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Developmental expression of synthetic cis-regulatory systems composed of spatial control elements from two different genes

(transcriptional control/regulatory modules/*Endo16*/*SM50*)

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ABSTRACT Synthetic cis-regulatory systems consisting of positively and negatively acting cis-regulatory modules of the *Endo16* gene were combined with the lineage-specific regulatory element of the *SM50* gene associated with a reporter and injected into eggs of sea urchins. We show here that synthetic cis-regulatory systems consisting of the positive *Endo16* regulatory elements linked with the *SM50* regulatory element are expressed spatially exactly as the sum of the individual endodermal and skeletogenic expression patterns. In combination, both lineage-specific positive regulatory elements function autonomously. However, addition of the *Endo16* regulatory module that represses ectopic skeletogenic expression of *Endo16* receptor constructs does not affect expression driven by the *SM50* regulatory elements in the same skeletogenic cells. The repression function of this element is thus dedicated to control of the positive spatial output of the *Endo16* regulatory system.

The question addressed in this paper is whether a predictable combination of embryonic spatial gene expression patterns can be generated by combining cis-regulatory elements from diverse genes. We generated chimeric cis-regulatory systems by combining spatial control elements of the sea urchin genes *Endo16* and *SM50*. These elements individually direct expression to the endoderm and skeletogenic domains of the embryo, respectively. When physically linked in single constructs, these elements were observed to function autonomously and additively, producing a novel combined spatial pattern of expression in the embryo.

In recent studies (1–5), we experimentally defined the cis-regulatory systems of the *SM50* and *Endo16* genes of *Strongylocentrotus purpuratus*. These are expressed, respectively, in the skeletogenic mesenchyme lineages and in the endoderm of the embryo, in entirely nonoverlapping patterns. *Endo16* encodes a secreted cell surface protein (6). The gene is transcribed in the vegetal plate of the early embryo and throughout the archenteron after invagination. Transcription is turned off in the secondary mesenchyme cells as they delaminate from the tip of the gut, and *Endo16* expression then disappears from the foregut and the hindgut, so that at the end of embryogenesis only the midgut expresses the gene (3, 6). The 2.3-kb cis-regulatory system of *Endo16* consists of at least six modules. Three of the *Endo16* modules, *G, B*, and *A* (see Fig. 1) act positively and synergistically to promote expression in the endodermal lineages of the embryo (5). Each displays a particular temporal pattern of expression which also causes a modest ectopic expression in ectoderm lineages surrounding the vegetal plate, and in skeletogenic lineages, as well as correct expression in endodermal lineages. The repressive spatial functions executed by the other three modules, *F, E*, and *DC*, are required to confine expression to the vegetal plate and its derivatives (5). *DC* shuts off the gene in the skeletogenic mesenchyme, thus setting the lower boundary of expression, and modules *E* or *F* suffice to turn off *Endo16* cis-regulatory activity in the tier of ectoderm cells adjacent to the endoderm, thus setting the upper boundary. The cis-regulatory system that controls *SM50* gene expression is considerably simpler. This gene encodes a spicule matrix protein. It is expressed exclusively in the skeletogenic mesenchyme lineages, and is controlled by an ≈500-bp upstream sequence (Fig. 1*A*) that responds to a few key activators which are presumably present, or active, only in skeletogenic lineages throughout embryogenesis (2). There is no evidence for negative spatial control elements in the *SM50* cis-regulatory system.

MATERIALS AND METHODS

Embryo Culture and Microinjection. *S. purpuratus* embryos were cultured and injected as described (7, 8). They were collected at the appropriate stage and processed for chloramphenicol acetyltransferase (CAT) measurements or whole mount *in situ* hybridization.

Construction of Reporter Gene Constructs. *Starting constructs.* The *SM50·CAT* construct (construct 1 of Fig. 1*B*) described in ref. 2, and the plasmids *GBA–Bp·CAT* and *GDCBA–Bp·CAT* described in ref. 5, were used as starting constructs (constructs 2 and 5 of Fig. 1*B*). These plasmids contain various fragments of regulatory DNA of *SM50* or *Endo16*, a transcription initiation site, 5′-leader sequences, a *CAT* fusion gene, a simian virus 40 (SV40) 3′-trailer, and a poly(A) adenylation site. *Bp* denotes the *Endo16* basal promoter (5).

