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Ontogenic expression of a *Cyl* actin fusion gene injected into sea urchin eggs

KAREN S. KATULA*, BARBARA R. HOUGH-EVANS, ROY J. BRITTEN and ERIC H. DAVIDSON

Division of Biology, California Institute of Technology, Pasadena, California 91125, USA

* Present address: Biology Department, West Virginia University, Morgantown, West Virginia 26506, USA

Summary

The 5′ terminus of the *Cyl* actin gene transcription unit of *Strongylocentrotus purpuratus* was located by primer extension and other procedures, and the flanking upstream region was partially sequenced and mapped. A fusion gene was constructed containing about 2·5 kb of 5′ flanking sequence, the transcribed leader sequence, and the first few codons of the *Cyl* gene ligated to the bacterial gene coding for chloramphenicol acetyl transferase (CAT). This was microinjected into the cytoplasm of *S. purpuratus* eggs, and CAT enzyme activity was measured at various stages of embryonic development. CAT synthesis was activated between 10 and 14 h postfertilization, the same time at which newly synthesized transcripts of the endogenous *Cyl* gene first appear. The exogenous CyI·CAT fusion DNA replicated actively during cleavage, as observed previously for other DNAs injected into sea urchin egg cytoplasm. Thus the absence of CAT activity prior to 10 h postfertilization could not be due to insufficient CyI·CAT genes. The amounts of CAT enzyme produced by embryos bearing CyI·CAT deletions that lack various regions of the *Cyl* sequence were measured. As little as 254 nucleotides of upstream *Cyl* sequence suffice for correct temporal activation of the fusion construct, although the level of CAT enzyme produced in embryos bearing any deletion retaining <850 nucleotides of upstream sequence was significantly lowered compared to controls bearing the complete CyI·CAT fusion construct.

Key words: gene transfer, cytoskeletal actin, developmental regulation, sea urchin, *Cyl*.

Introduction

Cloned genes microinjected into the cytoplasm of sea urchin eggs by the methods described previously (McMahon *et al.* 1985; Flytzanis *et al.* 1985) may be expressed appropriately during embryonic development. Most studies to date have been carried out with histone genes and with the *CyIIIa* cytoskeletal actin gene of *Strongylocentrotus purpuratus* (Davidson *et al.* 1985; Flytzanis, Britten & Davidson, 1986, 1987; Hough-Evans *et al.* 1987). The *CyIIIa* gene is normally activated at 10–12 h postfertilization in this species (Shott, Lee, Britten & Davidson, 1984; Cox *et al.* 1986) and exclusively in precursors of aboral ectoderm cells, to which its transcripts are confined throughout embryonic development (Angerer & Davidson, 1984; Cox *et al.* 1986). We showed earlier that after injection into unfertilized eggs a fusion gene construct containing *CyIIIa* 5′ flanking sequences ligated to sequences coding for the bacterial enzyme chloramphenicol acetyl transferase (CAT) is regulated normally, at least to a first approximation. That is, CAT enzyme begins to be synthesized in embryos deriving from the injected eggs at the stage of development when the endogenous *CyIIIa* gene is activated and there ensues at least a 100-fold accumulation of CAT enzyme protein (Flytzanis *et al.* 1987). The amount of CAT mRNA produced is at maximum a few times greater than the normal amount of CyIIIa mRNA. Moreover, in *S. purpuratus* embryos bearing the CyIIIa·CAT fusion gene, CAT mRNA is observed only in the aboral ectoderm cells (Hough-Evans *et al.* 1987).

In this paper, we describe experiments carried out with another CAT fusion, containing putative regulatory elements of the *Cyl* rather than the *CyIIIa* cytoskeletal actin gene. Our objects were to determine whether correct temporal regulation would

again be observed on injection into *S. purpuratus* egg cytoplasm of a construct including *CyI* control sequences; and to locate these sequences in the gene map, at least to a coarse level of resolution. This initially required the determination of the 5′ end of the *CyI* gene, which had not previously been reported. The *CyI* gene is activated at about the same stage of development as is the *CyIIIa* gene (Shott *et al.* 1984; Cox *et al.* 1986). However, by *in situ* hybridization it has been demonstrated that CyI actin message is present in a different set of cells, which in late embryos is essentially complementary to the set of cells expressing the *CyIIIa* gene. Thus at the pluteus stage *CyI* transcripts appear in the oral ectoderm and much of the gut, regions which are devoid of detectable *CyIIIa* transcripts, and *CyI* transcripts are absent from the aboral ectoderm where are located all of the *CyIIIa* transcripts (Angerer & Davidson, 1984; Cox *et al.* 1986). At the early blastula stage, when newly synthesized transcripts of both genes first appear, the CyI RNAs accumulate in all regions of the embryo. In contrast, at this stage newly synthesized *CyIIIa* transcripts are asymmetrically confined to the precursors of the future aboral ectoderm, then located on one side of the spherical embryo. *CyI* transcripts are also found in primary and secondary mesenchyme cells in gastrula-stage embryos, but ultimately disappear from these cell types, as they do from the presumptive aboral ectoderm. In respect to the succession of cell types in which the gene is activated, the spatial pattern of *CyI* gene expression is thus completely distinct from that of the *CyIIIa* gene. It has been pointed out (Lee *et al.* 1984; Shott *et al.* 1984) that the *cis*-regulatory *CyI* sequences are likely to be located in the vicinity of the gene or within it, since this gene is closely linked to another cytoskeletal actin gene, *CyIIa*, which displays a third pattern of lineage-specific cellular expression in the embryo (Cox *et al.* 1986). The cytoskeletal actins encoded by these differently utilized genes may perform diverse functions within the embryonic cells. Thus from the spatial and temporal distribution of its transcripts Cox *et al.* (1986) surmised that the *CyI* gene produces a cytoskeletal actin required in dividing cells, while the CyIIIa actin is believed to contribute to the structural characteristics of the relatively rigid, single cell thick aboral ectoderm wall of the larva (Akhurst *et al.* 1987). The important point for our present purposes is that although the time course of activation of the *CyI* and *CyIIIa* genes is similar, at least some of the molecular regulatory signals to which they respond must be distinct, since at any given stage they are expressed in different sets of cells.

Materials and methods

DNA sequencing

The DNA sequences reported were obtained by the dideoxy chain termination method (Sanger, Nicklen & Coulson, 1977) using the single-strand DNA phage M13 vectors mp10 and mp11 (Messing, Crea & Seeburg, 1981).

