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Green Fluorescent Protein in the sea urchin: new experimental approaches to transcriptional regulatory analysis in embryos and larvae

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SUMMARY

The use of Green Fluorescent Protein (GFP) as a reporter for expression transgenes opens the way to several new experimental strategies for the study of gene regulation in sea urchin development. A GFP coding sequence was associated with three different previously studied *cis*-regulatory systems, viz those of the *SM50* gene, expressed in skeletogenic mesenchyme, the *CyIIa* gene, expressed in archenteron, skeletogenic and secondary mesenchyme, and the *Endo16* gene, expressed in vegetal plate, archenteron and midgut. We demonstrate that the sensitivity with which expression can be detected is equal to or greater than that of whole-mount in situ hybridization applied to detection of CAT mRNA synthesized under the control of the same *cis*-regulatory systems. However, in addition to the important feature that it can be visualized nondestructively in living embryos, GFP has other advantages. First, it freely diffuses even within fine cytoplasmic cables, and thus reveals connections between cells, which in sea urchin embryos is particularly useful for observations on regulatory systems that operate in the syncytial skeletogenic mesenchyme. Second, GFP expression can be dramatically visualized in postembryonic larval tissues. This brings postembryonic larval developmental processes for the first time within the easy range of gene transfer analyses. Third, GFP permits identification and segregation of embryos in which the clonal incorporation of injected DNA has occurred in any particular desired region of the embryo. Thus, we show explicitly that, as expected, GFP transgenes are incorporated in the same nuclei together with other transgenes with which they are co-injected.

Key words: gene regulation, transgenic analysis, larval development, mosaic DNA incorporation, Green Fluorescent Protein (GFP), sea urchin

INTRODUCTION

The *cis*-regulatory systems that control developmental gene expression are highly organized and complex, typically requiring sequence-specific interactions with many different transcription factors (reviewed by Arnone and Davidson, 1997). Functional analysis of such *cis*-regulatory systems requires an easy and rapid gene transfer methodology, since the large number of interactions necessitates tests of many different experimental variants of the natural regulatory DNA sequence. Sea urchin embryos provide experimental advantages because of the ease of gene transfer into the eggs of these organisms, and also their accessibility to biochemical technologies for transcription factor purification (Calzone et al., 1991; Coffman et al., 1992, 1996). Sea urchins are enormously fecund creatures and the amounts of embryonic material that they produce enable transcription factors to be isolated by affinity chromatography and their mRNAs to be cloned. This is a crucial advantage, both for biochemical exploration of *cis*-regulatory functions, and for excursions into the upstream domains of the regulatory network. In this paper, we describe a significant extension of current methodology for gene transfer and expression analysis in sea urchin development, using a green fluorescent protein (GFP) reporter (Chalfie et al., 1994).

The gene transfer method used in sea urchins is injection of expression vectors into egg cytoplasm (Flytzanis et al., 1985; McMahon et al., 1985; Hough-Evans et al., 1988; Livant et al., 1991). This is an easy and rapid procedure, such that >10,000 eggs can be injected per day by a skilled investigator. Of these 60-90% will develop normally, and stably incorporate and express the injected transgene. This method has recently been exploited in a number of remarkably detailed and complete functional *cis*-regulatory studies on developmentally regulated genes (e.g., Gan and Klein, 1993; Mao et al., 1994; Makabe et al., 1995; Wei et al., 1995; Kirchhamer and Davidson, 1996; Kozlowski et al., 1996; Yuh and Davidson, 1996; Coffman et al., 1996, 1997; see Yuh et al., 1996; reviews of Kirchhamer et al., 1996a; Arnone and Davidson, 1997). The injected DNA is concatenated in the egg cytoplasm within minutes of injection (McMahon et al., 1985; Livant et al., 1991) and the concatenates are stably incorporated, usually in a single nucleus at 2nd, 3rd or 4th cleavage (Flytzanis et al., 1985; McMahon et al., 1985; Hough-Evans et al., 1988; Franks et al.,

1990; Livant et al., 1991). The mosaic pattern of incorporation that results is entirely random with respect to spatial territory and cell lineage in the embryo. Mosaic DNA incorporation has been observed after injection into egg cytoplasm in many other experimental animals as well, including the ascidians *Halocynthia* and *Ciona* (Hikosaka et al., 1992; Corbo et al., 1997), *Xenopus* (Etkin and Pearman, 1987), zebrafish (Amsterdam et al., 1995) and the gastropod mollusc *Patella* (Damen and Van Loon, 1996). In sea urchins, the extent of mosaicism can be reduced by multiple injections (Livant et al., 1991), but this is an inconvenient procedure in applications where large numbers of experimental embryos are required.