Experimental constructs. For the cloning of *GBA–SM50·CAT* and *GDCBA–SM50·CAT* (constructs 3 and 7, Fig. 1*B*), *SM50·CAT* was cut with *Hin*dIII and *Pst*I. The *GBA* and *GDCBA* fragments were amplified with primers mod1 and mod2, using *GBA–Bp·CAT* and *GDCBA–Bp·CAT* as templates, respectively. The primer sequences, with introduced *Hin*dIII and *Pst*I sites underlined, were as follows: mod1, 5′-GGAAAGCCTTCTTATTCTAATATCCAC-3′; and mod2, 5′-GGTCTGCAGAACAGTTTAACCCGG-3′. PCRs were carried out by using the conditions described in ref. 9, the fragments digested with *Hin*dIII and *Pst*I, and subcloned into *SM50·CAT*. For cloning of *DC–SM50·CAT* (construct 4, Fig. 1*B*), the *DC* fragment of *GDCBA–Bp* was amplified in a PCR by using primers DC1 and DC2. Their sequences, with the *Sph*I and *Bgl*II sites underlined, were as follows: DC1, 5′-CGGGCATGCGACTTCGAACTCATTT-3′; and DC2, 5′-CGGAGATCTGTATACCAATACCCGTT-3′. The PCR product was digested with *Sph*I and *Bgl*II and inserted into *SM50·CAT* cut at the same sites. Similarly, the *DC* fragment

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Abbreviations: CAT, chloramphenicol acetyltransferase; PMC, primary mesenchyme cell.

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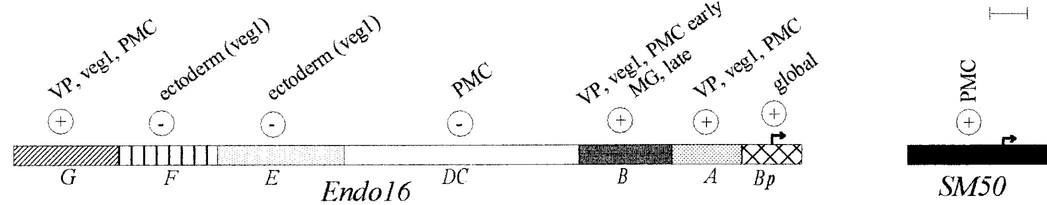
A. MODULAR FUNCTIONS



