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Differential stability of expression of similarly specified endogenous and exogenous genes in the sea urchin embryo

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Summary

The object of these experiments was to determine whether competitive titration *in vivo* of factors required for expression of the *CyIIIa·CAT* fusion gene would affect expression of the endogenous *CyIIIa* gene in the same embryos. Earlier work showed that expression of this fusion gene after injection into sea urchin eggs is stoichiometrically reduced when low molar excesses of DNA fragments containing only its regulatory domain are coinjected. In order to compare endogenous (i.e. *CyIIIa*) and exogenous (i.e. *CyIIIa·CAT*) expression simultaneously in embryos bearing excess competitor regulatory DNA, we developed, and here describe, a new procedure for generating transgenic sea urchin embryos in which all of the cells in many embryos, and most in others, bear the exogenous DNA. Such large reduction of mosaicism can be achieved by multiple injection of the exogenous DNA fragments into fertilized eggs. Using this method, we demonstrate that at a level of competitor DNA incorporation which reduces *CyIIIa·CAT* expression by 85%, endogenous *CyIIIa* mRNA levels are wholly unaffected. Nor is spatial expression of the endogenous *CyIIIa* gene disturbed. Since the *CyIIIa·CAT* genes are properly expressed under control of the *CyIIIa* regulatory sequences, they must participate in the same set of necessary DNA–protein interactions. However, we infer from the results that we report here that the regulatory complexes in the endogenous *CyIIIa* gene are greatly stabilized relative to those of the exogenous *CyIIIa·CAT* genes.

Key words: *in vivo* competition, commitment, specification, gene transfer, gene regulation in early development.

Introduction

The early blastula of the sea urchin *Strongylocentrotus purpuratus* consists of five embryonic territories, each composed of a unique set of clones, and each displaying a unique developmental fate (Davidson, 1989). The aboral ectoderm is at the molecular level one of the best known of these territories, and is composed of the progeny of 11 founder cells that segregate from other lineages at 3rd–6th cleavage (Cameron *et al.* 1987, 1990). An early molecular marker of aboral ectoderm specification is expression of the cytoskeletal *CyIIIa* actin gene, which begins at least as early as 8th cleavage (Hickey *et al.* 1987; Lee, 1986; Cox *et al.* 1986; Shott *et al.* 1984). The *CyIIIa* gene is expressed throughout the aboral ectoderm during the whole of embryogenesis (Cox *et al.* 1986), and continues to be active until metamorphosis (Cameron *et al.* 1989). However, *CyIIIa* transcripts are not detectable in the juvenile or adult sea urchin (Shott *et al.* 1984).

Regulatory interactions governing *CyIIIa* gene expression have been studied *in vivo* using a fusion construct, *CyIIIa CAT*, in which the *CyIIIa cis*-regulatory domain controls the expression of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene (Flytzanis *et al.* 1987; Hough-Evans *et al.* 1987, 1988, 1990; Franks *et al.* 1990). The *cis*-regulatory domain required to induce the expression of *CyIIIa·CAT* on the same temporal schedule, and in the same spatial elements of the embryo, as the endogenous *CyIIIa* gene extends about 2.3 kb upstream from the transcriptional start site. This domain includes about 20 specific sites of DNA–protein interaction, mediated by 12 distinct factors (Thézé *et al.* 1990; Calzone *et al.* 1988; Thiebaud *et al.* 1990). Many of these factors have been partially or extensively characterized biochemically. Some are known to function positively and some negatively; some are involved with spatial, some with temporal, and others with amplitude regulation of *CyIIIa* transcription (Franks *et al.* 1990; Hough-Evans *et al.* 1990; Calzone *et al.* 1991; Höög *et al.* 1991; reviewed by Davidson, 1989). An interesting experimental feature of the sea urchin embryo is that both positive and negative functions can be competitively

titrated *in vivo* by coinjection with *CyIIIa · CAT* of fragments of the regulatory domain containing target binding sites for given regulatory factors (Franks *et al.* 1990; Hough-Evans *et al.* 1990). If the whole *CyIIIa* regulatory domain needed for correct expression is co-injected with *CyIIIa · CAT*, CAT enzyme activity is decreased almost stoichiometrically, as a function of the injected molar ratio of competitor to *CyIIIa · CAT* (Livant *et al.* 1988). This result at once raises the issue of whether expression of the *endogenous CyIIIa* genes is correspondingly depressed on introduction of the excess regulatory target sites. In order to address this question experimentally, we had first to circumvent the extensive mosaicism resulting when DNA is introduced into sea urchin eggs by a single microinjection of several thousand molecules of linearized DNA, here *CyIIIa · CAT* DNA (McMahon *et al.* 1985; Flytzanis *et al.* 1985; Hough-Evans *et al.* 1988). We had shown that, after a single injection, in 75% of embryos the exogenous DNA is concatenated and stably incorporated into a blastomere following 2nd, 3rd or 4th cleavage, resulting in a number-average frequency of only about 6% of nuclei per embryo that contain exogenous DNA concatenates. In order to measure experimentally any effects of competition on expression of the endogenous *CyIIIa* gene the competitor must obviously be stably incorporated in a far greater fraction of cells. As described below, we discovered that, after 3 or 4 separate injections postfertilization, many embryos incorporate exogenous DNA in all their nuclei, and others in most nuclei. We provide an analysis of exogenous DNA incorporation mechanics in embryos subjected to the new multiple injection procedure, and show that *CyIIIa · CAT* expression in multiply injected embryos remains temporally and spatially correct. We then present simultaneous measurements of CAT enzyme and *CyIIIa* actin mRNA levels in the same multiply injected embryos. These measurements showed that under conditions in which 85% of control *CyIIIa · CAT* expression was abolished by competition with a several-fold excess of *CyIIIa* regulatory domain DNA, endogenous *CyIIIa* gene expression remained entirely unaffected. *In situ* hybridization confirms that the endogenous *CyIIIa* gene continues to be expressed in all aboral ectoderm cells, and no other cells, in the competition embryos. Since both endogenous and exogenous regulatory systems are specified correctly for expression, they both must participate in the same necessary regulatory interactions. However, these interactions are evidently greatly stabilized in the endogenous gene, by a mechanism that if understood might provide a clue to the phenomenon known to embryologists as 'commitment.'

Materials and methods

Eggs, embryos and multiple injection procedure

Eggs of *Strongylocentrotus purpuratus* were collected and prepared for injection as described previously (McMahon *et al.* 1985). Animals were induced to shed by 12.5 V electrical stimulation or intracoelomic injection of 0.5 M KCl. Eggs were filtered through gauze and treated for a few minutes with pH 5 filtered sea water (FSW) to remove egg jelly. Unfertilized eggs were then moved to Petri dishes that had been treated for 1 min with a 1% solution of protamine sulfate. The eggs were placed in rows in FSW containing 82 mg l^{-1} aminotriazole (ATA), an ovoperoxidase inhibitor, in order to prevent hardening of the fertilization membrane. Eggs were fertilized by the addition of activated (diluted) sperm immediately before microinjection. The zygotes were microinjected at intervals of 4 to 8 min with approximately 2 pl of DNA solution per injection containing 0.1 pg of DNA. After injection, ATA-containing FSW was replaced by FSW containing 20 units of penicillin and 50 μg of streptomycin per ml (PSSW). Embryo samples were taken at 8–24 h postfertilization for CAT assays, *in situ* hybridization to squashed blastula nuclear DNA, slot blot assays, and probe excess RNA titration. Embryos were cultured to 72 h (early pluteus) in PSSW at 15 °C for *in situ* hybridization to CAT mRNA.

Microinjection solutions contained 40% glycerol, 0.12 M KCl, the fusion gene *CyIIIa · CAT* (Flytzanis *et al.* 1987) and, in some experiments, *Sph*I-cut sea urchin DNA size selected to have an average length of 10 kb as carrier (Livant *et al.* 1988) and a 9.3 kb *Sal*I fragment from the CyIIIa gene. Injection solutions contained from 600 to 3000 molecules per pl of *CyIIIa · CAT* DNA linearized with *Sph*I. In the competition experiments, varying amounts of the 9.3 kb *Sal*I fragment containing the entire regulatory domain of the *CyIIIa · CAT* gene and enough carrier DNA to make the final DNA concentration 0.1 pg per 2 pl were also included in the injection solution.

Measurement of CAT enzyme activity and CyIIIa · CAT DNA content per embryo

CAT enzyme activity per embryo was measured in samples of 30 to 80 24 h blastulae, by a method derived from that of Gorman *et al.* (1982), as described by McMahon *et al.* (1984). The number of *CyIIIa · CAT* DNA molecules per embryo was measured in samples of 30 to 80 24 h blastulae by quantitative slot blot hybridizations as previously described (Flytzanis *et al.* 1987).

