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Ontogenic activation of a fusion gene introduced into sea urchin eggs

(gene transfer/cytoskeletal actin/developmental regulation)

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ABSTRACT **Regulatory sequences of a sea urchin cytoskeletal actin gene (*CyIIIa*) were ligated to the bacterial gene coding for chloramphenicol acetyltransferase (CAT; acetyl-CoA:chloramphenicol O^3-acetyltransferase, EC 2.3.1.28) and the construct was injected into unfertilized sea urchin eggs. CAT activity is detected at the early blastula stage, when transcripts of the endogenous *CyIIIa* gene normally appear. Our measurements show that during activation the amount of CAT enzyme increases at least 100-fold; that there are present in late blastula stage embryos about 5×10^5 molecules of CAT mRNA (i.e., ≈6 times the number of endogenous CyIIIa mRNAs); and that within the range studied the amount of CAT enzyme produced is independent of the number of *CyIIIa-CAT* genes incorporated per embryo, probably because the genes are present in excess of factors required for their activation. Activation of the *CyIIIa-CAT* construct is seriously inhibited, or abolished, by successive deletions of upstream *CyIIIa* sequences.**

Differential gene activation has been demonstated in several specified cell lineages of early blastula stage sea urchin embryos (reviewed in ref. 1). Among the best-known examples is the *CyIIIa* cytoskeletal actin gene, which is expressed only in aboral ectoderm cells (2). Transcripts of this gene appear in the progenitor cells of the aboral ectoderm at 10–12 hr after fertilization, when the embryo contains only 100–200 cells (1, 3). The *CyIIIa* gene is not detectably expressed in any cell type other than aboral ectoderm, and it is utilized exclusively during embryonic and larval life (2, 3). Thus its initial specification in the early embryo is also its final specification.

The problem we address in this paper is the mechanism responsible for the initial activation of the *CyIIIa* gene. By application of a recently developed system for transferring exogenous genes into sea urchin eggs (4, 5) we demonstrate that the 5′ flanking sequences of this gene include genetic regulatory information sufficient to specify its temporal pattern of activation and, in studies to be reported elsewhere, its spatial pattern of activation as well. Additional results imply that the *CyIIIa* gene is probably activated in consequence of interaction with sequence-specific trans-acting factor(s) that are present in limited quantity in the aboral ectoderm precursors of the early embryo.

METHODS

Chloramphenicol Acetyltransferase (CAT) Assays. Embryos were injected as described (4) and collected in a Microfuge (15,000 × *g* for 2 min). Pellets were mixed with ≈1,500 uninjected blastula stage embryos used as carrier and lysed in 100 μl of 250 mM Tris·HCl, pH 7.8, by three consecutive freeze-thaw cycles as described (6). Half of the lysate, representing 10–50 embryos, was used for assay of CAT (acetyl-CoA:chloramphenicol O^3-acetyltransferase, EC 2.3.1.28) enzyme activity (6). Percent acetylation was estimated by scintillation counting of appropriate regions of the TLC assay plates. The number of enzyme units present in each lysate was calculated by reference to a standard curve measured for each experiment. One unit of the bacterial CAT enzyme represents $\approx 2.6 \times 10^{11}$ molecules of protein.