B. REPORTER GENE CONSTRUCTS

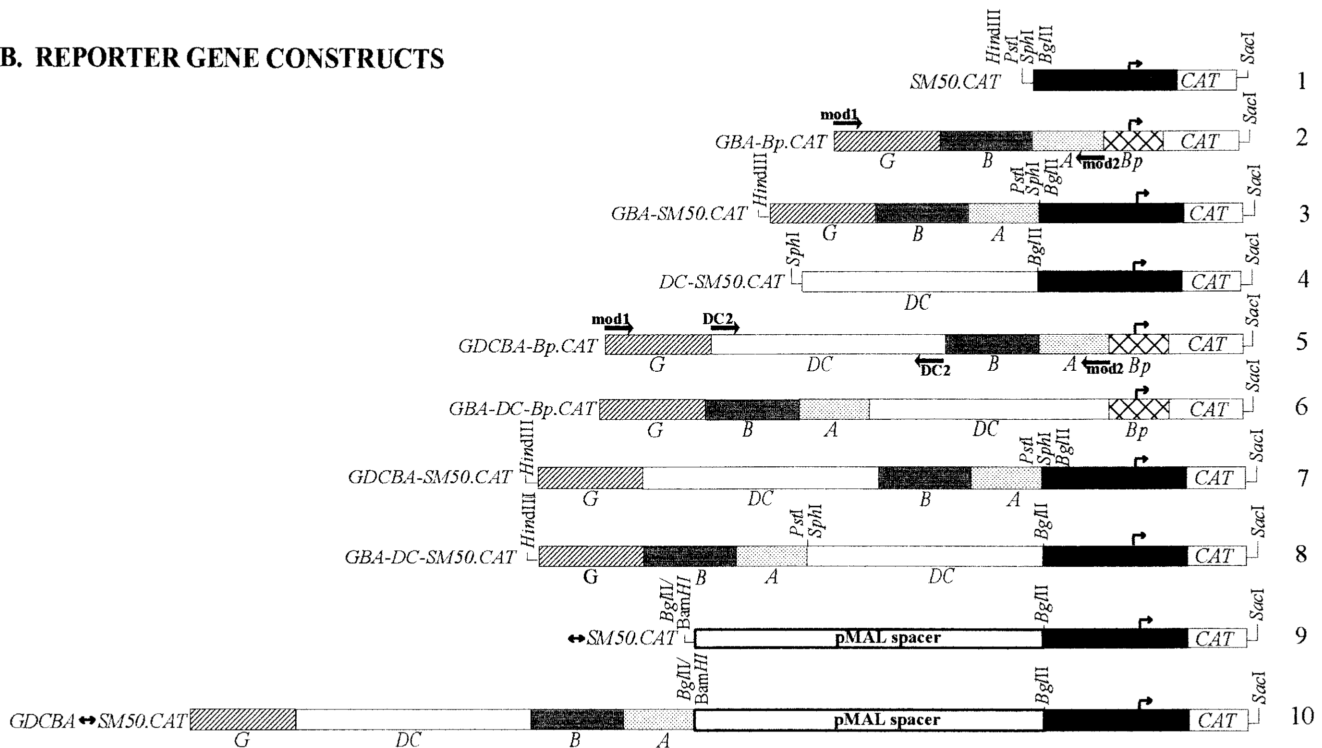


FIG. 1. Schematic illustration of the modular cis-regulatory regions of the sea urchin genes *Endo16* and *SM50*, and reporter gene constructs used in this study. VP, vegetal plate; veg_1, veg_1 tier; PMC, primary mesenchyme cells; MG, midgut; Bp, basal promoter. (*A*) Modules of the 5′ regulatory regions of *Endo16* and *SM50*. Regulatory modules are indicated by differently shaded or hatched boxes. The *Endo16* upstream region consists of six regulatory modules. Module *A* drives early expression in the vegetal plate, the adjoining skeletogenic mesenchyme and ectoderm early in development. The negatively acting modules, *DC* and *E* or *F*, repress gene expression in the skeletogenic mesenchyme and the adjacent veg_1 ectoderm, respectively. Thus, *E* or *F* set the upper, and *DC* the lower, boundary of expression. *B* is sufficient for midgut-specific late expression, and *G* helps both *A* and *B* in stepping up the amplitude of expression throughout development. In contrast, the *SM50* cis-regulatory region is organized in a single module and drives expression exclusively in the autonomously specified skeletogenic mesenchyme. (*B*) Schematic diagrams of reporter gene constructs. DNA fragments of the *Endo16* and *SM50* cis-regulatory regions contained in the constructs are shown as boxes. Restriction sites used to clone reporter gene constructs are indicated. Primers are shown as straight arrows, transcription start sites as bent arrows. Cis-regulatory sequences are drawn to scale, but not the *CAT* fusion gene or the primers. Vector sequences were omitted. (Bar = 100 bp.)

was inserted into *GBA–SM50·CAT* at the *Sph*I and *Bgl*II sites to obtain *GBA–DC–SM50·CAT* (line 8, Fig. 1*B*). To clone *GBA–DC–Bp·CAT* (construct 6, Fig. 1*B*), *GBA–DC–SM50·CAT* was digested with *Bgl*II, filled in, and cut with *Sac*I, which releases the *SM50* and *CAT* sequences, and the *Sma*I–*Sac*I fragment of *Bp·CAT* (5) was inserted. The *Bgl*II–*Bam*HI fragment of *pMAL-p2* (New England Biolabs) was used as a nonfunctional spacer. This consists of an ≈900-bp sequence encoding the bacterial maltose-binding protein. It was inserted in antisense orientation into the *Bgl*II site of *SM50·CAT* to yield plasmid *↔SM50·CAT* (construct 9, Fig. 1*B*). To create plasmid *GDCBA↔SM50·CAT* (line 10, Fig. 1*B*), the same *pMAL-p2* fragment (↔) was inserted into *GDCBA–SM50·CAT* using the single *Bgl*II site. Restriction sites and primers used for cloning are indicated in Fig. 1*B*. Plasmids were linearized for microinjection with *Sac*I.

CAT Measurements and Whole Mount *in Situ* Hybridization. CAT enzyme activity was determined as reported (7), and whole mount *in situ* hybridizations were carried out according to the procedure described in ref. 9. Embryos were analyzed and digitized with a Roche Instruments imaging system using PRORES software and a Zeiss Axioskop microscope. Representative examples were printed on a Nikon CP-300 Printer.

RESULTS

The spatial expression patterns of the experimental constructs used in this work were assessed by whole mount *in situ* hybridization of *CAT* mRNA. Incorporation of injected reporter constructs in sea urchins is mosaic (8, 10, 11), and thus only a subset of the cells of each territory in which a given construct is expressed are stained in any individual embryo. In this study the percentages of experimental embryos displaying expression of transgenes was 62–82%, as summarized in Table 1. The eggs were injected following fertilization, and the embryos were assayed at the late gastrula stage.

Combination of Cell-Type-Specific Modules Results in Expression in Both Territories. *G, B,* and *A,* the positive endoderm-specific modules of the *Endo16* gene (see above and Fig. 1*A*), were linked to the *SM50* regulatory element that is

Table 1. Whole mount *in situ* hybridization data for expression constructs containing *SM50* and *Endo16* regulatory elements, assayed at the late gastrula stage

Construct	Scored embryos*	Labeled/not labeled† (% expression‡)	Labeled, interpretable embryos	% gut expression§	% PMC expression§	% ectoderm expression§
SM50·CAT	608	418/190 (68.8)	385	3.6	97.6	5.0
GBA-Bp·CAT¶	413	258/155 (62.5)	248	94.4	9.7	9.3
GBA-SM50·CAT	591	471/120 (79.7)	436	72.2	92.9	8.7
DC-SM50·CAT	206	146/60 (70.9)	142	2.1	98.6	4.9
GDCBA-Bp·CAT¶	636	429/197 (67.5)	422	97.4	2.8	9.8
GBA-DC-Bp·CAT	163	126/37 (77.3)	119	96.6	3.4	10.9
GDCBA-SM50·CAT	839	692/147 (82.5)	654	79.5	88.2	11.0
GBA-DC-SM50·CAT	260	200/60 (76.9)	191	89.5	88.5	12.0
⇔*SM50·CAT*	226	150/76 (66.4)	141	2.1	100	5.7
GDCBA⇔*SM50·CAT*	272	211/61 (77.6)	187	86.6	80.2	10.2

Gastrulae were collected at 48–54 h postfertilization. PMC, primary mesenchyme cell.

