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An *in vivo* titration of regulatory factors required for expression of a fusion gene in transgenic sea urchin embryos

(gene transfer/*CyIIIa* actin gene/regulatory factor/competition *in vivo*)

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ABSTRACT **We report that endogenous regulatory factors mediating expression of a lineage-specific sea urchin embryo gene can be titrated *in vivo* by introduction of a sufficient molar excess of DNA-binding sites. Thus we obtain an estimate of the quantity of limiting factor(s) required for developmental activation and transcriptional expression, which can be compared with estimates of factor prevalence obtained by measurements *in vitro* carried out under equilibrium conditions. A fusion construct in which the bacterial gene for chloramphenicol acetyltransferase (CAT; acetyl-CoA:chloramphenicol O^3-acetyltransferase, EC 2.3.1.28) is controlled by cis-regulatory elements of the *CyIIIa* cytoskeletal actin gene (*CyIIIa–CAT*) was introduced in varying numbers of copies into sea urchin eggs. The activity of the *CyIIIa–CAT* fusion gene in 24-hr blastula-stage embryos was shown to saturate as the number of exogenous genes was increased. The mean number of *CyIIIa–CAT* fusion genes per nucleus at which half saturation was obtained was 105 ± 40 (mean ± SD). This result suggests that equilibrium parameters measured earlier *in vitro* may apply, at least approximately, within the embryo nuclei.**

The *CyIIIa* cytoskeletal actin gene of *Strongylocentrotus purpuratus* (1, 2) provides a marker of the process of differential gene expression by which the embryo constructs its initial sets of specialized cell lineages. The gene is transcriptionally activated 10–12 hr postfertilization (3–5) and is expressed throughout embryonic development, exclusively in the six cell lineages from which derive the aboral ectoderm of the completed pluteus (6–8). A fusion construct consisting of the chloramphenicol acetyltransferase (CAT; acetyl-CoA:chloramphenicol O^3-acetyltransferase, EC 2.3.1.28) reporter gene under the control of upstream *CyIIIa* sequences (*CyIIIa–CAT* fusion gene) has been microinjected into unfertilized eggs (9). A regulatory domain that includes necessary and sufficient cis-acting sites to promote CAT expression on the correct developmental schedule (10), and exclusively in cells of the correct lineages (11, 12), can thus be identified. The *CyIIIa* regulatory domain extends for about 2.5 kilobases (kb) upstream of the locus of transcription initiation (refs. 3 and 10; F. Calzone, N. Thézé, and E.H.D., unpublished data). Reactions carried out *in vitro* with probes spanning this region demonstrate at least 11 different sequence-specific interactions with DNA-binding proteins present in extracts from nuclei of blastula-stage embryos in which the *CyIIIa* gene is active (ref. 13; N. Thézé, F. Calzone, and E.H.D., unpublished data). The stoichiometry of DNA–protein complex formation in these extracts provides estimates of the equilibrium parameters that govern these interactions *in vitro* and also of the approximate minimum prevalences in the blastula nuclear extracts for each of the respective DNA-binding factors (13–15). The biological significance of several of these specific interactions is indicated by additional gene transfer experiments in which excess quantities of individual subfragments of the regulatory domain were coinjected with the complete *CyIIIa–CAT* fusion construct (R. Franks, R. Anderson, B. Hough-Evans, and E.H.D., unpublished data). Thus competition by certain subfragments that include particular interaction sites identified *in vitro* severely depresses CAT expression in transgenic embryos and, in at least one case, appears to affect spatial regulation as well. To summarize, current evidence supports the conclusion that some of the DNA–protein interactions observed *in vitro* constitute the casual events that produce the temporal and spatial regulation of the *CyIIIa* gene.

