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

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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 Cyl-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 Cyl-CAT genes. The amounts of CAT enzyme produced by embryos bearing Cyl-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 Cyl-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 Cyl 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 Cyl gene, which had not previously been reported. The Cyl gene is activated at about the same stage of development as is the CylIIIa gene (Shott et al. 1984; Cox et al. 1986). However, by in situ hybridization it has been demonstrated that Cyl actin message is present in a different set of cells, which in late embryos is essentially complementary to the set of cells expressing the CylIIIa gene. Thus at the pluteus stage Cyl transcripts appear in the oral ectoderm and much of the gut, regions which are devoid of detectable CylIIIa transcripts, and Cyl transcripts are absent from the aboral ectoderm where are located all of the CylIIIa transcripts (Angerer & Davidson, 1984; Cox et al. 1986). At the early blastula stage, when newly synthesized transcripts of both genes first appear, the Cyl RNAs accumulate in all regions of the embryo. In contrast, at this stage newly synthesized CylIIIa transcripts are asymmetrically confined to the precursors of the future aboral ectoderm, then located on one side of the spherical embryo. Cyl 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 Cyl gene expression is thus completely distinct from that of the CylIIIa gene. It has been pointed out (Lee et al. 1984; Shott et al. 1984) that the cis-regulatory Cyl 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, CylIIIa, 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 Cyl gene produces a cytoskeletal actin required in dividing cells, while the CylIIIa 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 Cyl and CylIIIa 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).

SI nuclease protection assays

The various DNA fragments chosen for SI 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 SI 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 32P at the 5' end using T4 polynucleotide kinase (IBI). The primer fragment was gel purified on an 8M-urea-10% acrylamide gel for electrophoresis. 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).

Construction of the Cyl-CAT fusion gene

Cyl 5' upstream sequences were obtained from the plasmid pCIP5 (Fig. 1), which includes about 6 kb of Cyl sequence 5' to the Cyl ATG signal, plus 42 nt of actin coding sequence fused to the gene for neomycin phosphotransferase. The original sources of the sea urchin Cyl 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 Cyl-CAT fusion gene was constructed as outlined in Fig. 1A. The HindIII site located 31 nt upstream from the CAT ATG in the plasmid pSVO-CAT (Shott-Akhurst, Calzone, Britten & Davidson, 1984) was changed to a SalI site by digestion with HindIII, and ligation to SalI linkers. After extension of the recessed termini with Klenow polymerase I. The indicated BamHI–BclI Cyl fragment of pCIP5 was subcloned into the pUC18 vector, and the BamI–BamHI

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Fragment containing the remaining sea urchin sequences was then cloned into the BamHI site of this plasmid. The presence of a SalI site in the polylinker adjacent to the BclI–BamHI ligation site made it possible to remove from this plasmid a 4.4-kb SalI fragment which, as shown below, contains about 2.5 kb of 5' flanking sequence upstream of the Cyl cap site. This fragment was isolated and ligated into the SalI site of the altered pSVOCAT in the proper orientation. The resulting construct, called Cyl-CAT, contains the 2.5 kb 5' flanking sequence, exon I of the Cyl gene, the entire 5' leader intron and 42 nt of actin-coding sequence derived from exon II of the gene. The fusion between the Cyl sequences and the CAT coding region inserted a sequence between the Cyl ATG and the CAT ATG 96 nt in length. Thus if translation were to initiate at the Cyl 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 Cyl ATG, as shown in Fig. 1B.

Fig. 1. Construction of the Cyl-CAT plasmid. (A) Flow diagram of the steps used to generate the Cyl-CAT plasmid. The restriction enzyme sites shown are BamHI (B), SalI (S), BclI (Bc), HindIII (H). The hatched areas indicate sea urchin sequences. Details of the construction are given in Materials and Methods. (B) Sequence of the Cyl-CAT junction. A small fragment containing the junction between the Cyl 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 Cyl 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 [14C]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 2.6×10¹¹ 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 effectivley 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 Cyl 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 Cyl 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 Cyl gene

Though the complete nucleotide sequence of the coding region of the Cyl 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 CyIIa cytoskeletal sea urchin actin gene of S. purpuratus (Akhurst et al. 1987). This

Fig. 2. Structure of the 5′ end of the Cyl gene. (A) Map of the gene and location of leader sequence, introns and trailer sequence. The Cyl 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 EcoRI (R), BamHI (B), SalI (S), HindIII (H), HinClII (He). SsrI (Ss), BclI (Bc), PstI (P) and BglII (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 4121/122 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 Cyl 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 BclI site (see B) was labelled with 32P 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.
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fragment was labelled, and utilized as a probe to screen a λgt11 library constructed from 6h 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 BclI 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 BclI 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

![Image of a genetic sequence diagram]


![Image of a DNA sequence diagram]

5' ACGT 1

INTRO

1)

2)

3')

4)

5)

6)
sequence including the BglI 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 Cyl-CAT fusion gene during development

Shott et al. (1984) showed that transcripts of the endogenous Cyl 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 Cyl 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 Cyl gene is transcriptionally regulated during early development.