*Values represent data from at least four separate experiments carried out on independent batches of eggs.

†Embryos with more than two labeled cells were scored as labeled.

‡Percent expression = [Σ labeled embryos/Σ labeled + unlabeled embryos] × 100.

§Percent territorial expression = [Σ embryos labeled in given territory/Σ labeled, interpretable embryos].

¶Exactly comparable data were obtained in ref. 5; see table 2 therein. Specifically, ectopic expression generated by *GBA-Bp·CAT* was 9.4% in PMCs and 15.0% in ectoderm, while in *GDCBA-Bp·CAT* it was 1.4% in PMCs and 2.6% in ectoderm. Essentially the same data were obtained with the complete construct—i.e., *Endo16·CAT.* All of these constructs were expressed in endoderm in ≥94% of stained embryos, as in the present work.

necessary and sufficient to promote specific, high-level expression in skeletogenic mesenchyme (PMC) cells. At the gastrula stage when the observations were made these are the primary or skeletogenic mesenchyme cells. In an initial series of experiments we obtained the quantitative expression results expected for the *GBA* (5) and *SM50* (2) regulatory elements when these are tested separately. The control construct *SM50·CAT*, shown diagrammatically in Fig. 1*B*, carries the entire cis-regulatory element of the *SM50* gene. This construct is expressed almost exclusively in the skeletogenic mesenchyme cells in all specimens scored as stained. An example of this type of expression pattern, visualized by whole mount *in situ* hybridization, is shown in Fig. 2*A*, and data from embryos bearing *SM50·CAT* are given in Table 1. Ectopic gut and ectoderm expression are very low, equivalent to background observed with all injected constructs (3.6% and 5.0%, respectively; Table 1). These data are in very good accord with previous observations (1, 2). The second control plasmid, *GBA–Bp·CAT* (line 2 of Fig. 1*B*), is expressed in archenteron cells in ≈95% of all stained embryos (Table 1). However, *GBA–Bp·CAT* generates ≥10% ectopic expression in both the ectoderm and the skeletogenic mesenchyme as is shown in Table 1. These results are again almost the same as reported earlier (ref. 6; see Table 1). The *GBA* and *SM50* regulatory sequences were combined in the fusion gene *GBA–SM50·CAT* (line 3 of Fig. 1*B*). Expression of this construct is detected in both skeletogenic mesenchyme and gut cells in a high percentage of all labeled embryos (93% and 72%, respectively; Table 1). Ectoderm cells are ectopically stained with the same frequency as observed in embryos expressing *GBA–Bp·CAT* (Table 1).

The expression pattern generated by *GBA–SM50·CAT* is the sum of those generated by the two individual control plasmids *SM50·CAT* and *GBA–Bp·CAT*. The percentages of embryos displaying staining specific to the skeletogenic mesenchyme, or the gut, respectively, appear slightly higher for the control constructs *SM50·CAT* and *GBA–Bp·CAT* than for *GBA–SM50·CAT* (between 5 and 20%; compare data in Table 1 for *SM50·CAT* expression in the skeletogenic cells and *GBA–Bp·CAT* expression in archenteron to *GBA–SM50·CAT* expression). This apparent difference is due solely to the requirement of normalizing to the number of total labeled embryos in a system in which incorporation of exogenous DNA is mosaic. That is, the total number of embryos seen to be labeled with the *GBA–SM50·CAT* construct is higher (≈80% versus 65%; Table 1) because embryos in which either gut or PMCs alone contain the exogenous DNA both display labeling, while embryos bearing either *SM50·CAT* or *GBA–Bp·CAT* display labeling only if the exogenous DNA happens to be located in skeletogenic or endoderm lineages, respectively. If the fractions of embryos in the *GBA–Bp·CAT* or *SM50·CAT* samples are normalized to the larger fraction of stained embryos recorded for *GBA–SM50·CAT* in Table 1, the levels of expression achieved by the latter turns out to be about the same as the normalized control construct values. It follows that there is no inhibitory effect of any of the components interacting with *GBA* on the *SM50* regulatory system and *vice versa*. In fact, CAT assays show that the total amount of CAT enzyme produced in embryos expressing *GBA–SM50·CAT* is approximately one-third higher than the sum of CAT protein synthesized from *SM50·CAT* and *GBA–Bp·CAT* separately. This measurement is shown in Fig. 3 *Left*.

The experiments summarized in Table 1 show clearly that the *GBA* and *SM50* regulatory modules function autonomously when combined in a single construct. Each causes transgene expression in the appropriate spatial domains of the embryo. Neither interference effects nor unanticipated expression patterns are observed, and except for a slight enhancement of overall activity levels, the functions of the *Endo16* and *SM50* regulatory elements appear strictly additive.