The present work was undertaken to obtain estimates of the available concentration of the limiting factor(s) *in vivo*. We have varied the number of *CyIIIa–CAT* fusion genes stably incorporated per embryo nucleus and have measured the consequent output of CAT enzyme. We first show that, as implied by our earlier study (10), the regulatory system is effectively saturated when about 1500 copies of the *CyIIIa–CAT* gene are injected per egg. Thus CAT enzyme production in blastula-stage embryos raised from these eggs is almost stoichiometrically decreased by coinjection of various amounts of the upstream *CyIIIa* regulatory domain. We then report measurements of CAT enzyme production as a function of the number of copies of the *CyIIIa–CAT* gene incorporated for several different batches of eggs. These measurements demonstrate the saturation behavior of the limiting factor(s) *in vivo* and thus provide an approximate estimate of the number of such factor molecules available per nucleus. Finally, we compare this estimate with those obtained previously from *in vitro* measurements of factor prevalence in crude nuclear extracts of embryos expressing the *CyIIIa* gene (13).

MATERIALS AND METHODS

Microinjection of Eggs with Carrier and Competitor DNA Solutions. *S. purpuratus* eggs were microinjected and subsequently cultured as previously described (9, 10). The aggregate concentration of the injected DNAs was held constant within each experiment, at about 56 μg/ml. Sperm DNA prepared as previously described (1) was used as carrier. About 200 μg of DNA was digested with *Sph* I, concentrated by 1-butanol extraction, and loaded onto two preformed 10–40% sucrose step gradients in 1 M NaCl/20 mM Tris, pH 8/5 mM EDTA. The gradients were centrifuged at 20°C in an SW41 rotor at 28,000 rpm for 16 hr. Each gradient was divided into ≈20 fractions. Ten microliters was removed from each fraction; size analysis was performed by gel electrophoresis; and fractions containing fragments larger than 5 kb were pooled, dialyzed against 10 mM Tris, pH 8/0.1 mM EDTA, reconcentrated by 1-butanol extraction, and

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Abbreviation: CAT, chloramphenicol acetyltransferase.

precipitated. This protocol yielded carrier DNA with *Sph* I termini and with an average length of ≈10 kb, [i.e., only slightly below the length of the *CyIIIa–CAT* construct utilized (14 kb)]. The fragments of the *CyIIIa–CAT* used for competition experiments were prepared by digestion with the appropriate restriction enzyme, as indicated, followed by separation by electrophoresis through an 0.8% agarose gel and purified by elution from DE81 filters.

Other Procedures. All other procedures were carried out as previously described (10).

RESULTS

Near-Stoichiometric Competition *in Vivo* by *CyIIIa* Regulatory Sequences at a Saturating Level of *CyIIIa–CAT* Genes. The study of Flytzanis *et al.* (10) demonstrated that a more or less constant amount of CAT enzyme is produced by embryos raised from eggs injected with various quantities of *CyIIIa–CAT* DNA, ranging from a few hundred to thousands of molecules per egg. It was proposed that within this range the number of copies of the *CyIIIa* regulatory sequence incorporated is probably saturating for regulatory factors available at limited concentrations within the blastula nuclei (10). Linear DNA molecules injected into sea urchin egg cytoplasm are rapidly ligated into end-to-end concatenates, irrespective of the terminal sequences. In most embryos, these are stably incorporated into one or a few blastomere nuclei after the third to fifth cleavage, which results in a mosaic pattern of exogenous DNA retention that is apparently random with respect to embryonic cell lineage (9, 10, 12, 16, 17). After incorporation, the exogenous DNA replicates at approximately the rate of the genomes of the host cells. Significant loss of DNA does not occur during embryogenesis. Thus, if several thousand molecules of DNA are injected into the unfertilized egg, this number of copies of the injected sequence may be stably retained in all the cells descendant from the blastomere(s) in which the initial incorporation event took place.