When the Cyl-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 Cyl 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 Cyl-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 Cyl-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
are expressed at peak levels at about 10 h postfertilization. Therefore, lack of Cyl•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 Cyl•CAT genes present per average embryo at 10 h, i.e. $\approx 4 \times 10^4$ (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 Cyl•la-CAT fusion genes introduced in exactly the same manner (Flytzanis et al. 1987), and in normal mid-blastula stage embryos the quantity of Cyl IIIa mRNA is over twofold greater than the quantity of Cyl mRNA (Lee et al. 1986). While we cannot yet be certain of this interpretation in respect to the behaviour of the Cyl•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 Cyl•CAT construct suffice for positive temporal regulation, on the normal Cyl schedule.

Cyl•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 Cyl 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 Cyl 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 BglI 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 Cyl sequence decreases expression by a small amount, a factor often $<2$. Thus, in other experiments not included in Table 2 the ratios of ΔSHc/Cyl•CAT activity were 0.56, 0.62 and 0.73. This minor effect could indicate the presence of a distant sequence that potentiates Cyl•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 Cyl•la-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 Cyl•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

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

<table>
<thead>
<tr>
<th>Hours postfertilization</th>
<th>CAT enzyme†</th>
<th>CAT DNA‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Experiment</td>
</tr>
<tr>
<td></td>
<td>1</td>
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</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>5.0×10⁵</td>
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<td>3.0×10⁵</td>
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<tr>
<td>48</td>
<td>6.0×10⁵</td>
<td>3.2×10⁵</td>
</tr>
<tr>
<td>72</td>
<td>5.4×10⁵</td>
<td>7.7×10⁵</td>
</tr>
</tbody>
</table>

*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 Cyl•CAT (see Fig. 3). Thus anything less than an average of about $5 \times 10^6$ 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 [¹⁴C]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.
Fig. 4. Deletion constructs injected into eggs, and representative CAT assays. (A) Restriction maps of Cyl-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 Cyl sequences; SV40 and plasmid sequences are indicated. The filled-in regions denote, respectively, the first exon of the Cyl leader sequence, and a portion of the second exon, consisting of the remained of the leader sequence plus a small amount of Cyl coding region upstream of the SalI 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: BamHI (B), BclI (Bc), HindIII (Hc), PstI (P), SalI (S), and BglII (Bgl). 

(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, 1×10⁻¹ 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 Cyl-CAT and various other constructs

<table>
<thead>
<tr>
<th>Expt</th>
<th>Construct</th>
<th>Av. CAT enzyme molecules per embryo</th>
<th>Average CAT DNA genes per embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cyl-CAT</td>
<td>2.6 × 10^6</td>
<td>5.1 × 10^3</td>
</tr>
<tr>
<td></td>
<td>ΔSHc</td>
<td>6.3 × 10^5</td>
<td>2.9 × 10^5</td>
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<tr>
<td></td>
<td>ΔSP</td>
<td>2.7 × 10^5</td>
<td>5.5 × 10^5</td>
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<td></td>
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</tr>
<tr>
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<td>Cyl-CAT</td>
<td>2.8 × 10^6</td>
<td>5.9 × 10^4</td>
</tr>
<tr>
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</tr>
<tr>
<td>3a</td>
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</tr>
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<td></td>
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<td>7.8 × 10^5</td>
<td>2.5 × 10^5</td>
</tr>
<tr>
<td>b</td>
<td>CylIIa-CAT</td>
<td>2.8 × 10^6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyl-CAT</td>
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<td></td>
</tr>
<tr>
<td>c</td>
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<tr>
<td></td>
<td>Cyl-CAT</td>
<td>3.3 × 10^6</td>
<td></td>
</tr>
</tbody>
</table>

* 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.

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 CylIa actin gene suffice to regulate both spatial and temporal embryonic expression of a similar fusion construct. The cis-regulatory sequences of the Cyl 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 Cyl expression occurs in different cells than does CylIa 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 CylIa actin gene (Davidson et al. 1985; Flytzanis et al. 1987) and the Cyl actin gene (this work), all of S. purpuratus. The S. purpuratus CylIa actin gene regulatory sequences have also been found to function on the correct timetable when CylIa-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 CylIa 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 ontogenetically 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 Cyilla 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 Cyl·CAT construct. Several short sequence elements lying within the region between +1 and −254 display homology with 5' sequence elements of the Cyilla gene, though overall the upstream sequences of these two genes are wholly dissimilar (Akhurst et al. 1987). Since Cyilla 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 Cyilla 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 Cyilla 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 Cyilla·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 Cyilla 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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References


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