Measurement of *CAT* DNA Content per Embryo. The other half of the lysate (50 μl) was used to estimate the number of exogenous DNA molecules in each group of pooled embryos, as follows: an equal volume (50 μl) of 0.1 M EDTA, pH 8.0/1% $NaDodSO_4$ was added and the mixture was incubated with 20 μg of proteinase K at 55°C for 2 hr. After extractions with phenol and Sevag solution [chloroform/isoamyl alcohol, 24:1 (vol/vol)], one-fifth of the volume was removed and diluted 1:4 with a solution of 4′,6-diamidino-2-phenylindole (DAPI) in 100 mM NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.0. The total amount of DNA recovered was determined fluorometrically by the DNA-specific fluorochrome DAPI reaction (7). The remaining solution was treated with 0.4 M NaOH for 1 hr at 65°C and filtered onto nitrocellulose using a Schleicher & Schuell Minifold II apparatus. Each slot was cut in half, and the halves were hybridized to two different probes, a single-copy probe consisting of the 3′ noncoding trailer sequence of the *CyI* actin gene (8), which was used as a filter hybridization efficiency standard, and a *CAT* gene probe. Filter hybridization was carried out (5), using radioactively labeled RNA transcribed *in vitro* from Sp6 vectors. After autoradiography the individual half-slot filter samples were cut out and their radioactivity was determined by scintillation counting. The number of DNA molecules was estimated by reference to the specific activity of the RNA probe (usually $5–6 \times 10^8$ cpm/μg). The actual quantity of carrier DNA delivered to each slot in the experimental samples was known from the DAPI measurements. Hybridization efficiency was estimated by comparison of the number of *CyI* probe molecules hybridized with the number of *CyI* genes present per half slot, the probe specific activity, and the fact that there is one *CyI* gene per haploid genome (8) (i.e., per 0.8 pg). *CyI* hybridization efficiencies varied from about 30% to about 100%. The number of *CAT* DNA molecules present per half slot was finally computed in terms of *CAT* DNA molecules per embryo.

RNase Protection Assays. Embryos derived from injected eggs were collected 24 hr after fertilization and lysed in the presence of 20 μg of tRNA as carrier, using a urea/proteinase K protocol (9). Total RNA was isolated and hybridized to SP6 RNA probes transcribed *in vitro*. The hybridization reactions were carried out in solution for 3 hr at 50°C in 0.4 M NaCl/0.04 M Pipes, pH 6.7/1 mM EDTA/80% formamide.

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Abbreviation: CAT, chloramphenicol acetyltransferase.

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After incubation the RNA·RNA hybrids were treated with ribonuclease A (4.6 μg/ml) and ribonuclease T1 (48 units/ml) in the presence of tRNA (32 μg/ml) and separated on an 8% acrylamide/50% urea gel. Each RNA sample was divided in half and one half was hybridized to a *CAT* probe and the other, to a *CyIIIa*-specific antisense RNA probe. The autoradiograms were densitometrically scanned, and the amount of *CAT*-encoded transcript produced in injected embryos was compared in the same experiment to the amount of *CyIIIa*-encoded transcript present in similar numbers of injected and uninjected embryos. Normal *Strongylocentrotus purpuratus* embryos of this stage contain ≈8 × 10^4 molecules of CyIIIa mRNA (13).

RESULTS

Ontogenic Activation of the *CyIIIa-CAT* Fusion Gene. Davidson *et al.* (10) and Flytzanis *et al.* (11) have reported that CAT enzyme is generated at an appropriate stage in embryos developing from eggs injected with a fusion gene containing 5′ leader and upstream sequences of the *CyIIIa* gene ligated to the bacterial *CAT* gene. The fusion gene contained ≈7 kilobases of 5′ flanking *CyIIIa* sequence and ≈2.3 kb of transcribed 5′ leader sequence, most of which is included in a large intron (12). These sea urchin sequences were fused in phase with the *CAT* gene at a position 11 codons after the *CyIIIa* translation start signal (see ref. 10 for the sequence). The fusion point with respect to the *CAT* gene is 13 codons prior to its normal translation start. The construct also includes the simian virus 40 poly(A) addition site and a portion of plasmid pBR322. The *CyIIIa-CAT* fusion gene was injected after linearization at a unique *Sph* I site ≈2.5 kb prior to the location of the transcription initiation site of the *CyIIIa* gene (12). A random end-to-end concatenation of exogenous linear DNA molecules is rapidly formed in injected sea urchin eggs (4), and thus in 50% of junctions the original sequence organization is expected to be re-created.