If the amount of CAT expression in such embryos is indeed limited by regulatory factor availability and the *CyIIIa–CAT* genes are present in excess, then coinjection of additional copies of the upstream *CyIIIa* regulatory domain together with the complete *CyIIIa–CAT* reporter fusion construct should competitively reduce CAT enzyme expression. Furthermore, competition should occur at relatively low molar ratios of competitor to *CyIIIa–CAT* sequence, and the amount of competition should stoichiometrically reflect the incorporated competitor/fusion gene ratios. To explore these predicted consequences experimentally, we injected eggs with 1500 molecules of the *CyIIIa–CAT* fusion gene, together with varying numbers of molecules of subfragments containing the whole *CyIIIa* regulatory domain. Linearized carrier DNA terminating in *Sph* I ends, as does the linearized *CyIIIa–CAT* construct, were coinjected in quantities adjusted so that the total mass of injected DNA remained constant at 0.1 pg. Fig. 1*a* shows the restriction map of the *CyIIIa–CAT* gene and the locations of two different fragments that include the regulatory domain and that were used alternatively as competitor (namely, a 9.3-kb *Sal* I fragment and a 2.7-kb *Sph* I–*Bam*HI fragment). The 9.3-kb *Sal* I fragment contains the entire 5′ flanking region and the 5′ transcribed leader associated with the *CyIIIa–CAT* gene. The 2.7-kb *Sph* I–*Bam*HI fragment contains the most proximal 2.5 kb of 5′ flanking sequence as well as the origin of *CyIIIa* transcription and about 200 bp of 5′ transcribed leader. All known sites of interaction between nuclear factors and the *CyIIIa* upstream sequence and all known regions that in functional tests are of regulatory significance are included between the *Sph* I and *Bam*HI sites (refs. 10 and 13; R. Franks and E.H.D., unpublished data; see Fig. 1*a*). The results of these experiments are summarized in Fig. 1*b*, in which the average number of CAT enzyme molecules produced per embryo is given as a function of the molar ratio of competitor to *CyIIIa–CAT* genes injected (e.g., for the ratio of 5, 7500 molecules of the *Sph* I–*Bam*HI fragment were coinjected with 1500 molecules of the *CyIIIa–CAT* gene, together with about 6200 molecules of carrier DNA). Note that the two different 5′ *CyIIIa* gene fragments used compete similarly despite their >3-fold difference in length and the fact that the 2.7-kb *Sph* I–*Bam*HI fragment has a terminus homologous with the *CyIIIa–CAT* DNA termini (and the carrier DNA termini), whereas the 9.3-kb fragment does not. The dashed line in Fig. 1*b* shows the ideal results that would have been obtained had reduction in CAT activity occurred with perfect stoichiometry with respect to the number of *CyIIIa* 5′ flanking sequences present. The experimental data fit by the solid line indicate that the competition behaves as if about 40% of the competing DNA were effective, rather than all of it. There are several possible interpretations, the most likely of which is that in some embryos of each batch concatenates containing competing DNA and little or no *CyIIIa–CAT* DNA were formed and incorporated in different cells from the bulk of the *CyIIIa–CAT* DNA. It is also possible that for some reason the competing DNA was marginally less likely to be engaged in concatenates in the first place. This small difference from ideal stoichiometry aside, however, Fig. 1*b* provides clear evidence of systematic competition *in vivo*, at the modest competitor/reporter gene ratios expected. Thus, for example, at a competitor/reporter gene ratio of only 5:1, activity is reduced to about 30% of the control value. This behavior requires that the *CyIIIa–CAT* reporter gene is in fact close to, if not at, saturation with respect to at least the limiting intranuclear factor(s) required for CAT expression.