S1 nuclease protection assays

The various DNA fragments chosen for S1 analysis were subcloned into mp10 and mp11 vectors so that the strand complementary to the RNA could be transcribed. Preparation of the single-strand probes was essentially as described by Hu & Messing (1982), and S1 nuclease digestions were carried out by a method slightly modified from Berk & Sharp (1977). Details of these procedures as utilized in this work can be found in Akhurst *et al.* (1987).

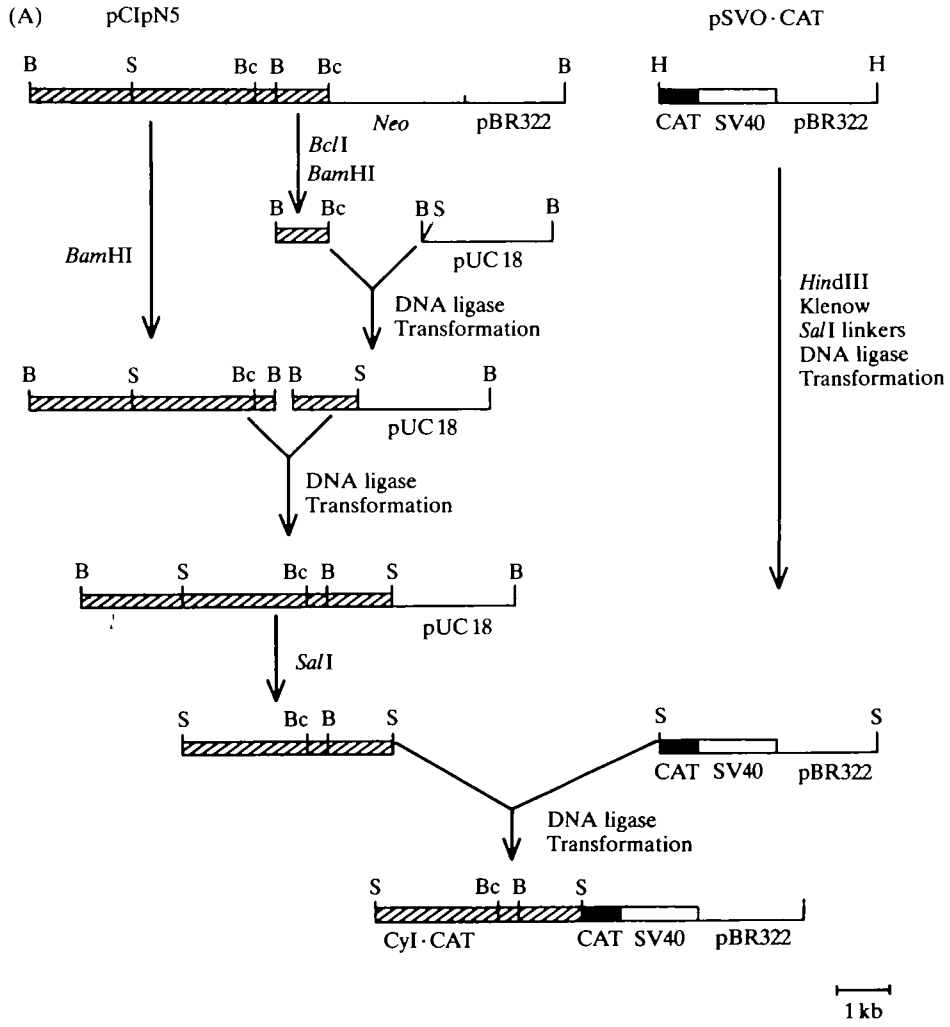
Primer extension

The 25 nucleotide (nt) primer used for the primer extension reaction was synthesized in the Caltech Microchemical Facility. The primer was labelled with ^{32}P at the 5′ end using T4 polynucleotide kinase (IBI). The primer fragment was gel purified on an 8 M-urea, 10% acrylamide gel (19:1 acrylamide:bisacrylamide). The labelled primer was hybridized to 5 μg of poly(A)-containing RNA isolated from 48 h embryos, or to 5 μg of yeast tRNA, in a total volume of 10 μl, consisting of 100 mM-Tris, pH 8·6, 10 mM-MgCl, 140 mM-KCl, and 20 mM-β-mercaptoethanol. After hybridization overnight at 37 °C, the reactions were diluted to 25 μl in the same buffer, containing 30 units of RNasin (Promega Biotec), 15 units of reverse transcriptase (Life Sciences, Inc.), 10 mM each of deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), and 1 μg of actinomycin D. After incubation at 37 °C for 1 h, the samples were extracted with phenol and with Sevag solution, (24:1 chloroform:isoamyl alcohol) and precipitated at −20 °C. The precipitated samples were spun down in a microfuge, washed with 70% ethanol and with 95% ethanol, and then air dried. The samples were resuspended in 6 μl of sequencing gel buffer (90% formamide, 10 mM-NaOH, 10 mM-EDTA, 0·25% bromophenol blue, 0·25% xylene–cyanol) and loaded on an 8 M-urea–10% acrylamide gel for electrophoresis.

Construction of the CyI·CAT fusion gene

CyI 5′ upstream sequences were obtained from the plasmid pCIpN5 (Fig. 1), which includes about 6 kb of *CyI* sequence 5′ to the CyI ATG signal, plus 42 nt of actin coding sequence fused to the gene for neomycin phosphotransferase. The original sources of the sea urchin *CyI* sequences were a lambda recombinant isolated from a sea urchin genomic library (Scheller *et al.* 1981) and a plasmid recombinant (Durica, Schloss & Crain, 1980). The CyI·CAT fusion gene was constructed as outlined in Fig. 1A. The *Hin*dIII site located 31 nt upstream from the CAT ATG in the plasmid pSVO·CAT (Shott-Akhurst, Calzone, Britten & Davidson, 1984) was changed to a *Sal*I site by digestion with *Hin*dIII, and ligation to *Sal*I linkers, after extension of the recessed termini with Klenow polymerase I. The indicated *Bam*HI–*Bcl*I CyI fragment of pCIpN5 was subcloned into the pUC18 vector, and the *Bam*I–*Bam*HI

fragment containing the remaining sea urchin sequences was then cloned into the *Bam*HI site of this plasmid. The presence of a *Sal*I site in the polylinker adjacent to the *Bcl*I–*Bam*HI ligation site made it possible to remove from this plasmid a 4·4 kb *Sal*I fragment which, as shown below, contains about 2·5 kb of 5′ flanking sequence upstream of the CyI cap site. This fragment was isolated and ligated into the *Sal*I site of the altered pSVO·CAT in the proper orientation. The resulting construct, called CyI·CAT, contains the 2·5 kb 5′ flanking sequence, exon I of the *CyI* gene, the entire 5′ leader intron and 42 nt of actin-coding sequence derived from exon II of the gene. The fusion between the *CyI* sequences and the CAT coding region inserted a sequence between the CyI ATG and the CAT ATG 96 nt in length. Thus if translation were to initiate at the CyI ATG, the CAT protein would contain an additional 33 amino acids, which would not be expected to decrease the enzymatic activity of the protein (Schottel, Sninsky & Cohen, 1984). The region between the two ATG's was sequenced to verify that the proper reading frame for the CAT protein would be initiated if translation does start at the CyI ATG, as shown in Fig. 1B.