The amount of CAT enzyme present per embryo at various times after fertilization is shown for two different experiments in Fig. 1. Autoradiographs of the CAT enzyme assay mixtures carried out on samples taken 5, 10, 15, 20, 25, and 45 hr after fertilization are reproduced in Fig. 1 (*Inset b*) for one of these experiments. As reported previously (10, 11), CAT activity is not detectable in hr 5 and hr 10 embryos, but shortly after this the enzyme appears in the embryo lysates and thereafter it accumulates rapidly. The exact timing of activation of CAT synthesis, as well as its maximal extent, varies slightly among different batches of embryos (cf. Fig. 1), and sometimes CAT appearance lags an hour or two behind *CyIIIa* activation in normal uninjected eggs (10). This is likely related to a 1- to 2-hr pause prior to the onset of cleavage that is often observed in injected eggs. The second set of CAT enzyme measurements shown in Fig. 1 was carried out on the embryos on which the *CAT* DNA measurements presented in this figure were obtained. The amount of *CAT* DNA increases exponentially, as does total embryonic DNA between about 5 hr and 12 hr after fertilization [i.e., from 8 cells to about 130 cells (≈7th cleavage)]. During this period the doubling time for cellular DNA at 15°C is about 1.67 hr, while the doubling time measured for the *CAT* DNA in the experiment of Fig. 1, which was carried out at 16°C, is 2.56 hr (1). This difference could mean that the *CAT* DNA replicates at only ≈60% of the rate of cellular DNA, or that a fraction of the *CAT* DNA is lost at each division, while the remainder replicates, for example, at the rate of cellular DNA. It is unlikely that the apparent difference in replication rates is due solely to experimental error, since a 2-fold higher rate of *CAT* DNA accumulation would require a systematic 4-fold error in all the data points shown in Fig. 1.

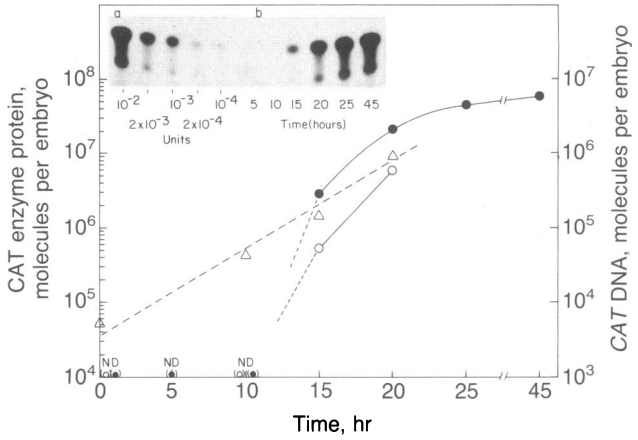

FIG. 1. Measurement of CAT enzyme (○ and ●) and CAT DNA (△) molecules in embryos developing from eggs injected with the *CyIIIa-CAT* fusion gene. Two experiments are shown, one in which 50 (○) and one in which 25 (●) embryos were pooled per sample, half of which were used for the CAT enzyme estimate. The quantities of *CyIIIa-CAT* molecules injected per egg (i.e., per ≈2 pl) were about 4 × 10^3 and 2 × 10^3 molecules, respectively, for the 50- and 25-embryo experiments. Average numbers of molecules were calculated on the basis of the total pooled samples and, since only 60–80% of embryos developing from cytoplasmically injected eggs retain and amplify the exogenous DNA (5), for these embryos the averages shown must be underestimates. ND, not detectable, for simplicity plotted along abscissa. The minimum levels detectable, which depend on sensitivity of the assay, and the number of embryos pooled, estimated as ≥2 times background, were about 1 × 10^5 (50-embryo sample) and 5 × 10^5 (25-embryo sample) molecules per embryo. (*Inset a*) Standards: acetylation of chloramphenicol by the bacterial CAT enzyme. (*Inset b*) Acetylation of chloramphenicol by embryo extracts in the 25-embryo experiment. Only the acetylated products are shown. Background was about 0.2% acetylation, and the highest values obtained were about 10% (20 hr, 50 embryo) and 29% (45 hr, 25 embryo).