***DC* Does Not Affect Expression of *SM50* Constructs in Skeletogenic Cells.** In the normal context of the *Endo16* control system repression of the weak ectopic skeletogenic expression that would otherwise be caused by the positive *GBA* regulators is accomplished by the *DC* module. Yuh and Davidson (5) showed that an interaction with module *A* is required in order for module *DC* to carry out this function. Since *DC* precludes *Endo16* expression in skeletogenic cells, we next asked whether this module would also effect the positive *SM50* regulators in the same cells. This would be the case if *DC* generates a dominant negative interaction with components of the transcription apparatus in these cells. Thus *DC* was linked to *SM50·CAT* (*DC–SM50·CAT,* line 4 of Fig. 1*B*), and its expression was compared with that of the control *SM50·CAT* construct. As illustrated in Fig. 2*B*, spatial expression of the *SM50·CAT* transgene is not affected at all by addition of *DC*. There is no apparent decrease in PMC expression. Thus 68.8% of injected embryos express detectable

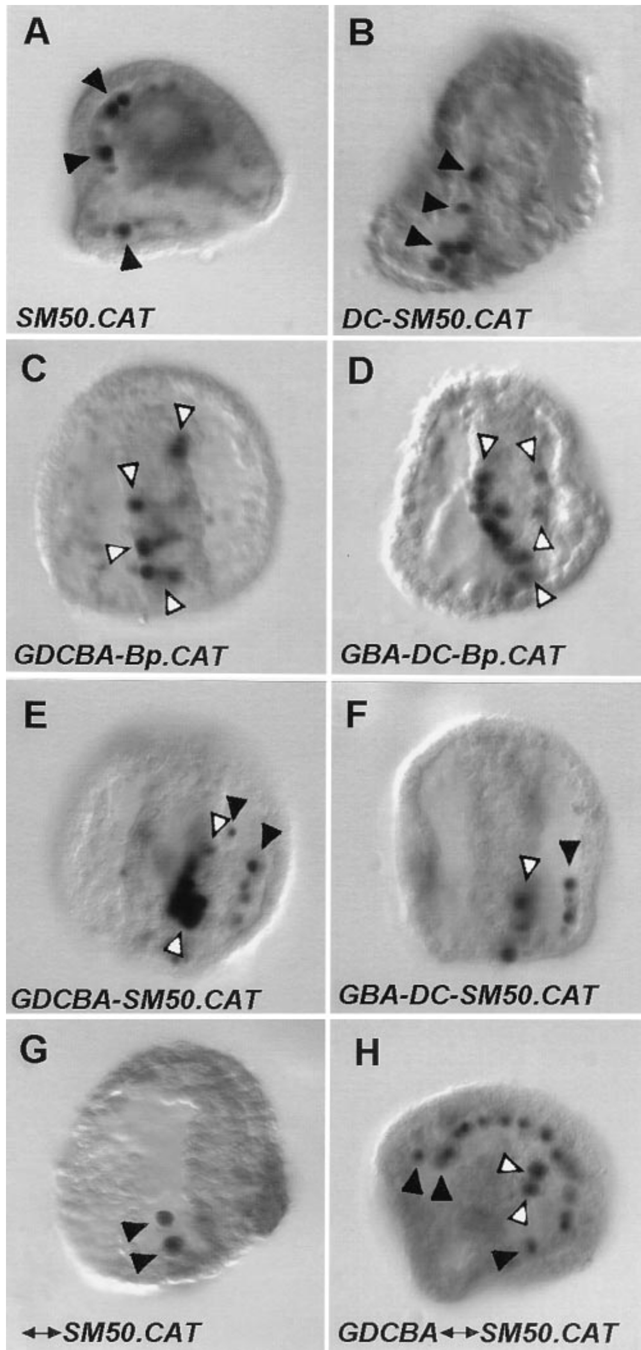


FIG. 2. Spatial expression patterns of various reporter constructs containing *SM50* and *Endo16* regulatory modules at the late gastrula stage. The spatial distribution of *CAT* transcripts was detected by whole mount *in situ* hybridization, using a digoxigenin-labeled antisense *CAT* RNA probe, and analyzed by means of differential interference contrast microscopy. The embryos in *A* and *H* are shown as optical sections perpendicular to the animal–vegetal axis. The oral ectoderm is positioned at the top of each panel, and the PMCs are external to the circular gut. The embryos in *C*, *E*, and *F* are displayed in frontal view, with the gut in the center and the PMCs lateral to the gut. *B*, *D*, and *G* show embryos in lateral view, with the oral side oriented to the right of the tube-shaped gut and the PMCs in the blastocoel. Black arrowheads indicate labeled PMCs, and white arrowheads indicate labeled gut cells. (*A*) Embryo showing normal *SM50·CAT* (line 1 of Fig. 1*B*) expression exclusively in the skeletogenic mesenchyme. (*B*) Embryo expressing *DC–SM50·CAT* (line 4 of Fig. 1*B*) in a clone of PMCs. The specimen is oriented with the plane of focus slightly oblique to the gut. Consequently, two of the labeled cells are superimposed on the contours of the gut. Changing the focal plane to a plane less useful for displaying the morphological features of the embryo demonstrates that all labeled cells are PMCs. (*C*) Embryo displaying appropriate *GDCBA–Bp·CAT* expression (line 5 of Fig. 1*B*) in the gut cells. (*D*) Embryo showing normal expression of *GBA–DC–Bp·CAT* (line 6 of Fig. 1*B*) in a large clone in the archenteron. (*E*) Embryo carrying *GDCBA–SM50·CAT* reporter constructs (line 7 of Fig. 1*B*), displaying patches of stained cells in both gut and skeletogenic mesenchyme. (*F*) Embryo expressing *GBA–DC–SM50·CAT* (line 8 of Fig. 1*B*) in skeletogenic mesenchyme and archenteron cells. (*G*) Embryo carrying reporter genes that contain a large, nonfunctional spacer element upstream of *SM50·CAT* (↔*SM50·CAT*, construct 9 of Fig. 1*B*) displaying CAT transcripts in PMCs only. (*H*) *GDCBA*↔*SM50·CAT* (line 10 of Fig. 1*B*) also locates reporter gene expression to the skeletogenic mesenchyme and gut.