Cytological preparations

Preparation of embryo sections and microscope slides were as described previously (Hough-Evans *et al.* 1987, based on the method of Cox *et al.* 1984). Embryos that developed from eggs that had been microinjected with *CyIIIa · CAT* were fixed at the 72 h pluteus stage in 2% paraformaldehyde, 0.5% glutaraldehyde in buffer containing 2.5% NaCl, 25 mM phosphate buffer pH 7.5. The embryos were enclosed in agarose boxes, embedded in Tissueprep 2 (Fisher Scientific), and sectioned at 5 μm. The serial sections were placed on polylysine-coated slides.

Squashes were prepared by placing blastulae in PSSW on a polylysine-coated slide and flattening them under a siliconized coverslip by gradually drawing off the sea water.

In situ *hybridization*

The single-stranded antisense CAT RNA hybridization probe used here was transcribed as described previously (Hough-Evans *et al.* 1987) from a fragment of CAT coding sequence inserted into the polylinker of the pSP65 vector, using ^{35}S-labeled UTP, specific activity 800 Ci mmol^{-1}. *In situ* hybridization was carried out as in earlier experiments by the procedures of Angerer and Angerer (1981) and Cox *et al.* (1984), modified for ^{35}S-labeled probes by the addition of 5 mM dithiothreitol and 1% β-mercaptoethanol to the hybridization solution and 1% β-mercaptoethanol to the

wash solutions. Hybridization was carried out in an N_2 atmosphere. The photographic emulsion (Kodak NTB-2) was diluted 1:1 with distilled water. Exposure was for 4 days.

For hybridization to the DNA of squashed blastulae, CAT coding sequence inserted in the opposite orientation in the pSP64 vector was used to transcribe a sense strand RNA probe labeled with ^{35}S-UTP. The hybridization procedure included deproteinization at 10 μg ml^{-1} of proteinase K (compared to 1 μg ml^{-1} in the RNA–RNA hybridization) and denaturation of the cellular DNA by immersing the slides in 95% formamide, 0.1×SSC for 15 min at 65°C (Hough-Evans *et al.* 1988). Cells labeled with five or more grains were considered to be positive. The fraction of cells that were labeled was estimated (as a percent) for each blastula (usually ~25) in every experiment. Variations in grain density are to be expected, due to local differences in thickness of the squash and/or the emulsion.

Probe excess RNA titration

Total RNA was isolated from 500–1000 blastulae, which had been counted, pelleted and frozen dry at −70°C, using a urea sarcosyl buffer (Lee *et al.* 1986) as described by Sucov *et al.* (1988). An Sp6-transcribed RNA probe labeled with 40 Ci mmol^{-1} ^{3}H-UTP corresponding to 1300 nucleotides of unique M actin 3′ sequence (described by Lee *et al.* 1984, 1986) was introduced at the urea solubilization step as an internal control for preparative yield of RNA. Probe excess titration was carried out as described by Lee *et al.* (1986) using an Sp6-transcribed RNA probe for *CyIIIa* mRNA. This 131 nucleotide probe is described by Lee *et al.* (1984, 1986).

Model for mechanism of exogenous DNA incorporation and mathematical treatment of incorporation data

To rationalize the exogenous DNA incorporation data shown in Figs 2 and 3, we formalized our hypothesis for the incorporation process. On the basis of evidence obtained from studies of singly-injected eggs, the following conditions are assumed: (*i*) that once incorporated, most exogenous DNA is retained in subsequent development in the nuclei of the lineage element descendant from a blastomere in which the incorporation event occurred, and that it replicates together with the host cell genomic DNA; (*ii*) that concatenates are rapidly formed and all the DNA injected is partitioned among them; and (*iii*) that individual incorporation events within an embryo, i.e. in different blastomeres, are independent and of low probability, so that the occurrence of these events can be described by a Poisson function. The incorporations apparently occur very early in cleavage, while our observations on stable incorporation frequencies were made on squashes of 24 h, 400-cell embryos. Thus, in the following treatment, we assume that all cell lineages divide essentially the same number of times during cleavage. While this is not precisely correct, the deviations are quantitatively insignificant for our purposes (see Davidson, 1986, pp. 213–218; Cameron *et al.* 1987).

We define the following parameters:

p: The probability of incorporation per concatenate, and per nucleus. **p** is treated as a batch-dependent biological characteristic. An analysis of single injection data (Hough-Evans *et al.* 1988) yields a value for **p** of ~0.034, whether incorporation occurred at 2nd, 3rd or 4th cleavage.

C: The number of concatenates per egg (formed after all three injections). **C** is conceived as a major variable distinguishing one batch of eggs from another.

X: The number of blastomeres of a cleavage stage embryo in which a nuclear incorporation event occurs; $\bar{\mathbf{X}}$ is the average number of cells/embryo in which an incorporation is detected in a batch of embryos.

n: The cleavage stage (i.e. 1st, 2nd, 3rd, etc.) at which given incorporation events occur; the number of cells or nuclei at this stage is $\mathbf{2^n}$.

G: The fraction of cells in an embryo at any given stage after incorporation has occurred which display exogenous DNA; $\bar{\mathbf{G}}$ is the average fraction of cells displaying exogenous DNA per batch.

I: Number of molecules of exogenous DNA initially injected (total for all injections) per egg.

$\bar{\mathbf{D}}$: Average number of molecules of exogenous DNA recovered per cell that contains exogenous DNA; for a given batch of embryos $\bar{\mathbf{D}}$ is calculated as average total exogenous DNA per 24 h embryo, measured by slot blot hybridization, normalized to the parameter (400 $\bar{\mathbf{G}}$). Here $\bar{\mathbf{G}}$ is calculated from measurements on squashed 24 h embryos.

From the definitions of **p**, **C** and **n**, the mean frequency of an incorporation at the **nth** cleavage is:

$$\bar{\mathbf{X}} = \mathbf{pC2^n} \tag{1}$$

and for a given batch and a given cleavage at which incorporation occurs, the numerical *distribution* of embryos containing 2^n cells, displaying exogenous DNA in different numbers of these cells, i.e, **P(X)** is approximated by:

$$\mathbf{P(x)} = \frac{\mathbf{e^{-pC\cdot 2^n}(pC\cdot 2^n)^x}}{\mathbf{x!}} \tag{2}$$

I is set experimentally. Assumption *ii* and *iii* above, and our prior analyses of single injection embryos, indicate that in general only one concatenate will be stably incorporated per nucleus. Thus the measured value of $\bar{\mathbf{D}}$ is as follows:

$$\bar{\mathbf{D}} = \frac{\mathbf{I}}{\mathbf{C}}; \text{ or } \mathbf{C} = \frac{\mathbf{I}}{\bar{\mathbf{D}}} \tag{3}$$

The latter provides an estimate of the value of **C** for any given batch. However, for batches where there are a very large fraction of embryos in which all cells display the exogenous DNA, it is likely on random expectation that some of these cells will contain more than one stable integration. Thus the values of **C** calculated from eq.(3) for such cases may be larger than actually were present.

We wish to relate $\bar{\mathbf{D}}$ with $\bar{\mathbf{G}}$, in order to understand the relationship shown in Fig. 3. From the definition of $\bar{\mathbf{G}}$ and the assumptions of equivalent divisions in all lineages and no significant loss of incorporation DNA, at the nth cleavage when incorporation events occur:

$$\bar{\mathbf{G}} = \frac{\bar{\mathbf{X}}}{\mathbf{2^n}} \tag{4}$$

Substituting equation (3) into (4), we have

$$\bar{\mathbf{D}} = \frac{\mathbf{pI}}{\bar{\mathbf{G}}}, \tag{5}$$

which is of the form $\bar{\mathbf{D}} = \frac{\mathbf{b}}{\bar{\mathbf{G}}}$, eq.(1) of text.

Using equations (1) and (4), we find that the meaning of the proportionality constant **b** is:

$$\mathbf{b = pI} \tag{6}$$

This hyperbolic relation between $\bar{\mathbf{D}}$ and $\bar{\mathbf{G}}$ (Fig. 3) is expected, because as **I** is divided into more concatenates there is a proportional *decrease* in DNA/concatenate (eq. 3), but for

given **n** and **p** a proportional *increase* in the number of cells expected to undergo incorporation (eq. 1).

From eq. (3) $\frac{\mathbf{I}}{\mathbf{C}} = \frac{\mathbf{b}}{\mathbf{G}}$.