It can be seen in Fig. 1 that there are thousands of *CyIIIa-CAT* genes present per embryo in the period 0–10 hr, though no CAT enzyme activity whatsoever can be detected at this stage. We show below that the average number of *CAT* genes per embryo in this experiment at 10 hr (i.e., ≈4 × 10^4) is adequate for generation of maximum CAT enzyme activity in embryos that have developed for 24 hr. It is therefore very unlikely that the failure to produce detectable CAT enzyme at 10 hr or before is due to insufficient quantities of *CyIIIa-CAT* DNA. Nor is the exogenous DNA in a condition that is not permissive for transcription at 10 hr. In similar experiments carried out with an analogous construct in which the *CAT* gene is ligated to the 5′ regulatory region of an early histone H2A gene (10), CAT synthesis under the control of the H2A sequences was observed to be maximal during the period before it can be detected at all when regulated by *CyIIIa* sequences. In more extensive experiments (L. Vitelli, I. Kemler, M. Busslinger, and M. L. Birnstiel, personal communication) have shown that mRNA deriving from injected early histone genes attains its peak concentration at the expected stage for α-histone message—i.e., during early/midcleavage, when the *CyIIIa* genes (2, 3) are silent. Thus, since it cannot easily be attributed to change in either quantity or general availability of the exogenous DNA for transcription, the onset of detectable CAT enzyme synthesis after 10 hr appears to be an ontogenically regulated event. If there is any precocious synthesis of CAT enzyme at 10 hr or earlier it is below the level of detection (see legend to Fig. 1). Thus Fig. 1 shows that activation of the fusion gene results in at least 100-fold accumulation of the CAT enzyme between 10 and 20 hr after fertilization.

Estimation of CAT mRNA in Embryos Developing from Injected Eggs. RNAs extracted from control embryos harvested 24 hr after fertilization and from embryos of similar age that had developed from eggs injected with *CyIIIa-CAT* were used for measurement of CAT mRNA content by the RNase protection procedure. A CAT antisense RNA probe fragment of expected length was protected from RNase digestion after reaction with the injected embryo RNA but not after injection with the control embryo RNA (data not shown). The quantity of CAT mRNA was calculated from this experiment by densitometric comparison with the amount of CyIIIa mRNA sequence protected by a 3′ trailer probe specific for this message (13). As shown in Table 1, the experimental embryos contain about six times the number of CAT mRNA molecules as normal embryos contain of CyIIIa message. No significant difference in the number of endogenous CyIIIa mRNA molecules could be discerned, comparing embryos injected with *CyIIIa-CAT* and control embryos, though a systematic study directed at this point has not been carried out.

Table 1 also shows the average amount of CAT enzyme produced per embryo by the particular batches of embryos used for the probe protection experiment. If we presume that the translation efficiency of the fusion mRNA is typical for sea urchin embryo messages, which would seem reasonable since the leader sequence is of *CyIIIa* origin, the turnover rate of the CAT enzyme protein can be estimated (see legend). The enzyme is clearly unstable, with an estimated $t_{1/2}$ of 40 min (Table 1). It follows that the amount of CAT enzyme reports the activity of the *CyIIIa-CAT* fusion gene for the period just before the observation is made, rather than representing an accumulation of protein product that integrates over a period of many hours.

Competition Among the Amplified *CyIIIa-CAT* Sequences. Some batches of eggs amplify different amounts of injected DNA by the same factor, thus providing a means of producing embryo samples that contain various numbers of *CyIIIa-CAT* fusion genes (Fig. 2*a*). It can be seen from the slopes of the double-logarithmic plots for experiments A and D, which approach unity, that these embryos have almost equally amplified a wide range of injected DNA quantities. The average extent of amplification is 150 for experiment A and 130 for experiment D. The maximum that can be obtained is an average of 2×10^6 *CAT* genes per embryo, since most eggs injected with more than $1–2 \times 10^4$ *CyIIIa-CAT* genes fail to develop (cf. ref. 4). In contrast, the embryos of experiments G and H (Fig. 2*a*) behave differently, in that the extent of amplification decreases as the quantity of initially injected DNA increases. The basis for this difference is not understood but may be variation in the initial capacity of the eggs to ligate various quantities of injected DNA into concatenated molecules that promote subsequent incorporation into the nuclear compartment (4, 5, 14).