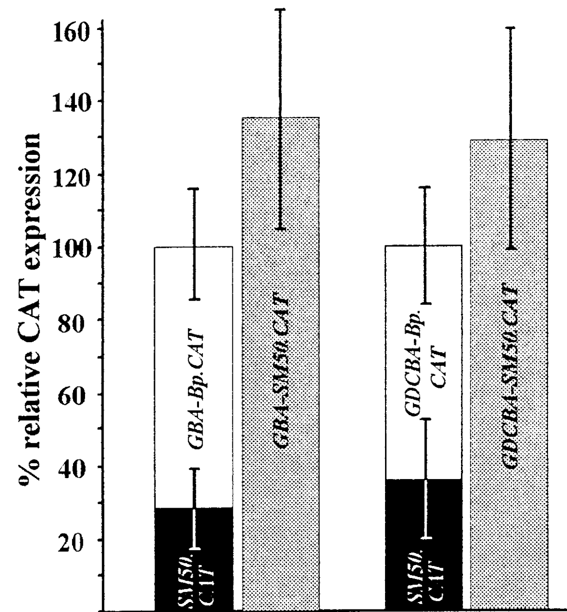


FIG. 3. Quantitative expression of various reporter gene constructs carrying *SM50* and *Endo16* regulatory modules at the late gastrula stage. The number of CAT enzyme molecules per embryo was determined in CAT assays by comparison of the samples to standards of known CAT protein concentration. All data are averages, and vertical lines through the bars represent standard deviations ($n = 4$). The values measured separately for *GBA–Bp·CAT* and *SM50·CAT* (*Left*) or *GDCBA–Bp·CAT* and *SM50·CAT* (*Right*) are shown as white and black bars, respectively. These values were added, and the totals set to 100%. Experimental values obtained with *GBA–SM50·CAT* and *GDCBA–SM50·CAT* were compared with these totals and also expressed as percentages (gray bars).

SM50·CAT and 70.9% express *DC–SM50·CAT* (Table 1); the fraction of embryos scoring positive by whole mount *in situ* hybridization is a sensitive measure of the level of expression (5, 9). On the other hand, when the same batches of embryos are injected with *GDCBA–Bp·CAT* (line 5 of Fig. 1*B*) the 10% ectopic PMC expression observed with *GBA–Bp·CAT* is eliminated, reducing skeletogenic expression to background levels (Table 1). An example of an embryo bearing *GDCBA–Bp·CAT* and displaying expression exclusively in the gut is shown in Fig. 2*C*. These data are again in excellent quantitative agreement with previous observations (6), which for comparison are reproduced in Table 1, and they confirm that *DC* is a repressor of ectopic *Endo16* expression in PMCs. Since *DC* does not affect expression of the *SM50* regulators in the *DC–SM50·CAT* construct, while it eliminates all but background expression of *GDCBA–Bp·CAT*, the repressive effects of this module are apparently dedicated to the *Endo16* regulatory system.

In construct *DC–SM50·CAT* module *DC* is directly upstream of the *SM50* regulatory sequences. To test whether the position of *DC* affects its function, we constructed plasmid *GBA–DC–Bp·CAT* (line 6 of Fig. 1*B*), which more closely reflects the

arrangement in *DC–SM50·CAT* with respect to the distance of *DC* to the start site compared with *GDCBA–Bp·CAT*. Table 1 shows that *GBA–DC–Bp·CAT* expression in the three embryonic territories monitored is indistinguishable from that of *GDCBA–Bp·CAT*. An embryo expressing *GBA–DC–Bp·CAT* in gut cells is illustrated in Fig. 2*D*.