Results

Exogenous DNA is incorporated in a large fraction of embryo nuclei after multiple injection into fertilized egg cytoplasm

In a series of preliminary experiments, we compared embryos from the same batch of eggs that developed after one, two or four rounds of microinjection. To determine the fraction of nuclei that had stably incorporated *CyIIIa · CAT* DNA after injection into the cytoplasm of fertilized or unfertilized eggs, the embryos were raised to mesenchyme blastula stage (24 h), squashed and the chromosomal DNA hybridized *in situ* with a *sense* strand RNA probe representing a portion of the CAT coding sequence (Hough-Evans *et al.* 1988; see Materials and methods). Fig. 1 displays squashes of four transgenic embryos. Those in panels A and B developed from fertilized eggs that had received a total of 0.4 pg of linearized *CyIIIa · CAT* DNA (the *CyIIIa · CAT* fusion is 14 kb long), delivered in four 0.1 pg injections. For comparison the typical embryo shown in panel C received about 0.1 pg of exogenous DNA, delivered in a single prefertilization injection. In this particular experiment, the injection aliquot contained *CyIIIa · CAT* DNA and ~10 kb long sea urchin DNA carrier in a molar ratio of 1:5. After a single injection, the exogenous DNA is very efficiently concatenated with the carrier, and incorporation frequency is not affected by the presence of carrier DNA (McMahon *et al.* 1985; Franks *et al.* 1988, 1990; Hough-Evans *et al.* 1988; data presented in this paper). Essentially all of the nuclei (>95 %) in the embryos of panel A are labeled with the ^{35}S-CAT probe and thus contain CyIIIa · CAT DNA. The embryo in panel B is about 50 % labeled, and the embryo shown in panel C is about 6 % labeled (19 out of ~320 nuclei). As shown in Table 1, less than 10 % of the injected DNA is actually incorporated and replicated in cells of the embryos that develop from multiply injected eggs.

The high frequency of stable nuclear incorporation of injected DNA shown in Fig. 1A and B is not unusual for embryos obtained from eggs injected three or four times postfertilization (hereafter designated as 3× or 4× injected eggs). These particular blastulae derived from the same batch of 4× injected eggs; 92 % of the nuclei in this batch of embryos displayed exogenous DNA at 24 h of development. At least three postfertilization cytoplasmic injections are necessary to obtain this significantly enhanced incorporation of exogenous DNA. Three injections produce maximum results, and the fraction of nuclei that display incorporated exogenous DNA at the blastula stage in 3× injected eggs is

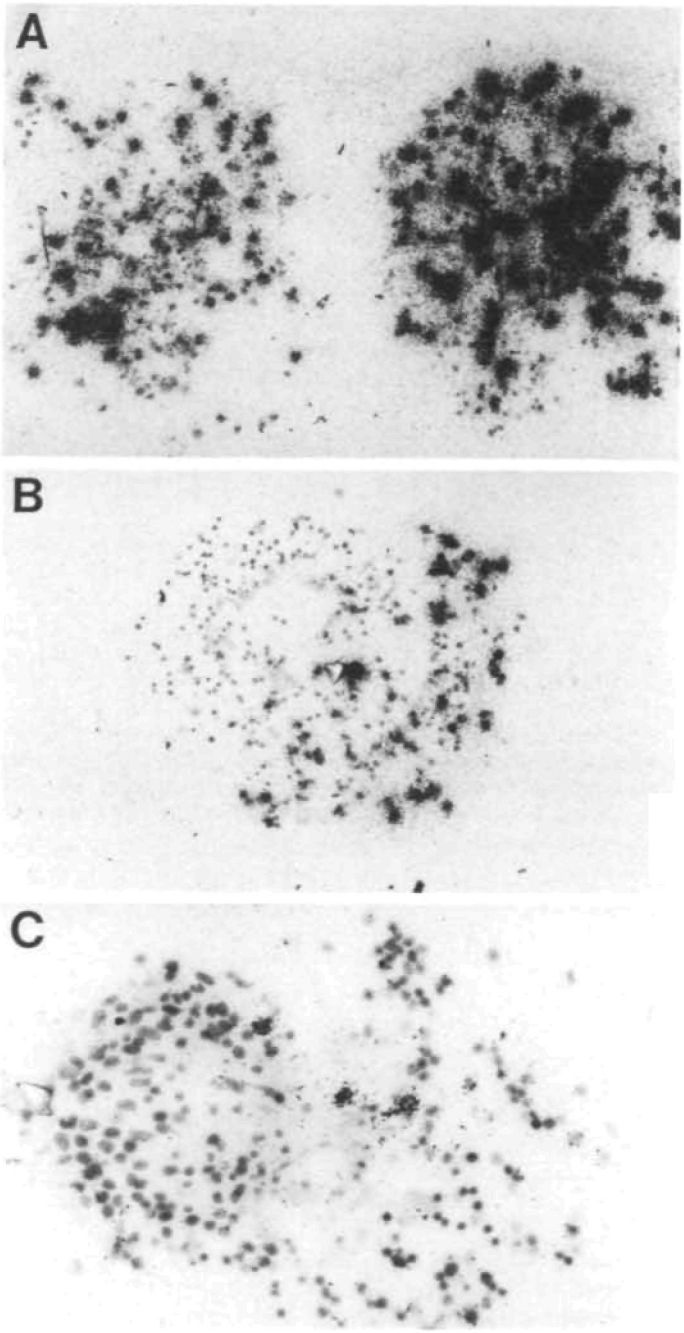

Fig. 1. Typical levels of mosaicism in blastulae raised from multiply *versus* singly injected eggs. Embryos were squashed, stained with hematoxylin and eosin, and hybridized as described in Materials and methods and by Hough-Evans *et al.* (1987). Embryos in panels A and B were obtained from eggs receiving four, 2 pl cytoplasmic injections approximately 5′ apart, beginning immediately after fertilization (see Materials and methods). The injection solution contained 3000 *Sph*I-linearized *CyIIIa · CAT* molecules per pl in 0.12 M KCl and 40 % glycerol. The embryo shown in panel C was obtained from an egg receiving one, 2 pl cytoplasmic injection prior to fertilization. The injection solution contained 750 *Sph*I-linearized *CyIIIa · CAT* molecules per pl and a five-fold molar excess of *Sph*I cut, 10 kb average length sea urchin carrier DNA in 40 % glycerol. Dark grains are visible over those nuclei in which the DNA hybridized to a *sense* riboprobe for CAT DNA sequences. Embryos were photographed at 200× magnification.

Table 1. *Parameters derived from measurements of frequency of incorporation of exogenous DNA, and amounts in 3× injected embryos*

Batch		I	$\bar{D}$	$\bar{G}$	C	p′	f	n
1	(C)	24000	590	0.93	40.7	0.14	1	1
2		32000	1550	0.24	20.6	0.017	1	1
3		32000	280	0.61	7.5	0.040	0.40	1
					185	0.040	0.60	1
4		24000	1550	0.45	26.9	0.033	0.34	2
					9.6	0.033	0.66	3
5		24000	880	0.17	2.7	0.077	1	2
6	(D)	24000	2100	0.34	11.4	0.034	1	2
7		24000	1350	0.63	43	0.12	0.35	1
					3.9	0.12	0.65	2
8		24000	1300	0.63	18.5	0.047	1	2
9		24000	260	0.86	92.3	0.036	1	1
10		24000	1350	0.72	17.8	0.11	1	1

The distribution of incorporation frequencies for Batch 1 is shown in Fig. 2C and for Batch 6 in Fig. 2D. All ten batches of 3× injected embryos for which there were available both nuclear incorporation (G) frequency distributions and average CAT DNA content per cell bearing CAT DNA ($\bar{D}$) are included in Table 1. The parameters heading the columns are defined in eq. (1)–(6) of Methods, except for the following: **f** indicates fraction of embryos in which an incorporation event occurred with the indicated C values (respectively, in cases where two **f** values are listed for a given sample). **p′** is the estimate of **p** (see eq. 1 of Methods) obtained from these least squares reductions of the measured *distribution* data (i.e., the fractions of each batch in which given fractions of nuclei bear exogenous DNA), though **p** as in eq. (1) might best be calculated from the batch average parameters by using eq. (6). In fact the two estimates agree reasonably except for the case of batch 1 where the incorporation frequency was so high that there were undoubtedly multiple incorporations per nucleus. Model calculations were carried out using $\mathbf{n}=1, 2, 3,$ or 4 for each set of embryos, and the best fit values of **pC** (see eq. 7 below) were obtained. Values of **C** were then calculated by eq. (3), and hence **p** and **C** were reported for each value of **n**. *Measured* ($\mathbf{f}_{(G)}$) distributions and *calculated* ($\mathbf{P}_{(G)}$) distributions were compared by grouping each distribution in $(\mathbf{2^n+1})$ equal bins (one bin for the zero incorporation class). To calculate $\mathbf{P}_{(G)}$ we used eq. (2), to derive $\mathbf{P}_{(x)}$ where $\mathbf{X}=0, 1, 2, 3, 4,$ etc., cells out of the 2^n cells present. In order to compare $\mathbf{f}_{(G)}$ **to** $\mathbf{P}_{(x)}$, both frequency distributions must sum to the same total value, and thus the reported $\mathbf{P}_{(G)}$ distributions were obtained by normalizing the $\mathbf{P}_{(x)}$ distribution by the sum of the $\mathbf{P}_{(x)}$ values, i.e.,

$$\mathbf{P}_{(G)} \equiv \frac{\mathbf{P}_{(x)}}{\sum_{x} (\mathbf{P}_{(x)})}.$$

