and inactive SpP3A2 by open circles.

Since we found no mutation or deletion that caused expression in vegetal plate derivatives, or in the skeletogenic mesenchyme prior to ingression, the key positive regulator of the proximal module must be active only in the ectodermal territories. The distribution of the activity of this regulator thus reflects the positions of future ectodermal fate along the prespecified animal/vegetal axis (Fig. 6, hatched lines). We show that this regulator is the factor binding to the P1 site, since as demonstrated in the experiments of Table 2, this site has the capacity to confer oral + aboral ectoderm expression when added to neutral enhancer constructs. Furthermore, the other positive regulatory factors binding in the proximal module are all known to be active elsewhere, and thus their activity cannot be localized in the embryo: SpOct1 and the CCAAT binding factor activate histone genes, which are globally expressed (Char et al., 1993; Barberis et al., 1987); the proximal module target sites (minus the P1 and P3A sites) also function to promote skeletogenic mesenchyme-specific gene expression when placed under control of the *SM50* locator sequence; and the SpGCF1 binding sites (i.e., P8 site) are also present in the *Endo16* regulatory system, which operates in the vegetal plate. Furthermore the experiments with the *RTBΔ5+1* construct show that the P5 site is not required for the process that locates expression in the ectoderm. The model in Fig. 6 thus illustrates the spatial *trans* information, in the form of transcription factor activity distributions, that the *CyIIIa cis*-regulatory system interprets as development begins.

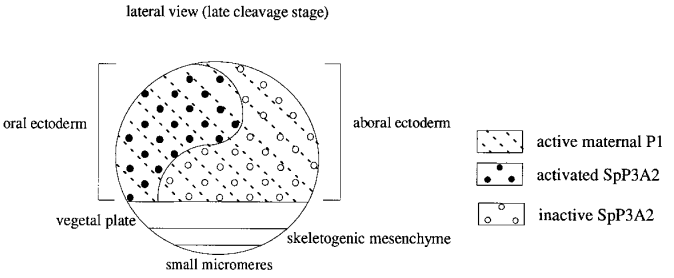


Fig. 6. Model for axial specification and institution of territorial *CyIIIa* expression in the aboral ectoderm. Spatial patterns of differential gene expression are established during the cleavage stage. At this time five embryonic territories are specified: *viz.* the primary skeletogenic mesenchyme, the vegetal plate, the oral ectoderm, the aboral ectoderm and the small micromeres, which play no significant role in embryogenesis. The approximate spatial arrangement of these territories in the late cleavage embryo is indicated by thin lines (cell boundaries are not shown; see Cameron and Davidson, 1991 for summary of territorial lineages). The key positive regulator to which the *CyIIIa* gene responds early in development is the P1 factor, as shown in this work. Maternal P1 factor (Calzone et al., 1988) is indicated throughout the oral and aboral ectodermal territories by the diagonal dotted lines. We propose that this factor is localized or is active *ab initio* in the regions of the egg that will be inherited by ectodermal founder cells; it is thus distributed with respect to the preformed animal/vegetal axis. Polarization of the ectoderm, resulting in the specification of oral *vs.* aboral ectodermal founder cells, occurs after fertilization (Davidson, 1989; Cameron et al., 1990). We show here that in the proximal module of the *CyIIIa* gene *cis*-regulatory system, the key regulator that detects oral/aboral polarity is a repressive factor binding to the P3A site. This is the transcription factor SpP3A2 (Calzone et al., 1990; Zeller et al., 1995c). SpP3A2 is also a maternal factor. Since any point on the circumference of the egg can be the oral pole, SpP3A2 must be present in both the oral and aboral ectodermal territories, as indicated by the small circles. We propose that an outcome of the cleavage stage specification functions is to activate SpP3A2 in the future oral ectoderm (closed circles), and/or inactivate it in the future aboral ectoderm (open circles).

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