Reporter constructs carrying a combination of *GDCBA* or *GBA–DC* plus *SM50* (*GDCBA–SM50·CAT* and *GBA–DC–SM50·CAT*, lines 7 and 8 of Fig. 1*B*, respectively), also express the *CAT* fusion gene in a very high fraction of both skeletogenic mesenchyme and gut cells. Representative examples displaying labeled cells in both these territories are shown in Fig. 2 *E* and *F*. Again, the overall percentage of PMC, and of gut expression is higher in embryos expressing controls *DC–SM50·CAT* or *GDCBA–Bp·CAT*, respectively, than in embryos expressing *GDCBA–SM50·CAT* or *GBA–DC–SM50·CAT* (Table 1). However, normalized to the total of scored embryos, there is no significant quantitative difference between controls and experimental samples, as above. Furthermore, quantitative measurements of CAT expression show that the sum of CAT enzyme production from *SM50·CAT* plus *GDCBA–Bp·CAT* is about 30% less than that of *GDCBA–SM50·CAT* (Fig. 3 *Right*). In summary, the experiments described in this paragraph show that the PMC activity of the *SM50* regulatory sequence is quantitatively impervious to repression by module *DC*, which eliminates the ectopic expression of *Endo16* constructs in the same cells.

The *Endo16* Regulatory System Functions in Combination with *SM50* Regulators When Separated from Them by a Spacer. A 900-bp spacer element was introduced between the *GDCBA* and the *SM50* sequences of *GDCBA–SM50·CAT*. This construct, *GDCBA↔SM50·CAT*, is shown in Fig. 1*B*, line 10. The fragment used as a spacer was a *pMAL-p2* element containing sequences of the bacterial maltose-binding protein. We first demonstrated that the spacer is not in itself functional—i.e., that it does not affect PMC expression when linked to *SM50·CAT* (construct *↔SM50·CAT* of Fig. 1*B*, line 9). Results are illustrated in Fig. 2*G* and summarized in Table 1 (compare with *SM50·CAT* of Table 1). Table 1 shows that *GDCBA↔SM50·CAT* is expressed exactly as is *GDCBA–SM50·CAT*. More than 86% of all embryos scored show gut expression, and around 80% display PMC staining, as compared with 80% gut, and 88% PMC labeling in embryos injected with *GDCBA–SM50·CAT* (Table 1). Fig. 2*H* displays an embryo expressing *GDCBA↔SM50·CAT* in gut and PMC cells. This experiment confirms the autonomous function of the *Endo16* regulatory system, and shows that its independent interaction with the basal transcription apparatus occurs efficiently over a considerable distance.

DISCUSSION

It seems remarkable that a novel gene regulatory system can be constructed simply by physically linking cis-regulatory elements from genes that function in different spatial domains. An earlier example was described by Levine and colleagues (12), who produced a synthetic cis-regulatory system by combining the modular stripe 2 element of the *Drosophila even-skipped* pair rule gene with an element from the gene rhomboid that produces bilateral stripes, generating a bilateral crossed pattern of expression. Here we describe the expression of a synthetic sea urchin embryo cis-regulatory system that was created by combining pieces of the *Endo16* and *SM50* cis-regulatory systems, and that faithfully functions in both the gut and the skeletogenic mesenchyme. In these constructs the basal promoter utilized is that of the *SM50* gene, which happens to have a TATA-less initiator. However, Makabe *et al.* (2) showed that the *SM50* cis-regulatory system functions just as well when the initiator sequence is replaced by a synthetic TATA box sequence. Active expression driven by the *Endo16* regulators when they are linked to *SM50·CAT* requires that the positive upstream modules of *Endo16*, *G*, *B*, and *A*, interact just as efficiently with the *SM50* basal transcription apparatus as with its own TATA box-containing promoter. This is shown by the results of Fig. 3, which demonstrate that the linked construct *GBA–SM50·CAT* has even more activity than the sum of the activities of *Endo16–Bp·CAT* (which contains the endogenous *Endo16* basal promoter, *Bp*), plus *SM50·CAT*. In spatial terms the combined construct expresses exactly as does the sum of the individual expression domains imposed by the separate *SM50* and *Endo16* regulatory systems. Furthermore, the *Endo16* cis-regulatory modules interact efficiently with the basal *SM50* promoter when placed about a kilobase further away. Earlier studies on both the *SM50* (2) and the *Endo16* (5) regulatory systems showed that both also function when associated with *SV40* basal promoters. The implication of these studies together with the present demonstration is clearly that the basal promoter apparatus is promiscuous; all the specificity for developmental function lies in the distal cis-regulatory systems, and each of those tested here has an autonomous capability of animating the basal promoter apparatus. This is the reason that it is possible to create synthetic spatial regulatory systems by combining upstream regulatory elements from different genes. To our knowledge, this is the first observation of this kind for a type 1 embryonic system (13), as the previous examples are confined to *Drosophila* (12).

The experiments utilizing the negative *DC* regulatory module of *Endo16* show that it does not affect expression driven by the *SM50* regulators in skeletogenic cells, though in these same cells it reduces expression driven by the *GBA* regulators of *Endo16* to background levels. *DC* is therefore not a dominant silencer of transcription in these cells. With respect to the *Endo16* and *SM50* regulatory systems it is likely that *DC* is dedicated to *Endo16*. This observation confirms and extends the previous finding that the function of *DC* requires interaction with module A of *Endo16* (5). However, we cannot rigorously exclude the possibility that *DC* fails to affect expression driven by the *SM50* regulators because of some particular characteristic of the *SM50* basal promoter, or because the DC repressor may bind too far away from the *SM50* activators, although we note that no repressive effect was observed when DC module was brought into direct apposition in the construct *DC–SM50·CAT*.