Saturation of *CyIIIa–CAT* Expression in Transgenic Embryos. Measurements of CAT expression in samples of 24-hr embryos bearing increasing numbers of *CyIIIa–CAT* genes were carried out on single batches of eggs. We were aware from earlier studies (10, 12) that given batches display variable abilities to concatenate, incorporate, and thence amplify the injected DNA. The eggs were all injected with a total mass of about 0.1 pg of DNA. Of this, at least 80% was carrier DNA (i.e., about 7500 molecules per egg at the nominal 10-kb length of the carrier preparation), and the remainder consisted of various amounts of the 14-kb *CyIIIa–CAT* fusion gene plus sufficient carrier to achieve the equivalent of 0.1 pg of injected DNA mass in all samples. Thus individual eggs received from 30 to 1500 copies of the *CyIIIa–CAT* construct, and all samples contained at least a 5-fold molar excess of carrier. The addition of a modest molar excess of carrier DNA has been found to increase CAT expression several fold in embryos bearing the *CyIIIa–CAT* fusion, possibly by providing spacers between adjacent *CyIIIa–CAT* molecules in the incorporated concatenates (ref. 17; R. Franks and E.H.D., unpublished data). Maximum expression is always attained at the ≥5-fold molar excess of carrier DNA/*CyIIIa–CAT* DNA utilized in these experiments. The experimental design thus ensures that the only systematic variable affecting CAT expression will be the number of incorporated *CyIIIa–CAT* genes in the various samples of each experiment. This was calculated from direct measurements of CAT DNA sequence content in aliquots of the experimental embryos, obtained by slot blot hybridization with a CAT sense-strand ^{32}P-labeled RNA probe (see legend to Fig. 2). CAT enzyme content was also measured in extracts of each sample of embryos, and the average amount of enzyme in the cells producing it (i.e., those aboral ectoderm cells bearing the exogenous DNA) was calculated. To derive this value, we applied a measurement recently obtained by Hough-Evans *et al.* (12), who found by *in situ* DNA hybridization that a mean of 6% of the total cells of 24-hr embryos grown from eggs cytoplasmically injected with *CyIIIa–CAT* bear stably incorporated exogenous DNA concatenates. When the small fraction of eggs in each sample that received or retained no