(B)

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...ATG TGT GAC GAC GAT GTT GCC GCT CTT GTC ATC GAC AAC GGA TCT GAT CCT CTA GAg tcg
   Met Cys Asp Asp Asp Val Ala Ala Leu Val Ile Asp Asn Gly Ser Asp Pro Leu Glu Ser

acg agc ttg gcg aga ttt tca gga gct aag gaa gct aaa atg ... CAT protein
Thr Arg Leu Ala Arg Phe Ser Gly Ala Lys Gly Ala Lys Met
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Fig. 1. Construction of the CyI·CAT plasmid. (A) Flow diagram of the steps used to generate the CyI·CAT plasmid. The restriction enzyme sites shown are *Bam*HI (B), *Sal*I (S), *Bcl*I (Bc), *Hin*dIII (H). The hatched areas indicate sea urchin sequences. Details of the construction are given in Materials and Methods. (B) Sequence of the CyI·CAT junction. A small fragment containing the junction between the CyI ATG and the CAT ATG was subcloned into the vector M13mp10 and sequenced. This sequence is shown along with the encoded amino acids. Capital letters represent CyI sequence and small characters the CAT sequence.

Injection of sea urchin eggs and CAT assays

Preparation and injection of sea urchin eggs were carried out as described by McMahon *et al.* (1985). The fertilized eggs were cultured at 15 °C and collected at the appropriate developmental stage by centrifugation for 5 min in a microfuge. The embryo pellets were taken up in 100 μl of 250 mM-Tris, pH 8·0, and to each sample approximately 1500 24 h noninjected embryos were added as carrier. The embryos were lysed by three consecutive freeze–thaw cycles using an ethanol–dry ice bath, and heating for 3 min at 65 °C. Half of the lysate was removed and stored at −70 °C for DNA determination. The remaining lysate was spun for 15 min in a microfuge. The supernatant was removed and assayed for CAT enzyme activity essentially as described by Gorman, Moffat & Howard (1982), with some minor changes (McMahon, Novak, Britten & Davidson, 1984). The final reaction, totalling 150 μl, contained 0·5 μCi of [^{14}C]chloramphenicol (45 mCi mmol^{-1}, Amersham) and 0·53 mM-acetyl CoA (Sigma). Bacterial CAT (Pharmacia P-L Biochemicals) was utilized to provide a standard curve. Assays were run for 2 h at 37 °C and the acetylated products were separated on an Eastman Kodak silica gel TLC plate. The TLC plates were exposed to Kodak XAR5 film at −70 °C. The calculated percent acetylation was estimated by scintillation counting of appropriate regions of the TLC assay plates. The number of enzyme units present in each sample was calculated by reference to the bacterial CAT standard curve. One unit of bacterial CAT enzyme activity represents approximately $2{\cdot}6\times10^{11}$ molecules of relatively pure enzyme protein (McMahon *et al.* 1984).

CAT DNA determination

An equal volume of 0·1 M-EDTA Tris, pH 8·0, containing 1 % SDS was added to the other half of the lysate for each sample. Proteinase K was added to a concentration of 20 μg ml^{-1}, and the samples were incubated at 55 °C for 2 h. Following extractions with phenol and Sevag solution, one fifth the volume was removed and diluted four times with DAPI buffer (DAPI buffer is 100 mM-NaCl, 10 mM-EDTA, 10 mM-Tris–HCl, pH 7·0). The total amount of DNA in the sample was then determined fluorometrically by the DAPI method (Brunk, Jones & James, 1979). The remainder of the solution was brought to 0·4 M-NaOH and heated for 1 h at 65 °C. This treatment effectively hydrolyses the RNA in the sample and denatures the DNA. The samples were neutralized and filtered onto nitrocellulose using the Schleicher and Schuell minifold II apparatus. Prior to hybridization, the experimental slots were cut exactly in half. One half was hybridized to a uniformly labelled single-stranded RNA probe consisting of CAT gene sequences transcribed *in vitro* from an Sp6 vector. The other half was hybridized to a single-copy probe specific to the 3′ non-coding trailer sequence of the *CyI* actin gene (Lee *et al.* 1986), also transcribed from an Sp6 vector. This serves as a hybridization efficiency standard, since the amount of DNA present is known (see above), and there is a single *CyI* gene per haploid genome (i.e. per 0·8 pg of DNA). The number of CAT DNA molecules calculated per half slot was then converted to *average* CAT DNA molecules per embryo.