Repressive elements that control spatial expression by dedicated interaction with particular positive regulatory elements within the cis-regulatory system may be a very general and essential feature of developmental gene regulation. To consider only some particularly well studied examples from embryonic spatial gene control systems, such locally acting negative regulators are known for *Drosophila*, in the *eve* stripe 2 (14, 15), and stripe 3 (16) modules; in the *rhomboid* lateral stripe element (17); and in the *Kruppel* embryonic expression element (18); and for the sea urchin, in the middle module of the *CyIIIa* cytoskeletal actin gene (9, 19) as well as in the *Endo16* gene (5). These are all examples of local dedicated interaction of negative regulatory elements with other elements that function positively, such that if they were not repressed in given embryonic territories, ectopic spatial expression would result. This is an important and general feature of the modular autonomy of cis-regulatory systems. It contributes to the flexibility with which spatial control elements can be combined in novel arrangements without abrogating function. That is, a high complexity of positive–negative interactions is afforded by the diverse arrays of upstream transcription factors, compared with a system in which expression is mediated only by direct interaction of the various repressors with the same basal transcription apparatus. In any case, in the example studied here, the *DC* skeletogenic repressor of *Endo16* has no dominant silencing activity in the cells in which it is active.

Of course the main implication of the experimental assembly of a synthetic regulatory system is that it emphasizes the likelihood that cis-regulatory recombination has been a major evolutionary mechanism for the generation of new developmental regulatory systems. This is scarcely a new idea (20). But it is only recently that it has become possible to demonstrate experimentally the remarkable lack of constraint in the identity of compatible elements, in spacing, in functionality, or in linear arrangement, that is a major characteristic of modular cis-regulatory organization.

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1. Sucov, H. M., Hough-Evans, B. R., Franks, R. R., Britten, R. J. & Davidson, E. H. (1988) *Genes Dev.* **2,** 1238–1250.
2. Makabe, K. W., Kirchhamer, C. V., Britten, R. J. & Davidson, E. H. (1995) *Development (Cambridge, U.K.)* **121,** 1957–1970.
3. Ransick, A., Ernst, S. G., Britten, R. J. & Davidson, E. H. (1993) *Mech. Dev.* **42,** 117–124.
4. Yuh, C.-H., Ransick, A., Martinez, P., Britten, R. J. & Davidson, E. H. (1994) *Mech. Dev.* **47,** 165–186.
5. Yuh, C.-H. & Davidson, E. H. (1996) *Development (Cambridge, U.K.)* **122,** 1069–1982.
6. Nocente-McGrath, C., Brenner, C. A. & Ernst, S. G. (1991) *Dev. Biol.* **136,** 264–272.
7. McMahon, A. P., Flytzanis, C. N., Hough-Evans, B. R., Katula, K. S., Britten, R. J. & Davidson, E. H. (1985) *Dev. Biol.* **108,** 420–430.
8. McMahon, A. P., Novak, T. J., Britten, R. J. & Davidson, E. H. (1985) *Proc. Natl. Acad. Sci. USA* **81,** 7490–7494.
9. Kirchhamer, C. V. & Davidson, E. H. (1996) *Development (Cambridge, U.K.)* **122,** 333–348.
10. Hough-Evans, B. R., Franks, R. R., Cameron, R. A., Britten, R. J. & Davidson, E. H. (1987) *Dev. Biol.* **121,** 576–579.
11. Franks, R. R., Anderson, R., Moore, J. G., Hough-Evans, B. R., Britten, R. J. & Davidson, E. H. (1990) *Development (Cambridge, U.K.)* **110,** 31–40.
12. Cai, H. N., Arnosti, D. N. & Levine, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9309–9314.
13. Davidson, E. H. (1991) *Development (Cambridge, U.K.)* **113,** 1–26.
14. Small, S., Blair, A. & Levine, M. (1992) *EMBO J.* **11,** 4047–4057.
15. Small, S., Blair, A. & Levine, M. (1996) *Dev. Biol.* **175,** 314–324.
16. Arnosti, D. N., Barolo, N., Levine, M. & Small, S. (1996) *Development (Cambridge, U.K.)* **122,** 205–214.
17. Ip, Y. T., Park, R. E., Kosman, D., Bia, E. & Levine, M. (1992) *Genes Dev.* **6,** 1728–1729.
18. Hoch, M., Gerwin, H., Taubert, H. & Jäckle, H. (1992) *Science* **256,** 94–97.
19. Coffman, J. A., Kirchhamer, C. V., Harrington, M. G. & Davidson, E. H. (1996) *Dev. Biol.* **174,** 43–54.
20. Britten, R. J. & Davidson, E. H. (1971) *Q. Rev. Biol.* **46,** 111–138.