This provides a direct comparison of the observed ($\mathbf{f}_{(G)}$) frequencies to the predicted approximate distribution $\mathbf{P}_{(x)}$. The main result is that in all but one batch in Table 1 incorporation of the exogeneous DNA at *either* first *or* second cleavage was the most consistent with the observed distributions. Usually this could immediately be determined by inspection, though we utilized a quantitative index of the deviation between measured $\mathbf{f}_{(G)}$ and calculated $\mathbf{P}_{(G)}$ distributions: $\delta=\sqrt{\Sigma(\mathbf{P}_{(G)}-\mathbf{f}_{(G)})}$. The values reported in Table 1 were those resulting in minimal values of δ. In batches 1, 2, 3, 7, 9 and 10 of Table 1 incorporation appeared to have occurred at 1st cleavage, though in batch 3 there were apparently two kinds of eggs, characterized by different proclivity to form concatenates (this is of course a statistical resolution and there could have been many intermediate subclasses in batch 3). In batches 5, 6 and 8 incorporation evidently occurred at second cleavage. Batches 4 and 7 required the assumption of two successive rounds of incorporation, as indicated.

indistinguishable from that of blastulae obtained from 4× injected eggs (data not shown). In other experiments (data not shown), we confirmed that enhanced incorporation does not occur after a single injection delivered 30 min *after* fertilization (4× injection of 50–100 eggs takes about 30 min), nor does increase in the mass of DNA injected from 0.1 to 0.4 pg in itself produce enhanced incorporation. Many batches of eggs are damaged by loads of injected DNA greater than this (McMahon *et al.* 1985). We also discovered that multiple injection of DNA *prior* to fertilization yields no enhancement in the frequency of nuclear incorporation compared to single injection (data not shown). The overall mortality of embryos developing from multiply injected eggs is similar to that of embryos developing from singly injected eggs, and many of the resulting larvae survive metamorphosis to become fertile adults (B.Evans and D.Livant, unpublished data).

The eggs of individual females respond differently to multiple injection. Fig. 2 describes the results of experiments on 10 separate batches of eggs obtained from different females and injected 3× or 4× postfertilization, with 0.1 pg of linearized *CyIIIa·CAT* and carrier per injection. As before, the blastulae were squashed at 24 h of development, and individually scored for the fraction of labeled nuclei displayed by *in situ* hybridization to the ^{35}S-labeled CAT probe. Panel A shows the composite nuclear incorporation profile obtained by combining all ten analyses. A total of 238 blastulae were assayed, and an *average* of 59% of the embryo nuclei contained *CyIIIa·CAT* DNA. Overall, 91% of the embryos scored had at least some nuclei containing exogenous DNA, and 29% of all blastulae contained *CyIIIa·CAT* DNA in >95% of nuclei. Similar results have been obtained with constructs other than *CyIIIa·CAT* (data not shown). For comparison, in Fig. 2B we show a similar composite nuclear incorporation profile, obtained from analyses of 24 h blastulae raised from several batches of eggs injected once prior to fertilization with about 0.1 pg of linearized *CyIIIa·CAT* plus carrier DNA (data from Hough-Evans *et al.* 1988). A total of 79 blastulae were assayed, and an average of 6% of the nuclei contained *CyIIIa·CAT* DNA. The *highest fraction* of nuclei bearing exogenous DNA in an embryo derived from a

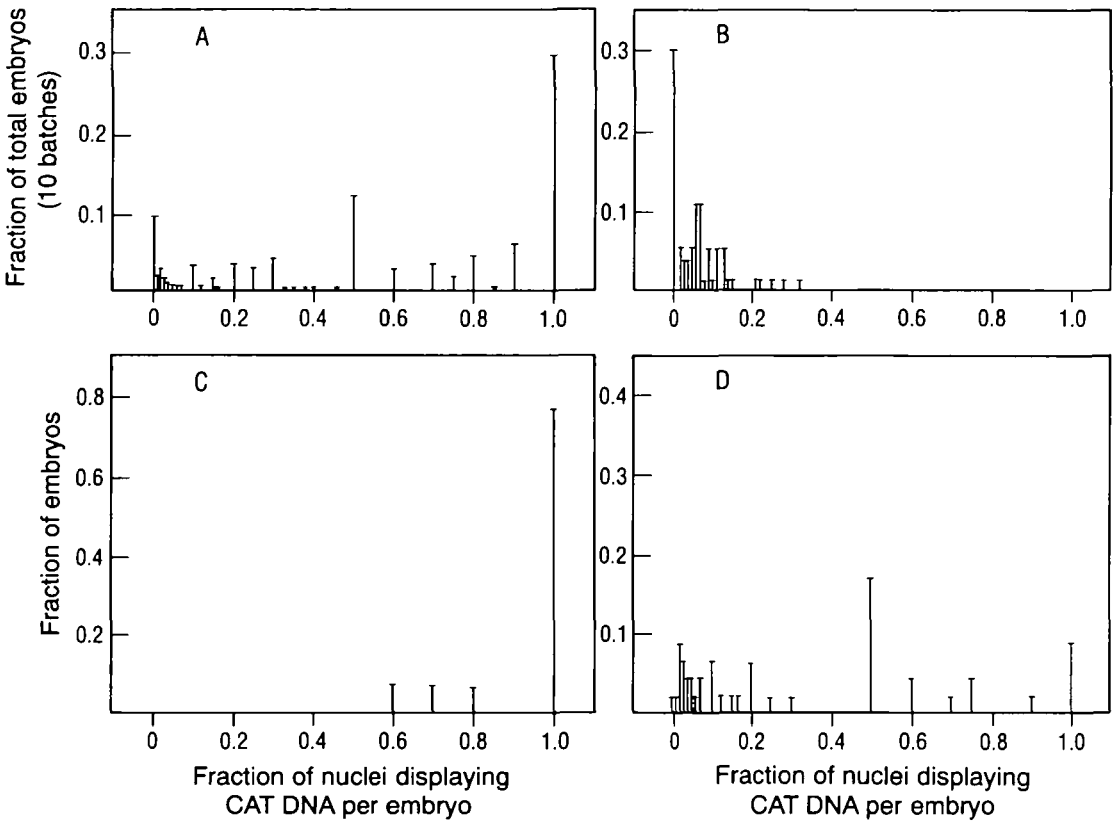

Fig. 2. Nuclear incorporation profiles for transgenic blastulae. In all four panels, the fraction of embryos obtained in each interval is plotted *versus* the fraction of nuclei displaying CAT DNA per embryo in intervals of 1%. (A) Composite profile for blastulae obtained from the 3× or 4× injected eggs of ten different females with each batch weighted equally. Eight of the ten batches received three postfertilization injections at approximately 5 min intervals each, of 2 pl. The injection solution used for these eight batches contained 600 *Sph*I-linearized CyIIIa · CAT molecules and 3360 molecules of *Sph*I linearized, 10 kb average size sea urchin carrier DNA per pl, in 0.12 M KCl and 40% glycerol. One batch received four postfertilization injections of the same injection solution, and one batch received four postfertilization injections of a solution containing 3000 *Sph*I linearized CyIIIa · CAT molecules per pl in 0.12 M KCl and 40% glycerol. (B) Composite profile for blastulae obtained from the singly injected eggs of five females. These eggs were injected with 2 pl of an injection solution containing 750 *Sph*I linearized CyIIIa · CAT molecules, and a fivefold molar excess of 10 kb carrier DNA per pl, in 40% glycerol. (C) Incorporation profile for one batch of blastulae obtained from 3× injected eggs. Thirteen blastulae were analyzed. (D)Incorporation profile for one batch of blastulae obtained from 3× injected eggs. Forty-five blastulae were analyzed.

singly-injected egg was 32%. Fig. 2 demonstrates that blastulae raised from 3× or 4× injected fertilized eggs have approximately ten times as many nuclei containing exogenous DNA as those obtained from singly-injected eggs. Figs 2C,D illustrate the batch variation that we observed in comparing the response of eggs of the 10 females to multiple injection. In the batch of blastulae represented in panel C, 78% of all embryos contained CAT DNA in >95% of nuclei, and the overall fraction of nuclei displaying the exogenous DNA was 93%. Panel D shows a batch of blastulae in which only 9% of all embryos contained exogenous DNA in >95% of nuclei, and here the overall fraction of positive nuclei was just 34%. In the ten batches of embryos assayed, the average fraction of nuclei displaying CAT DNA per embryo *varied* from 17% to 93%; and the calculated fraction of embryos in which >95% of nuclei contained exogenous DNA varied from 2.6% to 78%.