Table 1. Estimate of average CAT mRNA quantity and $t_{1/2}$ value of the CAT enzyme in hr 24 embryos developing from eggs injected with the *CyIIIa-CAT* construct

Endogenous CyIIIa mRNA, molecules per embryo	8×10^4 (13)
CAT mRNA, molecules per embryo	5×10^5
CAT enzyme, molecules per embryo	3×10^7
CAT enzyme/CAT mRNA	60
$t_{1/2}$ for CAT enzyme, min	≈40

Samples on which CAT mRNA content was measured contained 200 and 500 embryos. Similar results were obtained from these samples. The average *CAT* DNA content at 24 hr was 1.8×10^5 genes per embryo. We assume that the CAT RNA content is at steady state over the period around 24 hr that is relevant to the measurement (see Fig. 1 and the text). An average rate of translation for *S. purpuratus* embryos, measured both for several specific messages and for total protein, is about 1 polypeptide per mRNA released per min (ref. 15; reviewed in ref. 1). The $t_{1/2}$ value for the protein would be given by $\ln 2/k_d$ where k_d is the decay rate constant (min^{-1}) assuming stochastic first-order decay. We calculate k_d by taking 60 molecules of CAT enzyme protein per CAT mRNA as enzyme steady state; k_d = 60 ÷ rate of synthesis per CAT mRNA (i.e., ≈1 min^{-1}). Thus for the enzyme $t_{1/2} = \ln 2 \times 60/1\ min^{-1}$.

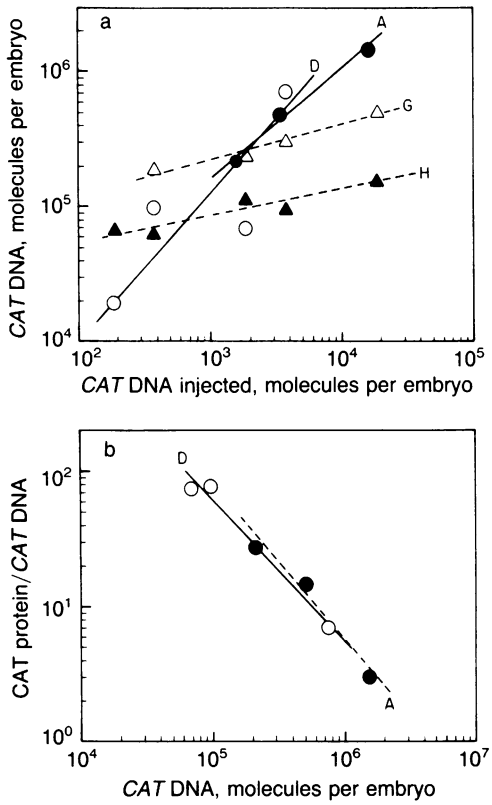

FIG. 2. Amplification of injected *CyIIIa-CAT* DNA and efficiency of CAT synthesis. (*a*) Number of CAT genes in hr 24 embryos developing from eggs injected with various numbers of *CyIIIa-CAT* molecules. Four experiments, A (●), D (○), G (△), and H (▲), are shown, each carried out with a single batch of eggs. All eggs were injected with a volume of ≈2 pl, and the nominal amount of *CyIIIa-CAT* delivered was varied by use of serial dilution of the *CyIIIa-CAT* preparation. The embryos of experiments A and D amplified the injected DNA almost proportionately: the slopes of the lines shown are 0.82 for experiment A and 0.90 for experiment D. The average amplification was 149 for experiment D, and 129 for experiment A. In experiments G and H, amplification decreased as the number of molecules of *CyIIIa-CAT* injected increased. Thus, for example, in experiment G the lowest amount of DNA injected was amplified almost 500-fold, while the highest amount was amplified ≈30-fold. (*b*) Efficiency of CAT enzyme synthesis, expressed as molecules of CAT enzyme per *CAT* gene. Samples of embryos from all of the points of experiments A and D plotted were used, except for the lowest point of experiment D, which failed to yield a reliable CAT enzyme estimate. The efficiency decreased in both experiments almost stoichiometrically with the number of *CAT* genes: the slopes of the least-squares double-logarithmic functions shown are −1.05 for experiment D and −1.15 for experiment A.