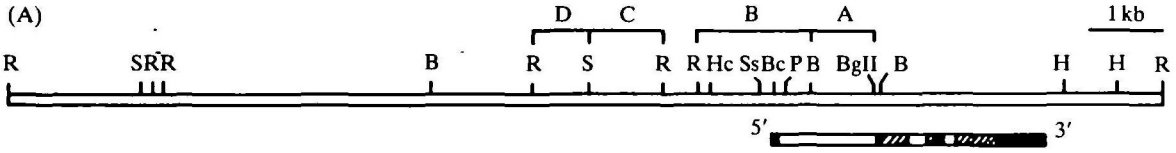
Results

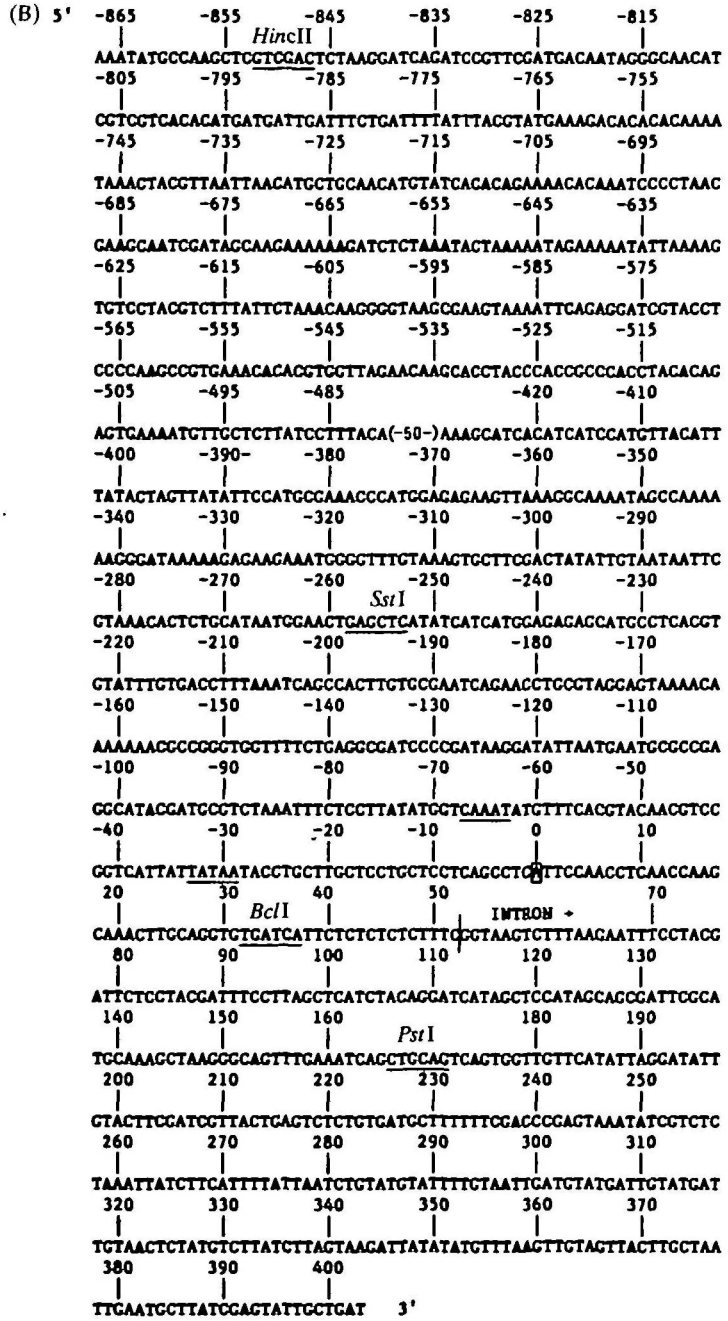
Structure of the CyI *gene*

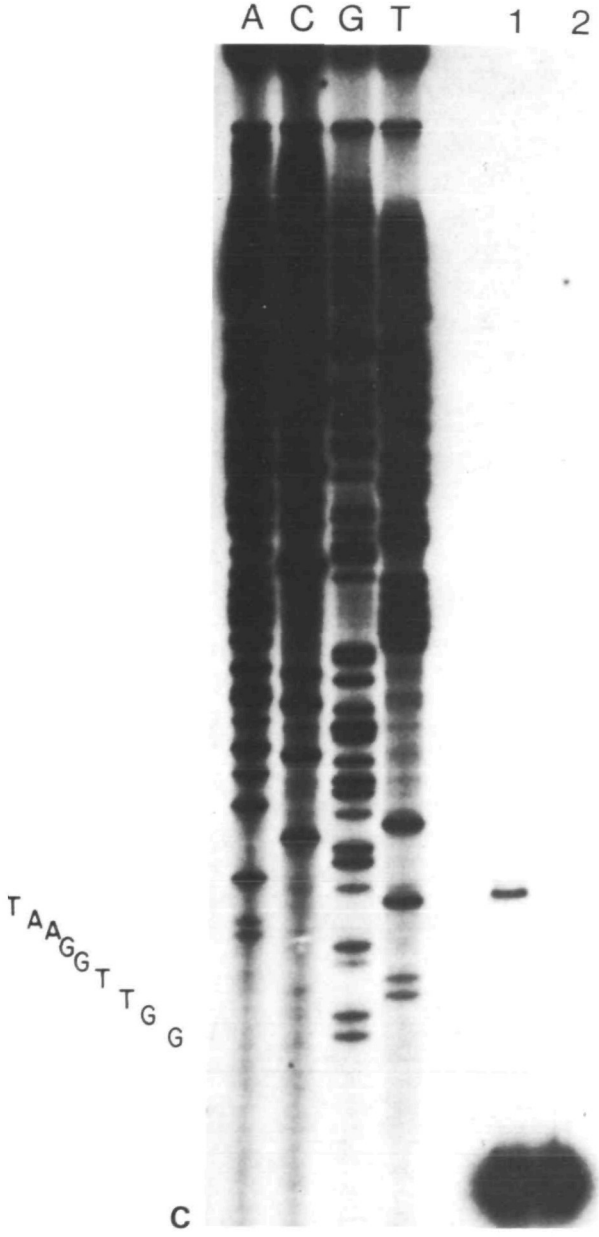
Though the complete nucleotide sequence of the coding region of the *CyI* gene had been reported by Cooper & Crain (1982), the location of the transcriptional start (cap) site, the organization of the 5′ end of the gene and the sequence of the 5′ flanking region were not known. A map of this region is shown in Fig. 2A. Restriction fragments A–D indicated on this figure were subcloned into M13 vectors and single-stranded DNA probes synthesized from these recombinants were reacted with embryo RNA and then treated with nuclease S1 (data not shown). These preliminary experiments indicated that the transcript begins in fragment B, which on reaction with RNA yielded a short protected subfragment similar in length to the leader exon that had previously been observed in the *CyIIIa* cytoskeletal sea urchin actin gene of *S. purpuratus* (Akhurst *et al.* 1987). This

Fig. 2. Structure of the 5′ end of the CyI gene. (A) Map of the gene and location of leader sequence, introns and trailer sequence. The *CyI* actin gene is shown beneath a partial restriction map of the λ-28 (Scheller *et al.* 1981) genomic recombinant DNA which includes this gene. The restriction enzyme sites shown are *Eco*RI (R), *Bam*HI (B), *Sal*I (S), *Hin*dIII (H), *Hin*cII (Hc), *Sst*I (Ss), *Bcl*I (Bc), *Pst*I (P) and *Bgl*II (BgII). Brackets indicate restriction fragments utilized for the S1 analysis referred to in text. The various regions of the gene are indicated as follows: filled areas represent noncoding leader and trailer sequences of the transcript; cross-hatched areas are coding regions, and open areas are introns. The two introns within the coding region occur at amino acid 121/122 and within the codon for amino acid 204 (Cooper & Crain, 1982). The position of the poly(A) addition site was determined by Lee *et al.* (1986). The location of the 5′ leader intron and start of transcription were determined in this work. (B) Genomic sequence including 5′ leader exon and flanking regions of the CyI gene. The sequence shown begins 351 nt within the leader intron and extends upstream for about 1300 nt. The start of transcription is indicated at the boxed 'A'. 'CAAT' and 'TATA' sequence elements are underlined at −67 and −33, respectively, from the start of transcription (0). The area in brackets indicates a region of approximately 50 nt that was not sequenced. The 5′ splice site for the leader intron, determined by comparison with a cDNA clone, is indicated by a vertical line. (C) Determination of the start of transcription by primer extension. A synthetic primer 25 nt long and including the *Bcl*I site (see B) was labelled with ^{32}P and hybridized with poly(A)-containing RNA isolated from 48 h embryos (lane 1) or yeast tRNA (lane 2), and extended with reverse transcriptase. The samples were denatured and analysed on an 8 M-urea–10 % polyacrylamide gel. The DNA sequence ladder shown was generated using the same synthetic primer.