Relation between amount of exogenous DNA incorporation and fraction of nuclei bearing exogenous DNA

DNA was extracted from samples obtained from 14 individual batches of embryos raised from multiply injected eggs, and hybridized with the CAT probe in order to determine the average quantity of exogenous DNA per embryo in each batch. These batches included the 10 represented in the composite summary of Fig. 2A. Twelve batches were derived from 3× injected eggs and two from 4× injected eggs. Embryos from each batch were also individually squashed and examined by *in situ* DNA hybridization to determine the distribution of fractions of cells per embryo bearing exogenous DNA in each batch. From these data, we could thus calculate for each batch the *average fraction of cells bearing exogenous DNA* ($\bar{G}$) and knowing the total cell number (400 cells/embryo) *the average*

amount of CyIIIa · CAT DNA per cell containing this DNA ($\bar{D}$). In Fig. 3 we show a plot of $\bar{D}$ *vs* $\bar{G}$, fit by a simple hyperbolic function, i.e.

$$\bar{D} = \frac{b}{\bar{G}} \quad (1)$$

The data from 12 out of the 13 3× injection batches are fit surprisingly well by this function (root mean square error is about 10% of the values of $\bar{D}$; we did not pool the 4× with the 3× injection data for the analysis shown, but all the data are evidently consistent with the form depicted). Thus it appears that there is a strong *inverse* proportionality between the (average) amount of CAT DNA incorporated per cell and the (average) number of cells bearing this DNA, so that the largest (average) amounts of CAT DNA per cell occur in those batches in which incorporation is least widespread. We present a plausible mechanism for the incorporation

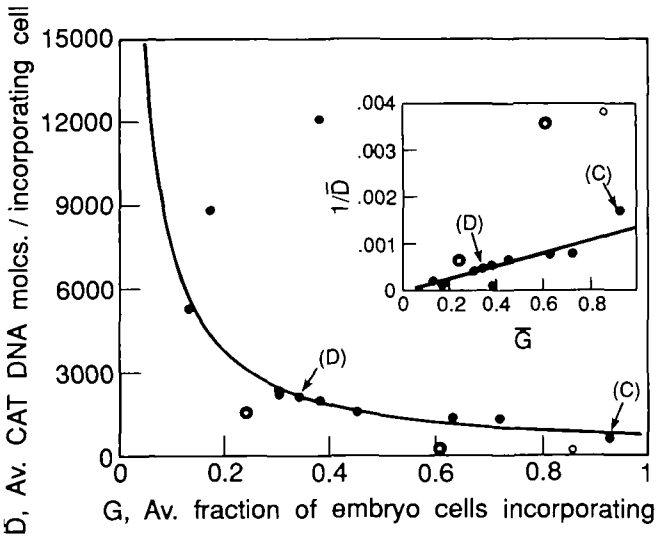

Fig. 3. Average CAT DNA content per cell displaying CAT DNA, $\bar{D}$, as a function of average frequency of cells displaying CAT DNA, $\bar{G}$. Each point represents measurements on one of the 15 individual batches of eggs studied (see text). The curve shown was fit by least squares to the linear transformation of eq.(1) of text, as shown in the insert. The three open points were not included in the fit; two represented the 4× injection batches (double circles; batches 2 and 3) and one unfilled (at $\bar{G}$=0.86; batch 9) perturbed the evaluation because it differed from the curve by >two standards of deviation. (For the algebraic meaning of the constant **b** in eq.(1) see Materials and methods.) However, even if all 15 points are included, the least squares solution changes little in either location or form from that shown. Points represented by the distributions shown in Fig. 2C and D are labeled 'C' and 'D' respectively. The batches of blastulae represented by filled-in points and the one unfilled point were obtained from eggs receiving three postfertilization injections, nominally 2 pl each. Double circled points represent batches of blastulae obtained from 4× injected eggs. Injection solutions contained 600 *Sph*I-linearized CyIIIa · CAT molecules, and 3360 10 kb, size-selected, *Sph*I-cut carrier DNA per pl in 0.12 M KCl and 40% glycerol.

process, and from this a derivation of eq.(1), in Methods. The parameters of this model are evaluated for ten multiply injected batches of embryos as summarized in Table 1. This analysis suggests that the two major variables among different batches of eggs are the number of concatenates formed from a given amount of injected DNA, and the probability of stable incorporation per concatenate nucleus. Most 3× injection batches may produce 20–100 concatenates per embryo, each having a probability of stable nuclear incorporation ranging from 0.02 to 0.14 (average 0.065) per concatenate nucleus. The most important conclusion is that for most batches, the data can be adequately accounted for on the assumption that incorporation first occurs in the nuclei of 2- to 4-cell embryos. It is unlikely that incorporation occurs before 1st cleavage because of the presence of the nuclear membrane. First or second cleavage incorporation of a concatenate should not preclude later incorporation events in the same embryos, particularly in those where large numbers of concatenates are present, but these events would involve relatively fewer cells and their effects would be superimposed on the quantitatively dominant effects of the earlier incorporation events.

Multiple injections probably produce embryos in which many or all cell nuclei bear exogenous DNA because large numbers of exogenous DNA concatenates are formed during the injection process. This might be due to physical disturbance of the egg cytoplasm, to the deposition of the DNA in several different locations in the egg, or to a physiological response to multiple injection that otherwise affects ligation efficiency. Possibly cytoskeletal changes occasioned by fertilization are important, since multiple injection into unfertilized eggs is ineffective in promoting high frequency incorporation. In any case, the result of postfertilization multiple injections is to greatly enhance the likelihood of incorporation in all or in a large fraction of early cleavage nuclei, though of smaller amounts of DNA per nucleus. The multiple injection procedure satisfies our requirement for a method that would enable exploration of the effects on endogenous genes of regulatory target site competition.

Temporal and spatial regulation of the CyIIIa · CAT gene in multiply injected embryos

The time course of normal *CyIIIa* transcript accumulation during embryonic development has been determined by RNA gel blot (Shott *et al.* 1984) and probe excess titration (Lee *et al.* 1986). Newly synthesized *CyIIIa* gene transcripts are undetectable until 10 h postfertilization, but thereafter they accumulate rapidly up to about 8×10^4 molecules per embryo (the unfertilized egg contains about 2×10^3 molecules of maternal *CyIIIa* mRNA). Flytzanis *et al.* (1987) showed that the time course of appearance of CAT enzyme activity in embryos raised from singly injected eggs parallels that of endogenous *CyIIIa* mRNA accumulation. To ascertain that this would also be the case for 3× injected eggs, we carried out the experiment shown in Fig. 4. Here it is shown that the kinetics with which

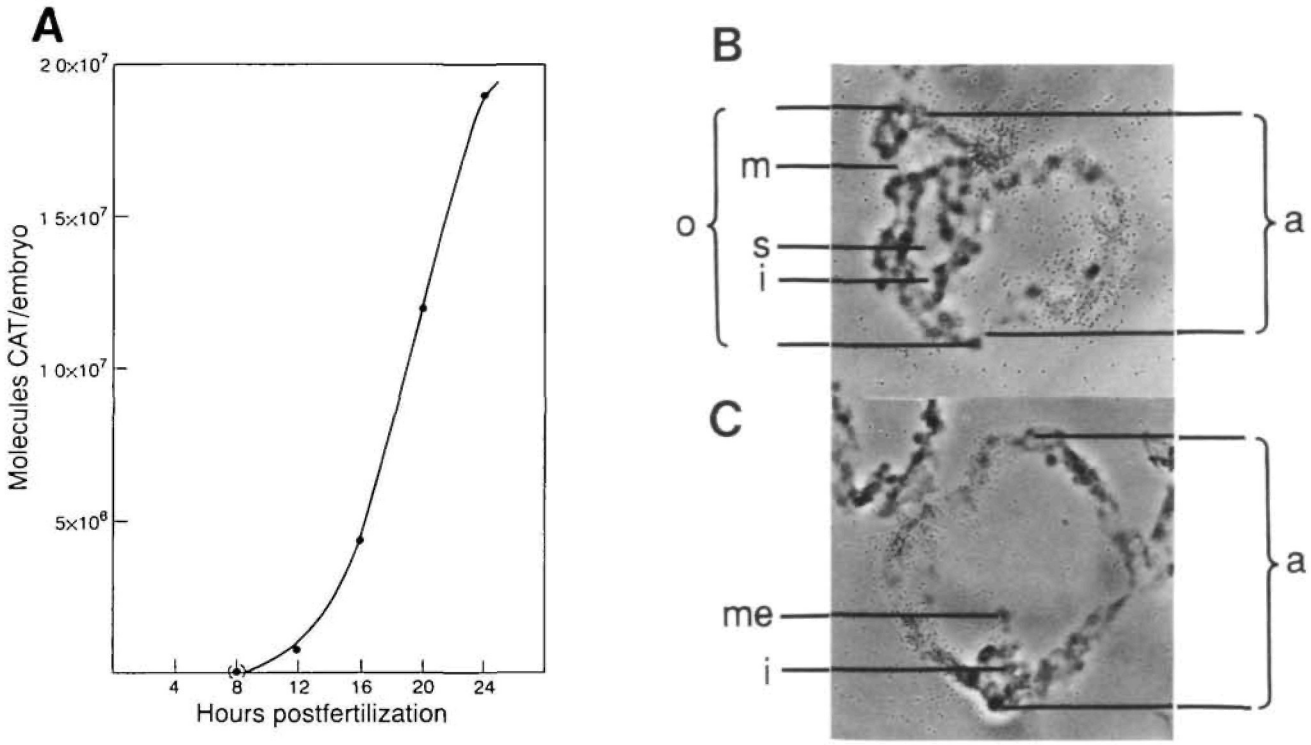

Fig. 4. Correct temporal specification of CyIIIa · CAT in multiply injected embryos. Time course of *CyIIIa · CAT* expression in embryos obtained from 3× injected eggs. Eggs received a total of 3600 *CyIIIa · CAT* molecules and 20400 carrier DNA molecules in three postfertilization injections. 181 embryos were collected and analyzed for CAT activity at 8 h, 38 at 12 h, 28 at 16 h, 18 at 20 h, and 26 at 24 h. The limit of detection was about 1.3×10^5 CAT molecules per embryo in this experiment.