To determine the efficiency with which the different quantities of *CyIIIa* sequences contained in these embryos were able to activate their associated *CAT* genes, we measured the amount of CAT enzyme produced in the various samples of experiments A and D (as shown, the embryos of experiments G and H displayed little variation in *CyIIIa-CAT* content). Over the range studied, from $\approx 6 \times 10^4$ *CAT* genes per embryo to almost 2×10^6 *CAT* genes per embryo, approximately the same amount of CAT enzyme was produced—i.e., $\approx 5 \times 10^6$ molecules of CAT enzyme per embryo. The almost exact correspondence between experi-

ments A and D in respect to CAT production per embryo is adventitious since different batches of embryos usually vary. Thus, for example, the embryos used in the experiments summarized in Table 1 produced about six times more CAT enzyme per embryo than did those in the experiment of Fig. 2. In any case, the important point is that within each experiment all the values of *CyIIIa-CAT* genes per embryo tested were saturating with respect to the ability of the batch of embryos to activate the *CAT* genes. Thus, as shown in Fig. 2*b*, the efficiency of CAT enzyme production per *CAT* gene varies inversely with the number of *CAT* genes. We conclude that the *CyIIIa* sequences in these embryos are present in excess of the available factors required for activation, and thus that they compete stoichiometrically with one another. There is little possibility that this competition is not sequence-specific. A general effect such as excess load on the embryonic translation system would seem implausible. The quantity of CAT mRNA produced in the experiment of Table 1, for example, is only ≈1% of the total polysomal embryo mRNA (1) and, by proportionality, that produced in the experiments of Fig. 2*b* would be only 0.15% of total mRNA. About half of the 400 cells of the hr 24 embryo express the *CyIIIa* gene, so that local concentrations of CAT mRNA would not be expected to be more than a few times higher. Furthermore, once they successfully undergo cleavage, most injected embryos develop normally through the blastula period studied in this work, which in the sea urchin embryo requires normal levels of transcription and translation (1).

The interpretation that the *CyIIIa* regulatory sequences of the *CyIIIa-CAT* construct compete for specific factors has been tested directly by coinjecting 5′ *CyIIIa* sequences in excess with respect to the *CyIIIa-CAT* construct. We invariably observe a decrease in CAT enzyme production in such experiments, sometimes directly proportional to the amount of *CyIIIa* sequence added, in comparison to control embryos receiving the same quantity of *CyIIIa-CAT* molecules plus a mass of random sea urchin DNA [or calf thymus DNA equal to that of the CyIIIa competitor sequence (C.N.F., R. Franks, and D. Livant, unpublished data)]. However, though the qualitative observation is reproducible that 5′ *CyIIIa* sequences compete with the homologous sequences mediating CAT synthesis in the *CyIIIa-CAT* fusion construct, further measurements are required to ascertain the stoichiometry of this competition and its effects, if any, on endogenous *CyIIIa* gene activity. Since only 60–80% of any batch of embryos retain the injected DNA (4, 5) and many embryos apparently incorporate the DNA in mosaic fashion, so that they include many cells lacking the fusion gene, competition with the endogenous *CyIIIa* gene might be difficult to measure.

Deletion Constructs Reveal Requirement for *CyIIIa* Regulatory Sequences. The experiments described above imply that *CyIIIa* cis-regulatory sequences are necessary for activation of the *CyIIIa-CAT* construct. To test this directly, the deletion constructs diagrammed in Fig. 3 were injected into unfertilized eggs, and CAT enzyme production was assayed after 24 hr of development. Table 2 presents the results of three experiments, each carried out with a single batch of eggs. In the first and third experiments, the amplification of the deletion constructs was about the same as for the corresponding complete *CyIIIa-CAT* fusion gene controls. In the second experiment, deletion ΔSB, the smallest of the injected fragments, was amplified about one-sixth as much as its control. Nonetheless, all of the amplified DNA quantities fall within the range shown to be saturating in the experiments of Fig. 2, and thus we do not expect *CAT* DNA quantity *per se* to affect CAT enzyme protein per embryo in any of the deletion construct samples. The average amounts of CAT enzyme per embryo produced in the control (i.e., *CyIIIa-CAT*) samples of experiments 1 and 2 of Table 2 lie between those observed in the study summarized in Table 1 (3×10^7 molecules of CAT per embryo) and those