fragment was labelled, and utilized as a probe to screen a λgt11 library constructed from 62 h embryo RNA (Sucov *et al.* 1987). The 5′ leader region of one of the four cDNA positive clones identified in this screen was sequenced and the restriction sites it contained determined by computer analysis. The presence of a unique *Bcl*I site also observed in fragment B provided a means of locating the 5′ leader sequence of the cDNA within this genomic DNA fragment. The primary sequence of the latter was then determined in both directions around the *Bcl*I site. This sequence is shown in Fig. 2B. Comparison with the cDNA sequences confirmed the location of these within fragment B and defined the position of a 1·35 kb intron wholly contained within the 5′ leader sequence. We determined the exact transcription start site by primer extension. A synthetic 25 nt long DNA primer complementary to a region of the leader







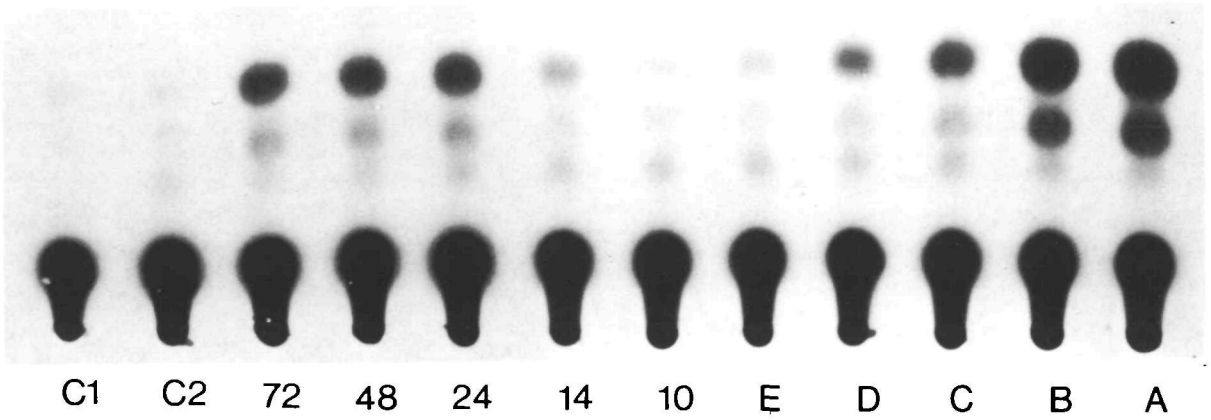


Fig. 3. Temporal expression of CyI·CAT in injected embryos. Approximately 3500 molecules per pL of linearized CyI·CAT DNA were injected into unfertilized eggs as described by McMahon *et al.* (1985); nominal injection vol. 2 pl. At 10, 14, 24, 48 and 72 h postfertilization, 75 embryos were collected and assayed for CAT activity. Bacterial CAT enzyme controls were assayed on the same TLC plate: lane A, 1×10^{-3} U; B, 5×10^{-4} U; C, 1×10^{-4} U; D, 5×10^{-5} U; E, 1×10^{-5} U. C1 is an assay using an extract from 75 uninjected embryos, and C2 is an assay reaction containing no extract. Data from this experiment are listed in expt. 2 of Table 1.

sequence including the *BclI* site was constructed and after reaction with embryo RNA the hybridization product was extended with reverse transcriptase. The result is shown in Fig. 2C, where it can be seen that the transcript begins with a sequence located 52 nt 5′ from the splice site of the leader intron (see Fig. 2B). A TATAA sequence is found at −33 with respect to the cap site and a CAAAT sequence begins at position −67. The overall structure of the gene, from this work and the prior studies carried out by Cooper & Crain (1982) and Schuler, McOsker & Keller (1983), is indicated below the restriction map in Fig. 2A.

Temporal expression of the CyI·CAT fusion gene during development

Shott *et al.* (1984) showed that transcripts of the endogenous *CyI* gene begin to accumulate between 10 and 14 h postfertilization (see also Durica & Crain, 1982; Lee *et al.* 1986). *In vivo* transcription rate studies as well as nuclear run-offs carried out on the *CyI* gene by Lee (1986) demonstrate that in late cleavage embryos, i.e. 7 h postfertilization, this gene is transcriptionally silent and show that the subsequent accumulation of message can be wholly accounted for by the rate of gene transcription following activation of the gene at the early blastula stage. Thus it is clear that the endogenous *CyI* gene is transcriptionally regulated during early development.

When the CyI·CAT construct was injected into unfertilized eggs and development then initiated, CAT enzyme also appeared between 10 and 14 h postfertilization. This result is illustrated qualitatively in the CAT assay series reproduced in Fig. 3, and in Table 1 are listed four separate sets of developmental measurements in which CAT enzyme protein content was estimated from the acetylation activity recovered in the embryo lysates. Table 1 indicates that in no case is any CAT activity detectable in 10 h embryos and that following activation the amount of CAT enzyme achieves a level at least 5–10× the minimum detectable (see Note 2 of Table 1). In interpreting this result, it is important to realize that in sea urchin embryos CAT mRNA and CAT enzyme are unstable. The estimated half-life of the enzyme is approx. 40 min (Flytzanis *et al.* 1987). Thus the sharp accumulation of CAT enzyme protein after 10 h postfertilization indicates an increased rate of transcription and consequent increased CAT mRNA content mediated by the CyI regulatory sequences included in the construct.