CAT activity increases again reproduces the measured kinetics of *CyIIIa* mRNA accumulation in normal embryos (Shott *et al.* 1984). Thus there is no detectable CAT activity at 8 h postfertilization; CAT expression becomes evident by 12 h; and CAT enzyme level, like *CyIIIa* mRNA, then continues to increase until about 24 h.

CyIIIa gene transcripts have been demonstrated by *in situ* hybridization in all aboral ectoderm cells and their precursors, but in no other embryonic cells (Cox *et al.* 1986). We assayed the spatial distribution of CAT transcripts by this method in 72 h plutei raised from 3× injected eggs. Each egg received a total of 3600 *CyIIIa · CAT* molecules, plus 20400 carrier DNA molecules. In this batch of plutei, an average of 64 % of the nuclei contained *CyIIIa · CAT* DNA. We scored complete serial sections of six >95 % transgenic embryos, four ~50 % transgenic embryos, and four ~25 % transgenic embryos, and a total of six ectopically labeled cells were found in different embryos, *viz* five single cells within the blastocoel (possibly mesenchyme cells) and one gut cell. Considering the large number of aboral ectoderm cells containing *CyIIIa · CAT* genes in these embryos, the ectopically labeled cells would increase the number of cells expressing *CyIIIa · CAT* by less than 0.2 %. This insignificant level of ectopic expression is similar to that seen previously in embryos reared from singly injected eggs (data are not shown here as the result is essentially the same as in previous publications [Hough-Evans *et al.* 1987, 1988; 1990]; additional observations demonstrating appropriate expression of *CyIIIa · CAT* in multiply injected eggs in which CAT enzyme is monitored immunologically are presented elsewhere [Zeller *et al.* in preparation]). We conclude from these experiments, and from Fig. 4, that as in mosaic embryos raised from singly injected eggs, in 3× injected eggs the *CyIIIa · CAT* construct is *correctly specified for expression, both spatially and temporally*. Furthermore, in the highly transgenic embryos *CyIIIa · CAT* is apparently expressed in all clones of which the aboral ectoderm is composed.

Unaltered transcription of endogenous CyIIIa genes in the presence of many excess copies of the CyIIIa regulatory domain

We assayed directly the transcriptional activity of the endogenous *CyIIIa* genes of living blastulae in the presence of a saturating amount of exogenous *CyIIIa · CAT*, and a several-fold molar excess of the *CyIIIa* regulatory domain. In order to make this measurement, we injected fertilized eggs 3× with solutions containing 600 molecules/pl of linearized *CyIIIa · CAT* and 0, 600, or 3360 molecules per pl of the 9.3 kb *SalI* fragment shown in Fig. 5A. This fragment is also present on the *CyIIIa · CAT* construct (Fig. 5A) and it contains the complete known regulatory domain of the *CyIIIa* gene. Thus the experimental samples contained a 0, 1, or 5.6-fold molar excess of competitor regulatory domain,

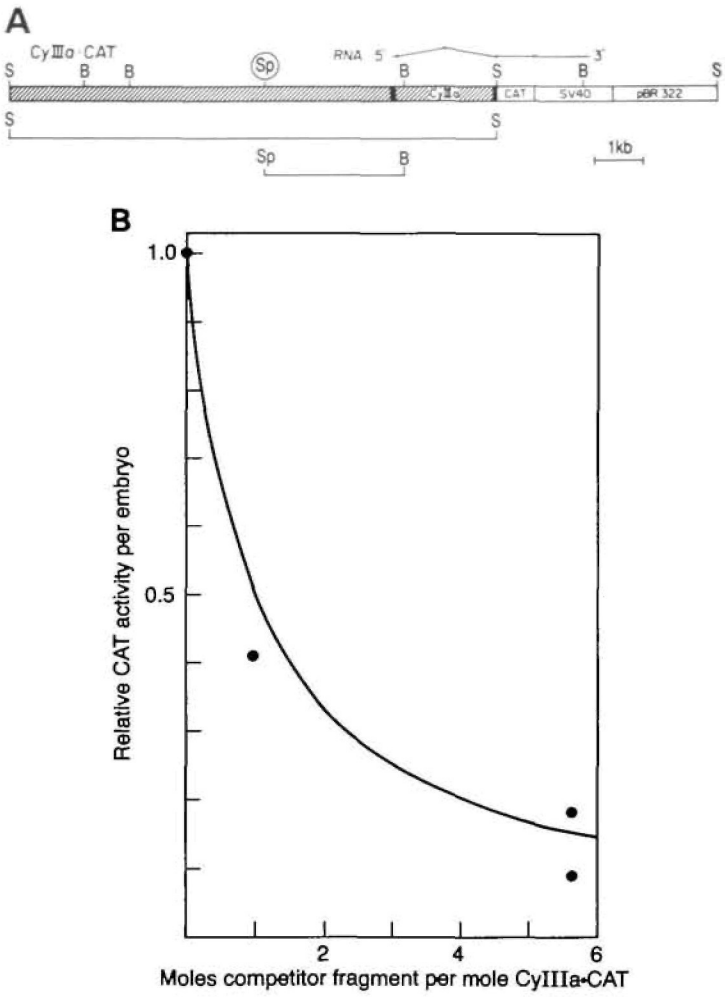

Fig. 5. Stoichiometric, competitive decrease in *CyIIIa · CAT* expression by coinjected excess CyIIIa regulatory domains. (A) Map of the *CyIIIa · CAT* construct. Restriction sites are as follows: B, *Bam*HI; S, *Sal*I; Sp, *Sph*I. The 9.3 kb *Sal*I fragment used for competition is indicated. The *CyIIIa* regulatory domain is located between the *Sph*I site and the adjacent *Bam*HI site on the 3′ side (Thézé *et al.* 1990; Calzone *et al.* 1988). The *CyIIIa* transcription initiation site, 5′ untranslated region, and the largest intron, are indicated. A naturally occurring in-frame *Sal*I site was ligated directly to the pSVO CAT *Sal*I site 1.3 codons following the translational initiation site (Flytzanis *et al.* 1987). (B) Relative average CAT activity per blastula, as a function of molar excess of coinjected competitor fragments with respect to *CyIIIa · CAT*. Eggs were injected 3× with 2 pl per injection of a solution containing 600 *CyIIIa · CAT* genes per pl, and 0, 600 or 3360, 9.3 kb *Sal*I fragments per pl. A constant mass of DNA was injected at each point; the balance was made up with 10 kb (average length) sea urchin carrier DNA prepared by size fractionation after digestion with *Sph*I. 20–50 embryos collected at 24 h postfertilization were assayed for each point. Relative CAT activity is the activity measured at each point normalized to the activity of the control embryos, which received no competitor DNA (set at 1.0. on ordinate).

with respect to *CyIIIa · CAT* genes. A constant total mass of DNA was introduced in all injections; the balance was made up with size-selected sea urchin carrier DNA. Blastulae reared from these injected eggs were collected 24 h postfertilization, the average CAT enzyme activity per embryo was measured, and the average number of *CyIIIa · CAT* genes per embryo (i.e. $\bar{\mathfrak{d}}$) was determined by hybridization with a CAT probe as in the preceding incorporation experiments (see Materials and methods). The average fraction of nuclei per embryo containing the injected DNA (i.e. $\bar{\mathfrak{G}}$) was also estimated as above, by *in situ* hybridization on embryo squashes. Finally, 728 blastulae of the same 5.6-fold molar excess competition sample were collected for the determination of their endogenous *CyIIIa* mRNA content. As a control, 742 blastulae reared from the same batch of eggs, but which had not been injected, were collected to provide a quantitative comparison of the endogenous *CyIIIa* mRNA content by the same methods. The value of $\bar{\mathfrak{d}}$ for the embryos of this experiment was 90 copies of *CyIIIa · CAT* DNA per nucleus containing this DNA, while the measurement of $\bar{\mathfrak{G}}$ indicated that an average of 93 % of the nuclei per embryo displayed CAT DNA. This sample of embryos was Batch 1 of Table 1, the incorporation distribution for which is shown in Fig. 2C, and the exogenous DNA content of which is compared to that of other batches in Fig. 3 (point C). We recall that the competitor fragments, the carrier DNA, and the *CyIIIa · CAT* DNA are all concatenated together, and in earlier competition experiments were in fact shown to be incorporated at the expected ratios (Livant *et al.* 1988; Franks *et al.* 1990). Thus we expect an average of 500 molecules of competitor regulatory domain per nucleus bearing *CyIIIa · CAT* DNA in the 5.6-fold molar excess competition samples used for the *CyIIIa* mRNA measurements. Note that Livant *et al.* (1988) showed that half saturation with respect to endogenous regulatory factors is achieved at about 100 copies of *CyIIIa · CAT* per nucleus, and 90 % saturation at about 1000 copies per nucleus. The total number of copies of exogenous *CyIIIa* regulatory domain per nucleus in these experimental embryos, i.e. about 590/nucleus, should therefore induce about 85 % saturation.