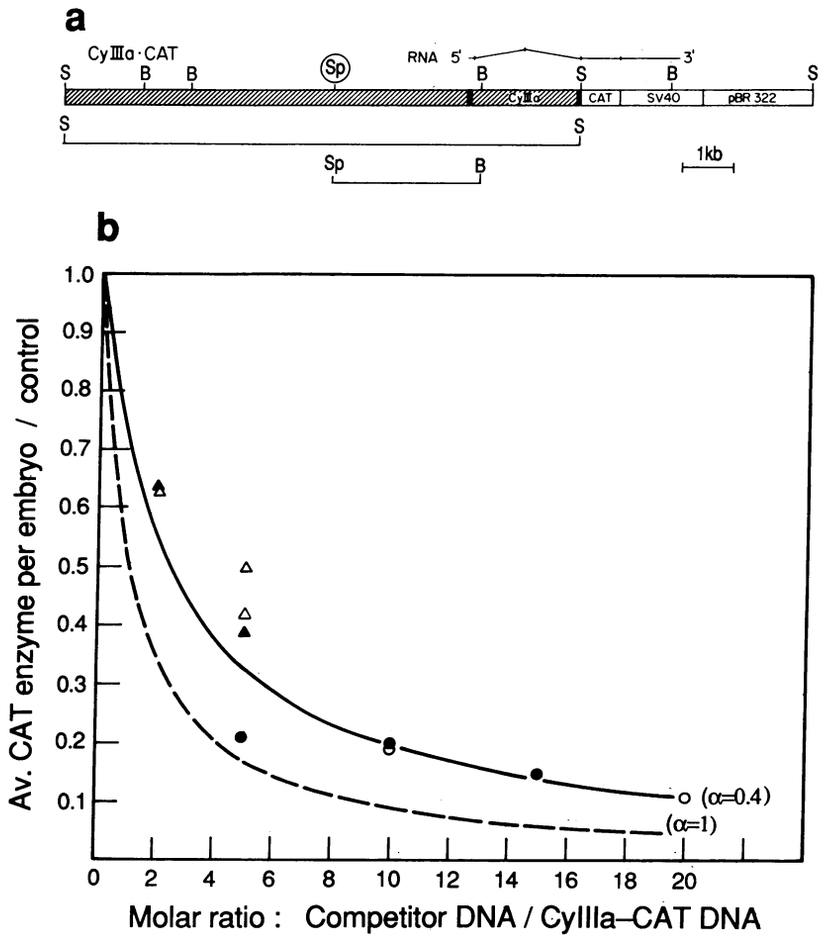

FIG. 1. *CyIIIa–CAT* fusion gene, competitor DNA fragments, and *in vivo* competition in transgenic embryos. (*a*) Map of the *CyIIIa* gene with the two upstream fragments containing all known *CyIIIa* regulatory elements used as competitor DNAs. Restriction enzyme sites are indicated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco* I; P, *Pst* I; Sp, *Sph* I. SV40, simian virus 40. (*b*) Competition by excess regulatory DNA fragments decreases CAT production from coinjected *CyIIIa–CAT* reporter genes. ▲ and △, Experiments with the 10-kb *Sal* I competitor fragment. ● and ○, Experiments with the 2.7-kb *Sph* I–*Bam*HI competitor fragment. About 1500 molecules of *CyIIIa–CAT* were injected at each point together with the indicated number of competitor fragments. The mass of DNA injected at each point was kept constant by the addition of varying amounts of sea urchin genomic DNA. Thirty to 90 embryos were pooled and assayed for CAT activity at each point. The relationship between CAT activity per embryo relative to the control in which no competitor DNA was included (y) and the molar ratio of competitor DNA molecules to *CyIIIa–CAT* genes (x) can be approximated as $y = (1 + ax)^{-1}$, where a is the fraction of competitor fragments actively functioning as competitor. Controls in these experiments averaged 5×10^6 CAT enzyme molecules per embryo (cf. refs. 10 and 17). The dashed curve indicates the ideal form (i.e., when $a = 1$). A least-squares fit to the experimental data shown yields a value for a of 0.41. Av., Average.

DNA whatsoever, due to unsuccessful injection, is included in the embryo pools as in this work, the mean fraction of cells in the total sample bearing the exogenous DNA is about 5%. This result was independently confirmed for two of the batches of eggs utilized for the present experiments, which were assayed by the same *in situ* DNA hybridization method (data not shown). Since ≈200 of the cells of the ≈500-cell 24-hr embryo belong to the aboral ectoderm lineages and are capable of expressing *CyIIIa–CAT* (6–8, 11, 12), CAT enzyme content per average expressing cell should be about 10% of the total CAT enzyme content per 24-hr embryo.

Saturation curves depicting CAT production as the number of *CyIIIa–CAT* genes is increased are shown in Fig. 2*a* for two separate batches of eggs obtained from a single *S. purpuratus* female. The data are fit to the form

$$C = \frac{C_0}{1 + kC_0/G}, \qquad [1]$$

where C is CAT enzyme activity per cell, G is the number of *CyIIIa–CAT* genes per cell as calculated from the slot blot hybridization measurements, C_0 is the saturation level of CAT enzyme per cell, and k is a proportionality constant (k is the inverse of the CAT enzyme output per gene at very small numbers of genes—i.e., far below saturation). Since it is impossible to inject enough DNA to achieve truly complete saturation without incurring lethality in the gene transfer process (9), the extrapolated saturation (C_0) values of the various experimental series are shown in Fig. 2*b* as double inverse plots; i.e., from Eq. 1,

$$\frac{1}{C} = \frac{1}{C_0} + k\frac{1}{G}. \qquad [2]$$

Here the saturation values can be obtained from the intercepts (i.e., $1/C_0$), and the slopes of the various lines (i.e., k) display directly the biological variations between different batches of eggs. Six such experiments (including the two shown in Fig. 2*a*, namely, experiments 5a and 5b) are coplotted in Fig. 2*b*, and the respective least-squares values of k and C_0 are shown in Table 1. Here it can be seen that the different batches of eggs vary considerably in their ability to produce CAT enzyme from high numbers of genes and hence in the values of CAT enzyme molecules per cell at saturation (C_0). However, the variability observed as well as the average of these saturation values is consistent with the typical CAT enzyme levels