Table 1 also presents two sets of measurements of CAT DNA carried out on samples from the same experimental embryos utilized for CAT enzyme assays. While the DNA continues to increase between 10 and 14 h, i.e. as the embryo is completing its cleavage divisions, this increase is relatively modest. During the 10–14 h postfertilization interval when production and transcription of the CyI·CAT construct is initiated, the amount of CAT DNA changes only about 30%, while the level of CAT enzyme increases many fold. This obviates the possibility that absence of CAT enzyme at 10 h results simply from the absence of adequate numbers of CAT genes at 10 h. Two observations reported by Davidson *et al.* (1985) and Flytzanis *et al.* (1987) support the conclusion that the results shown in Fig. 3 and Table 1 indicate correct temporal regulation of the CyI·CAT fusion. First, in similar experiments utilizing a CAT gene under the control of an α-histone promoter, appearance of CAT enzyme was observed at 10 h and earlier, as appropriate for the α-histone genes, which

Table 1. *Average CAT enzyme and CAT DNA molecules per embryo**

Hours postfertilization	CAT enzyme† Experiment				CAT DNA‡ Experiment	
	1	2	3	4	1	4
10	ND§	ND	ND	ND	$6{\cdot}5\times10^4$	$4{\cdot}2\times10^4$
14	$5{\cdot}0\times10^5$	$7{\cdot}7\times10^4$	$2{\cdot}6\times10^5$	$4{\cdot}0\times10^5$	$8{\cdot}8\times10^4$	$5{\cdot}4\times10^4$
24	$3{\cdot}1\times10^5$	$3{\cdot}0\times10^5$	$1{\cdot}2\times10^5$			
48	$6{\cdot}0\times10^5$	$3{\cdot}2\times10^5$	$2{\cdot}4\times10^5$			
72		$5{\cdot}4\times10^5$				

* Number of embryos assayed per point: expt. 1, 100; expt. 2, 75; expt. 3, 100; expt. 4, 150.

† The *maximum* amount of CAT activity that would not have been detectable was estimated from the level of background (approx. 0·1 % acetylation) in assay samples receiving no extract or extract from control embryos not injected with CyI · CAT (see Fig. 3). Thus anything less than an average of about 5×10^4 molecules of CAT enzyme per embryo would not have been detectable. Note that only 60–80 % of injected embryos successfully incorporate exogenous DNA (McMahon *et al.* 1985; Flytzanis *et al* 1985) and thus for the successful cases average number of CAT enzyme molecules per embryo is low by this factor. CAT activity (i.e. fraction of [^{14}C]chloramphenicol converted to monoacetylated form) was transformed to the number of enzyme molecules according to McMahon *et al.* (1984; see Materials and Methods).

‡ CAT DNA content per average embryo was obtained by slot blot hybridizations with embryo DNA with a CAT probe, with the aid of correction by an internal standard, as described in Materials and Methods.

§ ND, not detectable.

are expressed at peak levels at about 10 h postfertilization. Therefore, lack of CyI · CAT expression at 10 h cannot be due to any kind of general repression of exogenously introduced DNA at this stage. Second, the number of CyI · CAT genes present per average embryo at 10 h, i.e. $\geq4\times10^4$ (Table 1), is probably already greatly in excess of the number that can be activated at any time during embryonic development. This argument has been demonstrated for CyIIIa · CAT fusion genes introduced in exactly the same manner (Flytzanis *et al.* 1987), and in normal mid-blastula stage embryos the quantity of CyIIIa mRNA is over twofold greater than the quantity of CyI mRNA (Lee *et al.* 1986). While we cannot yet be certain of this interpretation in respect to the behaviour of the CyI · CAT fusion, it is consistent with the observation that all four sets of embryos shown in Table 1 produce about the same maximum level of CAT enzyme and that this level remains more or less unchanged after 24 h. It may in any case be concluded that the 2·5 kb of flanking sequence present in the CyI · CAT construct suffice for positive temporal regulation, on the normal CyI schedule.

CyI · CAT deletions

To obtain a crude map of the location of necessary *cis* regulatory sequences we constructed the series of deletions illustrated in Fig. 4A. Deletions ΔSHc, ΔSP and ΔSSs contain decreasing amounts of 5′ flanking sequence. ΔSSs′ contains the same *CyI* sequences as does ΔSSs except for the deletion of most of the large leader intron. The ΔSPP deletion serves as a promoterless control, as the *CyI* sequences included begin within this intron, 170 nt after the transcriptional start site. It should be noted that the deletions shown in Fig. 4A do not include identical amounts of plasmid sequences and the effects of this factor on the expression of the CAT fusion gene, if any, remains untested.

The deletion constructs were linearized at the *Bgl*I site of the cloning vector, injected into eggs as before, and embryos harvested and analysed for CAT enzyme and CAT DNA 24 h after fertilization. As shown in Fig. 4B and Table 2, deletion of the outer 1700 nt of *CyI* sequence decreases expression by a small amount, a factor often <2. Thus, in other experiments not included in Table 2 the ratios of ΔSHc/CyI · CAT activity were 0·56, 0·62 and 0·73. This minor effect could indicate the presence of a distant sequence that potentiates CyI · CAT function, but it could also merely be an effect of changing the overall structure of the concatenates that form from the exogenous DNA within the embryo, e.g. by bringing the vector plasmid sequence closer to the transcriptional start sites. Thus Flytzanis *et al.* (1987) showed in a similar series of experiments with CyIIIa · CAT deletion constructs that plasmid sequences exert a mild depressive effect on CAT activity. The further deletions ΔSP and ΔSSs reproducibly decrease activity to around 10–20 % of the CyI · CAT control (Fig. 4B). This result was obtained in three experiments in addition to that shown in Fig. 4 and Table 1. These effects cannot be attributed to differences in the amounts of exogenous DNA, since as shown in Table 1, the DNA of all of the constructs amplified to about the same extent.

The result shown in the final row of Experiment 1 of Table 2 (ΔSSs′) indicates that removal of all of the leader intron except for 118 nt at the 5′ end and 293 nt at the 3′ end does not affect the amount of CAT