Fig. 5B shows the decline in relative CAT activity in these transgenic blastulae as a function of the molar excess of competitor fragments injected. The solid curve represents the predicted decline in relative CAT activity were the competition by excess competitor fragment perfectly stoichiometric (see Livant *et al.* 1988). The experimentally determined points are entirely consistent with this function. Thus we may conclude that competitive titration of limiting DNA-binding proteins necessary for *CyIIIa · CAT* expression has taken place in these particular embryos, just as in the previous *in vivo* competition studies (Livant *et al.* 1988; Franks *et al.* 1990).

To measure quantitatively the amounts of *CyIIIa* mRNA in the 728 embryos bearing *CyIIIa · CAT* plus exogenous competitor, and in the 742 control uninjected embryos, we miniaturized RNA extraction and

single-strand probe excess titration procedures, and added an internal efficiency control. Different amounts of total RNA extracted from these embryos were reacted with at least a 10-fold molar excess of a ^{32}P-labeled riboprobe specific for the 3′ untranslated region of the *CyIIIa* message. These reactions achieved 95% completion, and the unreacted RNA was digested with RNAase. A known amount of a tritiated riboprobe approximately 1300 nt long derived from unique sequences 3′ of M actin mRNA (Lee *et al.* 1984) was introduced at the beginning of the isolation procedure as an internal standard, to provide a correction for any bias due to unequal yields of RNA in the preparation (see Materials and methods). In the event, an identical efficiency of RNA preparation (84%) was obtained for both samples. The results of the experiment are shown in Fig. 6. The points representing measurements from the transgenic embryos (filled symbols) and those from the control embryos (open symbols) fit the same line with a correlation coefficient of 0.92. Thus the *CyIIIa* mRNA contents of the two sets of embryos are indistinguishable. In absolute terms, the number of *CyIIIa* mRNAs is similar to that measured by Lee *et al.* (1986). Were the exogenous *CyIIIa* regulatory domains competing as efficiently with the endogenous *CyIIIa* regulatory domains for the necessary factors as they are with each other, we would expect to see the level of *CyIIIa* transcript indicated by the dashed line in Fig. 6. The result shown in Fig. 6 was also obtained in a similar experiment in which, however, the resolution was not as good because the exogenous DNA was present in only 25% of the nuclei. In this experiment, the slope of the control was 8% *less* than that of the injected embryos.

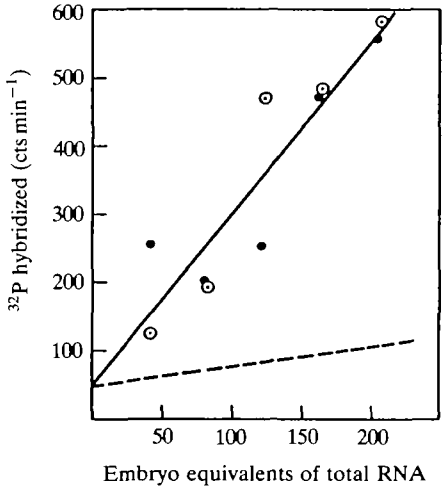

Fig. 6. Probe excess titration of *CyIIIa* transcripts in competition and control blastulae. Raw counts (obtained from a planar counter, and with background subtracted) are plotted *versus* the number of embryo equivalents of total RNA as calculated from the recoveries of the ^{3}H-M actin riboprobe (see text). Reactions of total RNA from blastulae containing an average of 590 exogenous *CyIIIa* regulatory domains per nucleus bearing exogenous DNA (90 *CyIIIa·CAT* molecules plus 500 copies of the 9.3 kb *Sal* fragment shown in Fig. 5A) are depicted by filled-in points. Titrations of total RNA from control blastulae obtained simultaneously from the eggs of the same batch are shown by open points. The experimental blastulae contained exogenous DNA in 93% of their nuclei (average). The dotted line indicates the predicted position of the curve obtained from these transgenic blastulae were stoichiometric competition to have occurred between the exogenous *CyIIIa* regulatory domains and the endogenous *CyIIIa* genes as it did with the *CyIIIa·CAT* genes (Fig. 5B). The standard error, ±9% of the mean was estimated from five simultaneous determinations of *CyIIIa* mRNA content by probe excess titration on samples of 700–800 uninjected 24 h blastulae (data not shown). Thus random error cannot account for the difference between the solid and dotted titration curves.

These measurements establish that the quantitative expression of the endogenous *CyIIIa* gene remains unaffected in the same embryos in which quantitative expression of the exogenous *CyIIIa·CAT* construct is severely depressed by regulatory competition. It remained possible that spatial expression of the endogenous *CyIIIa* gene was perturbed in the competition embryos. An *in situ* hybridization study of endogenous *CyIIIa* expression was carried out on embryos grown from 3× injected eggs as above, using the gene specific 3′ trailer sequence probe with which earlier studies of *CyIIIa* expression have been carried out (Cox *et al.* 1986; Lee *et al.* 1984, 1986). An antisense ^{35}S-RNA probe was prepared and hybridized to 5 μm sections of 72 h pluteus stage embryos, in which all of the cell types of the embryo are explicit, and the distinction between oral and aboral ectoderm is easily perceived, particularly in sagittal or parasagittal sections. *In situ* hybridization was performed as in Cox *et al.* (1986), with the minor modification introduced by Nisson *et al.* (1989). A sample of these embryos was removed in order to test the efficiency of the competition in this particular experiment. The competitor DNA was introduced at 5.6× molar excess with respect to *CyIIIa·CAT* DNA (cf. Fig. 5). The average CAT enzyme activity per embryo (99 embryos) was 15.8% of that in the control embryos (151 embryos) bearing *CyIIIa·CAT* but no competitor. An ideal competition would predict 15.2% (i.e. $[1+n]^{-1}$ where n is the ratio of competitor DNA to *CyIIIa·CAT* DNA. Thus a highly effective competition against *CyIIIa·CAT* expression was obtained. 46 of the same batch of embryos were scored for endogenous *CyIIIa* gene expression. Of these 32 embryos were represented by a complete set of serial sections, and 14 were represented by between 6 and 15 sections each (the complete series, depending on orientation of the pluteus, consisted of 12–18 sections). All aboral ectoderm cells included in all of these sections were found to be labeled by the *CyIIIa* probe; i.e. there were no 'holes' in the labelling pattern such as might have occurred were *CyIIIa* expression silenced in the large patches of cells containing competitor DNA. No ectopic *CyIIIa* expression was observed. In sagittal and parasagittal sections the oral ectoderm displayed no labelling whatsoever above background, and neither were gut, esophagus, intestine, mesenchyme cells nor coelomic

pouches labeled above background. We conclude that spatial expression of the endogenous *CyIIIa* gene is, so far as detection by *in situ* hybridization allows, entirely impervious to competition by exogenous regulatory DNA.

Discussion

The CyIIIa genes and the exogenous CyIIIa · CAT genes differ functionally in their response to competition