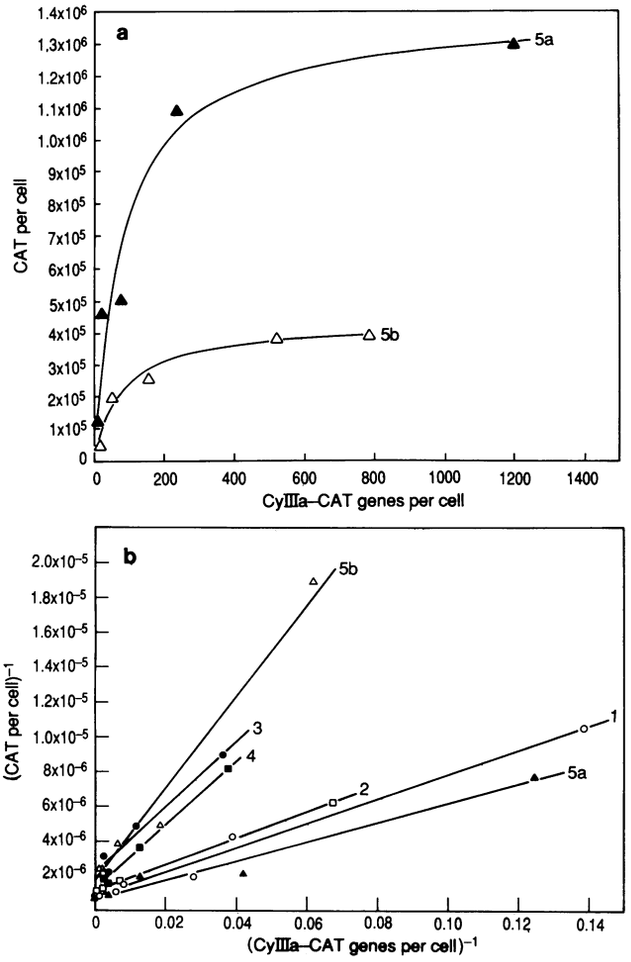

FIG. 2. Saturation of *CyIIIa–CAT* expression in different batches of transgenic *S. purpuratus* embryos. (*a*) CAT enzyme (molecules per embryo) produced by embryos grown from two batches of eggs obtained from the same female about 1 week apart. Increasing quantities of *CyIIIa–CAT* DNA plus carrier DNA, to a constant mass of 0.1 pg per egg, were injected, and the eggs were fertilized. At 24 hr, the blastulae were harvested. Forty to 80 embryos were analyzed for each point shown. *CyIIIa–CAT* DNA was measured for each sample by slot blot hybridization with a CAT sense-strand ^{32}P-labeled RNA probe, and CAT enzyme activity was assayed and converted to molecules of CAT enzyme as described (10, 18). Since concatenation and amplification of exogenous DNA in these embryos occur without reference to the sequence of the DNA (9, 16), to obtain the range of genes per embryo values shown for each experiment, it was only necessary to measure the actual CAT sequence content in samples of those embryos injected with the largest amounts of *CyIIIa–CAT* DNA. Thus the numbers of *CyIIIa–CAT* genes per cell shown are calculated for all but the final sample. The number of *CAT* DNA sequences in samples raised from eggs injected with lower *CyIIIa–CAT* DNA/carrier DNA ratios is thus given by the average amount present in the measured embryos multiplied by the ratio of the number of *CyIIIa–CAT* molecules injected in eggs of the given sample to that injected in eggs of the measured (i.e., highest) sample. The CAT enzyme per cell data points were fit by least-squares analysis to the form of Eq. **1** (solid lines). For the parameters obtained in these particular experiments see Table 1, samples 5a and 5b. (*b*) Double reciprocal plots according to Eq. **2** of six independent experiments, each carried out with a single batch of eggs (including the two experiments shown in *a*). Procedures were as described in *a*. Least-squares solutions for each data set are shown in Table 1.

reported in earlier studies using carrier DNA and near-saturating quantities of *CyIIIa–CAT* genes [i.e., taking into account that CAT enzyme levels per embryo will be about 10 times the CAT per cell values listed in Table 1 (for example, refs. 12 and 17)]. Table 1 shows that the half-saturating number of *CyIIIa–CAT* sequences per cell ($G_{0.5}$) is 105 ± 40 genes (mean ± SD). The variation in these data is little greater than the variation between the two sibling experiments shown in Fig. 2*a* (experiments 5a and 5b). The excellent fit of the data for all batches to the form of Eq. **1** or **2** demonstrates that as the number of *CyIIIa–CAT* genes per cell increases, the output of CAT enzyme indeed follows saturation behavior, and thus the incorporated genes can be considered to be titrating out cellular factor(s) needed to express this sequence. Since 50% saturation is approached at only about 10^2 genes per cell, whereas the same cells are probably expressing at least 1–2 × 10^4 diverse transcription units (18), it is obvious that the saturation observed is a phenomenon that pertains to *CyIIIa* or a small class of genes that might require the same factors. It follows that the saturation functions shown in Fig. 2 represent titrations of a limiting set of sequence-specific factors required for *CyIIIa* expression.