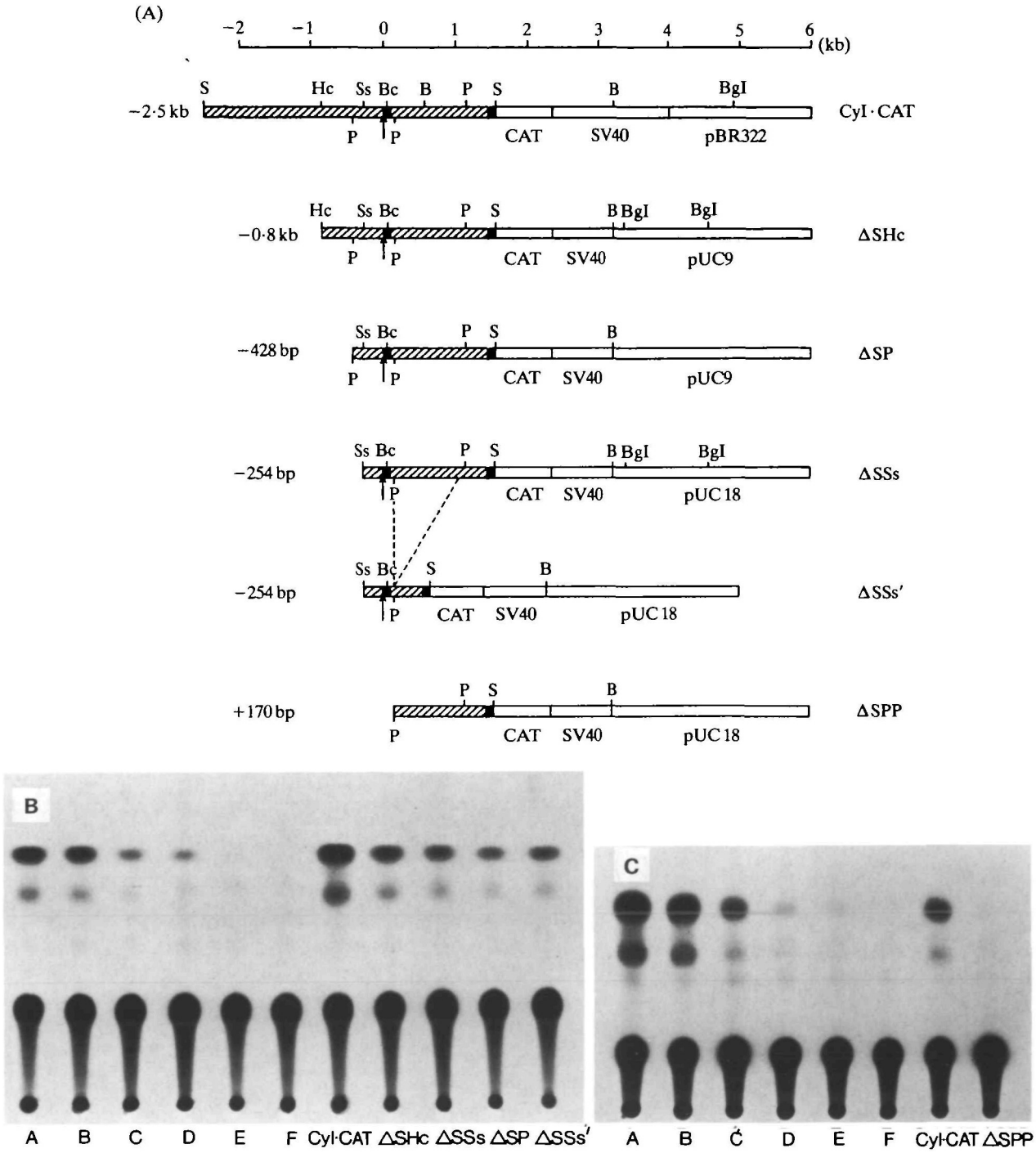


Fig. 4. Deletion constructs injected into eggs, and representative CAT assays. (A) Restriction maps of CyI·CAT deletion constructs. The arrow indicates the start of transcription. ΔSSs′ was derived from ΔSSs by removing the sequences indicated by the dashed lines. Hatched and filled-in areas are *CyI* sequences; SV40 and plasmid sequences are indicated. The filled-in regions denote, respectively, the first exon of the CyI leader sequence, and a portion of the second exon, consisting of the remained of the leader sequence plus a small amount of CyI coding region upstream of the *Sal*I restriction site located at the junction of the CAT and sea urchin sequences (see Fig. 1). The number to the left of each diagram indicates the number of ntp upstream from the start of transcription (0) remaining in the fusion construct. The restriction sites shown are: *Bam*HI (B), *Bcl*I (Bc), *Hin*cII (Hc), *Pst*I (P), *Sal*I (S), and *Bgl*I (BgI). (B) and (C), CAT assays of embryos injected with indicated deletions. Approximately 3500 molecules per pL of each construct were injected into unfertilized eggs, and at 24 h postfertilization 150 embryos were collected for each sample and assayed for CAT activity. Bacterial CAT enzyme controls were as follows: A, 1×10^{-3} U; B, 5×10^{-4} U; C, 1×10^{-4} U; D, 5×10^{-5} U; E, 1×10^{-5}; and F, No enzyme. Data for these experiments are shown in Table 2 (experiments 1 and 2).

Table 2. *CAT enzyme accumulation and CAT DNA per average embryo after injection of CyI·CAT and various other constructs**

Expt.	Construct	Av. CAT enzyme molecules per embryo	Average CAT DNA genes per embryo
1	CyI·CAT	$2{\cdot}6\times10^{6}$	$5{\cdot}1\times10^{5}$
	ΔSHc	$6{\cdot}3\times10^{5}$	$2{\cdot}9\times10^{5}$
	ΔSP	$2{\cdot}7\times10^{5}$	$3{\cdot}5\times10^{5}$
	ΔSSs	$2{\cdot}7\times10^{5}$	$3{\cdot}2\times10^{5}$
	ΔSSs′	$3{\cdot}3\times10^{5}$	$3{\cdot}5\times10^{5}$
2	CyI·CAT	$2{\cdot}8\times10^{5}$	$5{\cdot}9\times10^{4}$
	ΔSPP	0	$7{\cdot}0\times10^{4}$
3a	CyIIIa·CAT	$3{\cdot}1\times10^{6}$	$1{\cdot}2\times10^{5}$
	CyI·CAT	$7{\cdot}8\times10^{5}$	$2{\cdot}5\times10^{5}$
b	CyIIIa·CAT	$2{\cdot}8\times10^{7}$	
	CyI·CAT	$4{\cdot}1\times10^{5}$	
c	CyIIIa·CAT	$1{\cdot}8\times10^{6}$	
	CyI·CAT	$3{\cdot}3\times10^{4}$	

* Each batch of eggs displays a different level of CAT activity, which is reproducible within the batch (Flytzanis *et al.* 1986; unpublished data). The experiments shown in the table were done with different batches of eggs each obtained from a single female.

enzyme generated. This experiment provides no support for the possibility that the long leader introns common to both the *CyI* and *CyIIIa* sea urchin actin genes (Akhurst *et al.* 1987) might contain regulatory sequence, though it of course does not exclude this possibility, if such elements are redundant with others located in the 5′ flanking sequences. The promoterless construct tested in experiment 2 of Table 1 (ΔSPP) failed to display any activity (Fig. 4C). Thus, it is very unlikely that the productive transcription observed with the other constructs could derive from cryptic promoters active within the sea urchin cells, e.g. in the plasmid or SV40 DNA sequences.