This work began with our discovery of a way to introduce exogenous DNA into much larger fractions of sea urchin embryo cells than the average 6 % of embryo cells that stably incorporate these sequences by our previous methods. As described in the initial sections, we demonstrate that multiple injection of exogenous DNA into fertilized eggs produces many embryos in which >95 % of cells contain the injected sequences. The new method was then applied to the problem of determining whether introduction of a several-fold molar excess of the *CyIIIa* regulatory domain would affect endogenous *CyIIIa* transcription, in the same embryos in which it stoichiometrically depresses exogenous *CyIIIa · CAT* expression. The coinjected regulatory domain utilized in these experiments includes all of the ~20 known factor binding sites for the *CyIIIa* gene (Calzone *et al.* 1988; Thézé *et al.* 1990), and earlier evidence showed that this domain suffices to promote accurate specification of the *CyIIIa · CAT* construct in the early embryo, both qualitatively and quantitatively. Thus the spatial expression of *CyIIIa · CAT* is appropriately confined to aboral ectoderm (Hough-Evans *et al.* 1987, 1988, 1990); the temporal pattern of expression displays the same late cleavage activation and subsequent kinetics as does the normal *CyIIIa* gene (Flytzanis *et al.* 1987); and when subsaturating numbers of these genes are introduced, the *amount* of *CyIIIa · CAT* transcript per exogenous gene is approximately equivalent to the amount of *CyIIIa* mRNA per gene in normal embryos (Livant *et al.* 1988). We confirm the normal spatial and temporal patterns of expression in embryos raised from eggs injected 3× with *CyIIIa · CAT* in this paper. Nonetheless, we show that the endogenous *CyIIIa* regulatory system and that driving the exogenous *CyIIIa · CAT* construct are functionally different, in an unexpected way: both quantitative and spatial expression of the endogenous *CyIIIa* gene are impervious to competition. This result is consistent with the often repeated observation that no adverse developmental phenotype is associated with introduction of competitively effective levels of *CyIIIa* regulatory DNA (Livant *et al.* 1988; Franks *et al.* 1990; B. Evans, D. Livant, unpublished data). However, in itself the absence of a phenotypic effect is not interpretable. The other cytoskeletal actins could function redundantly with respect to *CyIIIa* actin, and thus could have substituted for newly synthesized *CyIIIa* protein even were the synthesis of the latter depressed; or the egg could be endowed with a sufficient maternal store of *CyIIIa* protein. Our finding differs from that of Vitelli *et al.* (1988), who reported that microinjected exogenous early histone genes cause a reduction in the expression of the endogenous early H2A in the sea urchin *Paracentrotus lividus*. This difference might be understood in terms of the contrasting developmental roles of these genes. Early histone genes are actively transcribed in all the cells of the embryo during early cleavage, and are then 'turned off.' Thus they do not become committed to a state of activity. The *CyIIIa* gene is activated in late cleavage in one particular set of cell lineages, and continues to be expressed in these lineages as long as the aboral ectoderm exists. Thus *CyIIIa* mRNA is found throughout larval life up to metamorphosis when the aboral ectoderm cytolyzes.

Competition and reversibility of factor-site interactions in vivo

The competitor regulatory and *CyIIIa · CAT* reporter sequences are present in the nuclei from early in cleavage, i.e. long before *CyIIIa* or *CyIIIa · CAT* activation. This is indicated explicitly by the analysis of exogenous DNA incorporation included in this paper (see also Hough-Evans *et al.* 1988). The accurate temporal activation of *CyIIIa · CAT* gene expression that we have observed (e.g. Fig. 4) is probably mediated by a steep developmental rise in intranuclear concentration of certain positively acting regulatory factors, whose function is specifically temporal control, and which service a number of different genes (Calzone *et al.* 1988; Davidson, 1989; Thiebaud *et al.* 1990). Among these is the factor **P5**. Competition with the **P5** binding site (Franks *et al.* 1990), or deletion of this site (Flytzanis *et al.* 1987), obliterates *CyIIIa · CAT* expression. Almost stoichiometric competition is observed when excess **P5** target site alone is injected, as also on introduction of excess quantities of each of several other individual factor binding sites that compete effectively, or of the whole regulatory domain (e.g. Fig. 5B). This means that the relevant factor molecules are more or less equally partitioned amongst the competitive sites and the sites linked directly to the CAT reporter in the *CyIIIa · CAT* gene. The factors are thus *titrated* away from the *CyIIIa · CAT* genes, as a function of intranuclear [competitor:*CyIIIa · CAT*] molar ratio. The endogenous and exogenous genes are expected to have the same initial access to diffusible regulatory factors. The fact that the exogenous genes function normally, in itself shows that these factors are indeed diffusible, since the exogenous sequences are not in general incorporated at the same genomic loci as the endogenous *CyIIIa* genes. In contrast, once bound to *endogenous* gene target sites, the necessary regulatory factors apparently do *not* thereafter partition amongst the excess *exogenous* competitor sequences. Two conclusions follow. First, the immunity to competition of the endogenous genes means that factor-site interactions occurring in these genes must be *greatly stabilized* (probably >10-fold) relative to the same interactions in the exogenous genes in the same nuclei. Second, the factors continue to redistribute amongst

the exogenous sites during development, perhaps according to equilibrium parameters similar in value to those measured *in vitro* for many of these factors (e.g. Calzone *et al.* 1988). These measurements suggest that the specific factor-site complexes have a half-life of minutes to an hour or so. *Continuing equilibrium redistribution of necessary factors amongst the exogenous genes is required.* Thus, per nucleus the specific sites are always in excess in the competition embryo nuclei, and the exogenous DNA replicates successively along with the genomic DNA during cleavage (McMahon *et al.* 1985; Franks *et al.* 1988; Flytzanis *et al.* 1985). The difference between endogenous and exogenous genes is maintained throughout cleavage and thereafter, since we observe this difference at the 24 h blastula stage. Thus the feature that *enhances stability of factor-site interactions in the endogenous CyIIIa genes* must be one that is reproduced following mitosis.

Interpretations

In embryos in which exogenous *CyIIIa · CAT* expression is stoichiometrically competed by excess regulatory target sites, both spatial and quantitative expression of the endogenous *CyIIIa* gene appear to remain perfectly normal. This is shown by the *in situ* hybridization experiment which revealed *CyIIIa* mRNA in all aboral ectoderm cells, but in cells of no other cell type or lineage, and by the mRNA titrations of Fig. 6, and ancillary experiments. We argue above that the imperviousness of the endogenous *CyIIIa* gene to levels of regulatory competitor sequence that effectively titrate factors away from the exogenous *CyIIIa · CAT* gene, requires an enhanced stability of the DNA–protein complexes specifying expression in the endogenous *CyIIIa* gene. What could explain this difference between the exogenous and endogenous gene regulatory systems? The more likely interpretations fall into two classes, that in abbreviated form could be described as 'historical' and 'locational' explanations. Enhanced stability of the endogenous gene regulatory complexes could conceivably be affected by the developmental *history* of the target site if during gametogenesis the DNA had been modified in a way that can be reproducibly propagated at cell division, e.g. by methylation. The alternative is that the genomic *locus* in which the endogenous *CyIIIa* gene resides includes a distant site (or sites) not contained in the *CyIIIa · CAT* construct, the function of which is to 'lock in' the factor-site complexes required for proper activation of *CyIIIa* expression. For example such a site could bind a protein that interacts cooperatively with other factors required for expression, or it could mediate a change in chromatin conformation that causes additional protein–protein interactions.

Observations on 'genomic imprinting' in the mouse provide a precedent for the first of these possibilities. Imprinting was discovered because the two parental genomes are not functionally equivalent in the mouse, in that both are required for normal development (Surani *et al.* 1984, 1986; McGrath and Solter, 1984), and specific chromosomal regions influence development according to their parental origin (reviewed by Cattanach, 1986). Genomic DNA is methylated differentially in male and female gametes, and these differences persist in early development (Sanford *et al.* 1987). Furthermore, Sapienza *et al.* (1989) showed that methylation of an exogenous sequence in a transgenic mouse depends on the sex of the parent from which it is inherited. Studies of Groudine and Conklin (1985) similarly demonstrated that in the chicken *de novo* methylation of specific genes occurs during spermatogenesis, generating developmentally persistent patterns, in that sites of undermethylation in sperm correspond to hypersensitive sites in the vicinity of these genes in somatic cells. However, sexually differential genomic imprinting is unlikely in the sea urchin, since normal parthenogenic development can be obtained, though only at relatively low frequency (Brandriff *et al.* 1975). Furthermore, the frequency of successful parthenogenic development could be affected by a phenomenon entirely distinct from differential genomic imprinting, *viz* that in sea urchins both centrosomes are normally contributed by the sperm (Boveri, 1900; Paweletz *et al.* 1987). The possibility remains that the genomes of *both* sexes are modified in sea urchins during gametogenesis, including the *CyIIIa* regulatory sequences, but excluding the identical exogenous *CyIIIa · CAT* (and competitor) sequences because these are introduced only after gametogenesis. To test this category of ideas we are constructing germ line transgenics bearing *CyIIIa · CAT*. In these animals, the exogenous genes will have undergone the same gametogenic 'experience', but are expected to be located in regions of the genome other than the normal domain of the endogenous *CyIIIa* gene. If in embryos derived from such transgenic animals *CyIIIa · CAT* expression proves to be impervious to competition by excess *CyIIIa* regulatory sequences an 'imprinting' mechanism would be implied, while if it can be competed, a distant additional regulatory sequence at the *CyIIIa* locus that is missing from the *CyIIIa · CAT* construct would be indicated. In either case there evidently exists a mechanism, the specific function of which is to superimpose a *developmental condition of irreversibility* on the specification of *CyIIIa* expression.

CyIIIa gene expression occurs only in the aboral ectoderm lineages, and continues throughout the lifetime of these lineages. Our experiment shows that specification of *CyIIIa · CAT* expression may occur normally, but remain abnormally reversible. Thus this experiment empirically separates *specification* of expression *per se* from *commitment*, i.e. irreversibility of the state of specification (cf. Davidson, 1986, p.195; Davidson, 1990). Perhaps these results will lead to a molecular definition of commitment, as opposed to specification, for the *CyIIIa* gene.

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