Table 1. Saturation parameters for *CyIIIa–CAT* expression in transgenic sea urchins

Female*	k, genes per CAT	$G_{0.5}$,† genes per cell	$G_{0.9}$,† genes per cell	C_0,‡ CAT per cell (sat.)
1	7.0×10^{-5}	88	990	1.25×10^6
2	7.7×10^{-5}	70	620	9.1×10^5
3	1.8×10^{-4}	82	760	4.5×10^5
4	1.9×10^{-4}	158	1860	8.3×10^5
5a	5.4×10^{-5}	79	700	1.45×10^6
5b	2.6×10^{-4}	153	1400	5.9×10^5
Mean ± SD	1.4×10^{-4} ± 8.3×10^{-5}	105 ± 40	1055 ± 480	1.1×10^6 ± 4.4×10^5

CAT, CAT enzyme molecules; sat., saturation.
*Each batch of eggs was obtained from a different female except for 5a and 5b, which both derived from the same female. The batches of eggs (female number) correspond to the experiments in Fig. 2*a*. See Figs. 1 and 2 and *Materials and Methods* for experimental details.
†$G_{0.5}$ and $G_{0.9}$ represent genes per cell (average) for 50% and 90% saturation, respectively, given the least-squares solutions for C_0 and k and assuming Eq. **1**.
‡Parameters derived from linear least-squares reductions of the data shown assuming Eq. **2**.

DISCUSSION

The titration described here provides an absolute index of the molecular availability of the limiting factors required for *CyIIIa–CAT* expression. We assume in the following that these are sequence-specific transcription factors, since the coinjected upstream regulatory sequences of the *CyIIIa* gene compete efficiently with the *CyIIIa–CAT* fusion gene. Similar results were obtained from other unpublished experiments (cited in the Introduction) in which small subfragments of the upstream sequence that include no transcribed or translated sequence whatsoever were coinjected. However, there is no formal proof that the results of the titration experiments are not affected by competition for specific, rare, translation factors, a possibility that we nonetheless discard on grounds of improbability. The efficient competition shown in Fig. 1 obviously cannot be explained on this basis since all samples contain the same amount of translatable sequence. Competition for general translation as well as transcription factors is precluded because the CAT transcripts are only a few percent of the total nuclear transcripts and, similarly, because CAT protein translation per se represents only a few percent of the total embryo protein synthesis. Thus Flytzanis *et al.* (10) calculated that the half-life of CAT enzyme in sea urchin embryos is about 40 min, and, from this and the maximum steady-state levels of CAT enzyme per cell shown in Table 1,

the synthesis rate should be about 1.9×10^4 CAT enzyme molecules per min per cell. This may be compared to about 2×10^5 protein molecules synthesized per min per cell in late blastula-stage sea urchin embryos, by assuming the polysomal mRNA contents and translation rates measured for *S. purpuratus* embryos (18).