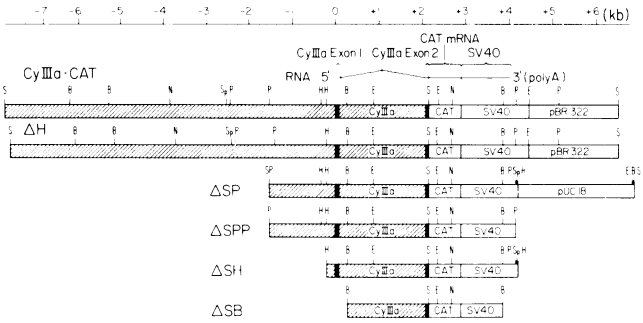

FIG. 3. Deletion constructs of the *CyIIIa-CAT* fusion gene. Restriction enzyme sites are indicated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco* I; P, *Pst* I; S, *Sal* I; Sp, *Sph* I. SV40, simian virus 40; kb, kilobase(s).

measured in the experiments of Fig. 2*b* (5×10^6 molecules of CAT per embryo), while the CAT enzyme level of the control sample in experiment 3 is slightly higher. Thus, none of the control CAT enzyme levels shown in Table 2 are atypical. The results obtained with the deletion constructs demonstrate drastic effects on the levels of CAT synthesis. Deletion of all upstream sequences beyond the *Hin*dIII site at about −250 with respect to the cap site reduces CAT activity to undetectable levels (experiment 1, ΔSH) as effectively as does deletion of the cap site itself plus all upstream sequences (experiment 2, ΔSB). Deletion of sequences to about −1500 decreases CAT synthesis to about 4% of the control level (experiment 1, ΔSPP). A deletion that differs from ΔSPP only in that it still contains the plasmid sequences of *CyIIIa-CAT* displays even less activity (experiment 1, ΔSP), suggesting that the plasmid sequences exercise a negative effect on transcription such as has been observed in other systems. In summary, experiments 1 and 2 of Table 2 suggest that sequences required for activation of *CAT* transcription are located both distally and proximally to the *Pst* I site at −1500. Finally, the fact that removal of the 140-nucleotide *Hin*dIII fragment between −220 and −360 (experiment 3, ΔH) causes a more than 85% decrease in the activity of the otherwise unchanged construct may have further localized one such regulatory region.

DISCUSSION

Activation of the *CyIIIa-CAT* Fusion Gene. The measurements presented here quantify our earlier report (11) that sequences derived from the 5′ region of the *CyIIIa* actin gene suffice to promote ontogenically regulated activation of a

Table 2. CAT enzyme levels after injection of 5′ *CyIIIa-CAT* deletion constructs

Construct*	*CAT* DNA, genes per embryo	CAT enzyme, molecules per embryo	*CyIIIa-CAT* activity, %
	Experiment 1		
CyIIIa-CAT	1.9×10^5	9.5×10^6	100
ΔSP	0.9×10^5	7.1×10^4	0.75
ΔSPP	1.4×10^5	3.7×10^5	3.9
ΔSH	1.1×10^5	$<10^4$	<0.1
	Experiment 2		
CyIIIa-CAT	3.5×10^5	2.6×10^7	100
ΔSB	5.5×10^4	$<10^4$	<0.04
	Experiment 3		
CyIIIA-CAT	1.4×10^6	4.5×10^7	100
ΔH	1.8×10^6	6.2×10^6	14

Approximately 2000 molecules of each construct were injected per egg. About 100 embryos were used per sample. CAT enzyme activity was assayed 24 hr after fertilization.
*See Fig. 3.

CAT "reporter" gene to which they have been ligated. In normal embryos this cytoskeletal actin gene remains silent until after the first 10 hr of development, and it is then transcriptionally activated (refs. 2, 3, 13; J. Lee and F. Calzone, personal communication). We report here that in embryos developing from eggs injected with the *CyIIIa-CAT* construct the CAT enzyme appears at about the time that synthesis of endogenous CyIIIa mRNA begins, that after activation the quantity of CAT enzyme rapidly increases to a level at least 100 times the minimum detectable amount, and that the quantity of CAT mRNA generated from the incorporated *CyIIIa-CAT* genes is several times the quantity of endogenous CyIIIa mRNA (13). The *CyIIIa* gene is normally expressed only in aboral ectoderm cells (2), and *in situ* hybridizations using a CAT antisense RNA probe show that the *CyIIIa-CAT* gene is also expressed only in this cell type (B. Hough-Evans and E.H.D., unpublished work). To a first approximation developmental regulation of the *CyIIIa-CAT* fusion gene would thus seem to provide a valid *in vivo* model for one of the earliest differential gene activations so far observed.