CAT synthesis from the same deletion constructs was also examined at 10 h of development. In no case could any enzyme activity be detected. It follows that the 254 nt of 5′ flanking sequence remaining in the ΔSSs construct suffice for *temporally regulated* expression, albeit at a relatively low level. At least one additional positively acting *cis*-regulatory element must exist in the region between −408 and approx. −800, since, as discussed, the ΔSP deletion to −428 results in 80–90% decrease in activity, relative to CyI·CAT.

Experiment 3 of Table 2 displays comparisons of CAT activity generated by the analogous CyIIIa·CAT construct (Davidson *et al.* 1985; Flytzanis *et al.* 1987) with that generated by CyI·CAT, after injection into the same batches of eggs. The normal ratio of CyIIIa to CyI mRNAs in the 24 h embryo is about 2·7 (Lee *et al.* 1986). The ratios of CAT produced from the CyIIIa construct to CAT produced from the CyI construct were approx. 4, 70 and 50 in the three experiments shown. The first of these values is probably abnormally low, due to a level of CyIIIa·CAT activity much below that usually obtained (e.g. Flytzanis *et al.* 1987; unpublished measurements). In any case, since CAT mRNA and protein are unstable in sea urchin cells (Flytzanis *et al.* 1987) these results suggest a significant difference in the fusion gene promoter activities, much exceeding the respective difference in the endogenous CyI and CyIIIa gene promoters (Lee *et al.* 1986; Lee, 1986). One possible explanation is that additional positively acting control elements are located elsewhere than within the 2·5 kb of 5′ flanking sequence included in the CyI·CAT construct.

Discussion

Regulatory activity of exogenous gene sequences injected into sea urchin eggs

These results demonstrate that *cis*-regulatory sequences of a second actin gene are capable of directing correct temporal activation of a reporter CAT coding sequence in the developing sea urchin embryo. As reviewed above, upstream sequences of the *CyIIIa* actin gene suffice to regulate both spatial and temporal embryonic expression of a similar fusion construct. The *cis*-regulatory sequences of the *CyI* actin gene that are responsible for its activation in the blastula stage embryo must interact with at least a partially different set of *trans*-activator molecules, since *CyI* expression occurs in different cells than does *CyIIIa* expression (see Introduction).

Regulatory sequences of at least eight different sea urchin genes have now been shown to function during embryonic development when injected into unfertilized eggs. These are the early *H2a* gene (Davidson *et al.* 1985); the early *H2b* and late *H2b* genes (R. Maxson, personal communication); the *Spec1* gene coding for a Ca^{2+}-binding protein (W. Klein, personal communication); the *CyIIIa* actin gene (Davidson *et al.* 1985; Flytzanis *et al.* 1987) and the *CyI* actin gene (this work), all of *S. purpuratus*. The *S. purpuratus CyIIIa* actin gene regulatory sequences have also been found to function on the correct timetable when CyIIIa·CAT is injected into *Lytechinus variegatus* eggs, i.e. when the host embryos attain the equivalent early blastula stage. At the higher temperature of culture (22°C), this occurs several hours prior to the time when the *CyIIIa* gene is activated in *S. purpuratus* embryos (R. Franks *et al.* unpublished data). In addition, L. Vitelli (personal communication)

showed that an injected sperm H2b histone is *not* transcribed at all in the embryo, again as expected on the premise of faithful regulation, since this gene is not utilized except during spermiogenesis. Except for the late *H2b* histone gene (and of course the sperm histone gene) the genes so far examined for activity after injection into the egg are normally all transcribed at maximal rates early in development, i.e. by the blastula stage. It remains to be determined whether other genes that are ontogenically activated at much later stages will also be expressed appropriately on injection into sea urchin eggs. Since incorporation of exogenous DNA after cytoplasmic injection tends to occur in a mosaic fashion (Flytzanis *et al.* 1985; Hough-Evans *et al.* 1987), the response observed in such cases might be quantitatively less impressive. Nonetheless, it is now evident that, at least for genes utilized early on, the cytoplasmic gene transfer system of McMahon *et al.* (1985) provides an excellent functional test system for the examination of gene regulation in early sea urchin development.

The deletion constructs provide a coarse localization of Cyl regulatory sequences

Unlike the *CyIIIa* gene, the *Cyl* gene is functional in various adult tissues as well as in the embryo (Shott *et al.* 1984) and the present studies provide no evidence as to the location of the sequences required for adult expression. The location of at least some of the regulatory sequences required for blastula stage activation is indicated on a coarse scale by the deletion experiments summarized in Fig. 4 and Table 2. We find that inclusion of a 254 nt sequence 5′ from the transcription initiation site suffices for temporally correct, though low-level expression. An additional positively acting element or elements apparently exist(s) between −428 and −800 and, as discussed in Results, there may be other *cis*-active sequences not included in the CyI·CAT construct. Several short sequence elements lying within the region between +1 and −254 display homology with 5′ sequence elements of the *CyIIIa* gene, though overall the upstream sequences of these two genes are wholly dissimilar (Akhurst *et al.* 1987). Since *CyIIIa* and *Cyl* are activated at about the same time, these shared sequence elements could be functional, but since they are activated in different sets of cells there are likely to be other distinct positive or negative regulatory sequences in addition. At least eight sites at which proteins bind specifically to upstream regions of CyIIIa DNA have now been identified (Calzone *et al.* 1987; unpublished data). Much additional analysis, utilizing both *in vitro* DNA–protein interactions and the gene transfer system applied in this study, will be required before the particular *cis*-regulatory sequences responsible for spatial and temporal activation of the *CyIIIa* and *Cyl* actin genes can be identified.

In a more general sense, this work contributes to the body of experimental evidence suggesting that activation of genes in the early embryo is mediated by *cis–trans* interactions. Such is the direct implication of the now multiple observations that exogenous sequences introduced into embryo nuclei in abnormal numbers of copies, and undoubtedly in abnormal positions, are nonetheless activated at the proper developmental stages. For CyIIIa·CAT the exogenous regulatory sequences have been shown to suffice as well for correct spatial expression (Hough-Evans *et al.* 1987), as would be expected on the assumption of regulation by *cis–trans* interactions. The simple consequence is that the spatial pattern of activation of genes that begin to be expressed in the early embryo must reflect the *regional presentation or activation of their* trans *regulators*. For genes such as *Cyl* and *CyIIIa* this is now a testable theory that could constitute an explanation of the means by which genomic information begins to be differentially expressed in the various cells of the early embryo.

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