By assuming then that the titrations we report measure the number of bound regulatory factors of the limiting species per nucleus *in vivo*, we proceed to a comparison with the results of DNA–protein binding studies carried out in vitro with the same *CyIIIa* regulatory domain (13). We adopt the $G_{0.9}$ values of Table 1 (on the basis that one protein molecule of a given limiting species binds per site) as an approximate index of the minimum number of factor molecules available for binding within the aboral ectoderm nuclei. That is, we assume that about 1055 sites per nucleus bind 90% of the available factor(s) of the limiting species, since this number of specific exogenous incorporated sites results in 90% of the possible gene expression. The number of these factors per embryo must then be at least 2.1×10^5, since there are 200 aboral ectoderm cells, and this could be up to a 2.5-fold underestimate, as the factors in question might be present in other cells as well. However, there are also factor molecules that would be unavailable because they are at any one moment bound to some of the huge number of nonspecific DNA sites present in the nuclear genome. As an extreme estimate of these, we consider the total internucleosomal DNA, or about 30% of the nucleotide pairs in the genome. For the *CyIIIa* regulatory factors, Calzone *et al.* (13) measured binding preferences, or K_r values, for specific as compared to nonspecific DNA sites that ranged from about 3×10^4 to about 2×10^6 ($K_r = K_s/K_n$, where K_s and K_n are, respectively, the equilibrium constants for the specific and nonspecific reactions of the binding proteins; ref. 15). The ratio of the amount of factor bound to nonspecific vs. specific sites is, from the definition of the equilibrium constants, given by D_n/K_rD_s, where D_n is the concentration of nonspecific sites in the nucleus and D_s is the concentration of specific sites. Here D_n is the molar concentration of 30% of 1.6×10^9 nucleotide pairs in the diploid nucleus, and D_s is the molar concentration of the 1055 specific sites at 90% saturation. Thus for the range of K_r values observed *in vitro*, most of which fall between 5×10^4 and 10^6, 90% and 50%, respectively, of the factor(s) would be found associated with the nonspecific sites, were equilibrium conditions to prevail. For example were $K_r = 10^6$, the total number of factor molecules of that species would be about 2100 per nucleus, of which half (1055) are bound to specific and half to nonspecific sites. However, the total amount of available nonspecific DNA per nucleus is most probably much less than 30% of the total base pairs in the diploid genome (14, 15), and thus the fraction of factor molecules bound to nonspecific DNA will in actuality be less. In any case, this heuristic argument shows that the amount of factor per nucleus should fall within a few fold of the total number of sites actually bound per nucleus as the saturation curves in our experiments approach their maximum. Our previous measurements of factor prevalence in nuclear extracts *in vitro* (13) yielded an average, for 11 different *CyIIIa* regulatory interactions studied, of about 3.2×10^3 molecules of factor per nucleus, by assuming distribution to 200 cells per embryo. This value seems unexpectedly similar to the value we estimate here from *in vivo* saturation measurements.

This result implies that DNA-binding factor prevalences, measured *in vitro* for DNA–protein interactions at equilibrium, may be referred to regulatory binding interactions in life. The general implication, which remains to be directly tested, is that the concentrations of bound and free regulatory sites and nonspecific sites are all at equilibrium within the nucleus. Our experiments show that there is no very large reservoir of extractable binding factor irreversibly associated with nonspecific sites (i.e., beyond those factor molecules that can be stolen from nonspecific sites as high-affinity sites are added). Calzone *et al.* (13) estimated a half-life for the protein–nonspecific DNA complexes bound by the *CyIIIa* factors *in vitro* of the order of a few milliseconds, and these complexes must be highly unstable *in vivo* as well. Thus, it is reasonable to conclude that the K_r values *in vivo* are not greatly lower than those we measured *in vitro*. They could be higher, however, and thus it is not yet clear whether once formed the specific DNA–protein complexes in vivo are also as unstable as observed *in vitro*. As measured in the crude nuclear extracts, the half-lives of these ranged from several minutes to close to an hour, and on present evidence it cannot be excluded that in living nuclei such complexes are "locked" into more stable configurations. However, the results we report permit the possibility that the specific as well as the nonspecific interactions within the nucleus behave more or less as expected from equilibrium considerations *in vitro*. The active state of a gene would thus be maintained by the continuous presence of a sufficient reservoir of binding factors, rather than by persistence of nondissociable complexes. Any given active gene at any given moment might be disengaged from its regulatory factors and re-engaged the next moment and, thus, with respect to dissociation of active complexes, DNA replication might not be a particularly unique event. In an equilibrium system, control would be dynamic rather than exclusively structural, and the cessation or quantitative modulation of activity might be governed, with a brief time lag dependent only on complex half-life, by an increase or decrease in the ambient concentration of active factor.

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