A deduction reported in Table 1 significantly supports this conclusion. The $t_{1/2}$ estimation shown indicates that within the internal milieu of sea urchin embryo cells the CAT enzyme is unstable. The form of the enzyme accumulation curve in Fig. 1 is consistent with this interpretation, and independent confirmation is provided by the early histone gene transfer studies cited above. Thus, after midcleavage the mRNA synthesized from exogenously introduced early histone genes decays and disappears, just as do the endogenous α-histone mRNAs (reviewed in ref. 1). When the histone coding sequence is replaced by the *CAT* gene, the CAT enzyme activity produced at midcleavage similarly disappears (10). It follows that the sharp rise in CAT enzyme shown in Fig. 1 represents a rising rate of synthesis of the enzyme, resulting from translation of an increasing pool of CAT mRNA that is transcribed under control of the *CyIIIa* regulatory sequences. Furthermore, if the enzyme is detectably unstable, to maintain the measured steady state, CAT mRNA translation must occur with an efficiency that is at least a significant fraction of the typical value assumed (1 polypeptide per average mRNA·min; cf. Table 1). This precludes an alternative interpretation for the results reported in Fig. 2*b*—i.e., that the limited, constant amounts of CAT enzyme produced by samples of embryos bearing very different numbers of *CAT* genes could be due to inability of the protein synthesis apparatus to translate this particular message except at extremely low average efficiency.

The Nature of the Activation Mechanism. The following four arguments indicate that the *CyIIIa-CAT* construct and therefore the endogenous *CyIIIa* gene are transcriptionally activated in consequence of interactions with diffusible trans-regulators. First, appropriate expression of the *CyIIIa-CAT* gene, in time and space, occurs despite its undoubtedly ectopic location in the host cell nuclei. As in other animal gene transfer systems the exogenous DNA is very unlikely to be integrated at homologous sequences (5); most of the incorporated genes in any case occupy internal positions in large concatenated molecules, where they are flanked by other such genes (4, 5); and in the early-stage embryos used in this work the exogenous DNA may not yet have integrated at all. Second, the *in vivo* competition experiment shown in Fig. 2*b* and the observation that coinjected *CyIIIa* sequences compete directly with *CyIIIa-CAT* genes to reduce their output of CAT enzyme directly imply the existence of trans-activators that are present in limited quantities compared to the number of exogenous *CyIIIa* sequences per cell. At the lower limit of the curve shown in Fig. 2*b*, where there are $\approx 6 \times 10^4$ *CyIIIa-CAT* genes per embryo, the average number of these genes per functioning aboral ectoderm cell would be ≥ 100 [at 24 hr, half the cells are included in the prospective aboral ectoderm and there are ≈ 200 such cells (1, 2)]. Third, the results of the deletions summarized in Table 2 specify regions of the 5′ *CyIIIa* flanking sequences that are required for activation; i.e., they demonstrate the existence of cis-regulatory elements. Fourth, although we as yet know little about them, we have localized by exonuclease III and DNase I footprint methods several regions of the upstream *CyIIIa* sequence that bind in a specific manner to hr 24 embryo nuclear proteins (16). Some of these regions lie within fragments shown by the deletion experiments of Table 2 to be required for transcriptional activation.

Institution of Differential Patterns of Gene Expression. The genomes of the early embryo nuclei can be regarded as developmentally naive and functionally equivalent (reviewed in ref. 1). If the implications of the present work may be generalized, the initial pattern of differential gene expression would seem likely to depend on the spatial distribution of sequence-specific trans-regulatory factors. These must in some cases be presented exclusively in specific lineage founder cells or in the clonal progeny of these cells. Such factors could be of maternal origin and might be localized before or during early cleavage in given regions of the egg cytoplasm. Or, whether they derive from maternally or zygotically expressed regulatory genes, they could be released or activated by means of inductive intercell interactions. In principle the observations reported here suggest an experimental approach to the exploration of this simple and rather obvious view of the process by which functional cellular diversity first arises, at the outset of the life cycle. That is, this problem may be resolved into the exercise of determining the temporal and spatial distribution and the origins of trans-regulators that are localized to given cells or regions during cleavage.

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