Clonal or mosaic incorporation of transgenes can be exploited to experimental advantage, as shown brilliantly by the application of *flp* transgenesis in *Drosophila* (e.g., Struhl and Basler, 1993; Basler and Struhl, 1994). The requirement is that the investigator be able to recognize the cells constituting the clone of interest. Here we demonstrate that co-injection of a transgene of experimental interest, together with a marker expression construct in which GFP is driven by a broadly active *cis*-regulatory element, serves this purpose, since the exogenous DNAs are incorporated together into the same clones of cells. We also establish the sensitivity with which expression of GFP reporters can be detected and, in addition, demonstrate that GFP can be used to study transgene expression in postembryonic larval development. This brings the processes of larval development and adult body plan formation within the range of experimental gene regulation molecular biology, for the first time in any organism that displays the maximal form of indirect development (Davidson et al., 1995; Peterson et al., 1997).

MATERIALS AND METHODS

Embryo culture, microinjection and whole-mount in situ hybridization

Strongylocentrotus purpuratus gametes were collected and embryos injected as previously described (McMahon et al., 1985) using linearized plasmid DNA. A total of approximately 1500 molecules of plasmid DNA were injected together with carrier DNA (a five-fold mass excess of *Hin*dIII-digested *S. purpuratus* DNA) per egg in a 2 pl volume of 20% glycerol and 0.12 M KCl (Franks et al., 1990). Embryos were cultured at 15°C in 20 units/ml penicillin, 50 μg/ml streptomycin in Millipore-filtered sea water (MFSW). The embryos were collected at the appropriate stage and processed for whole-mount in situ hybridization, following the procedures described by Ransick et al. (1993) and modified by Kirchhamer and Davidson (1996). Alternatively, they were directly mounted in MFSW under a coverslip for observation by fluorescence microscopy. Except where otherwise indicated, blastulae were collected at 24 hours, gastrulae at 48 hours and plutei at 72 hours postfertilization. Larvae were cultured in 24-well tissue culture plates at three to five plutei per well and were fed with *Rhodomonas lens*, essentially as described previously (Cameron and Hinegardner, 1978; Leahy, 1987).

Construction of reporter gene plasmids

The four GFP fusion constructs used in this study contain the Green Lantern™ (Gibco BRL) cDNA as reporter sequence herein designated (in the names of the expression constructs) 'GL'. Schematic diagrams of these constructs are shown in Fig. 1. The *SM50-GL* plasmid was constructed by PCR of the *SM50 cis*-regulatory system using oligos SMGL5 (5′-GGGCTGCAGGCCGTGGAAAATAACTAGGCA) and SMGL3 (5′-GGGGGTCGACTCCAGGGAACGCGCATGTTGAA) which amplify the entire *SM50* regulatory region (Sucov et al., 1988; Makabe et al., 1995), i.e., from −430 to +198. This 448 bp fragment was cloned in frame into the GreenLantern2 plasmid (Gibco BRL) at the *Pst*I and *Sal*I sites. For injection experiments, the resulting plasmid was linearized with *Pst*I.

To construct *Endo16-GL*, the regulatory domain of the *Endo16* gene, extending from −2235 to +26 relative to the transcription initiation site, was cloned into the *Sma*I site of p-Green Lantern-1 (Gibco BRL). This is the sequence included in the expression construct GFEDCBABp-CAT (*Endo16-CAT*) described by Yuh and Davidson (1996). First the *Eco*RV-*Sma*I fragment (from −2235 to −123) of *Endo16-CAT* was inserted into the plasmid. Subsequently, the fragment from −123 to +26 was amplified by PCR, using oligos ENDO25/26 (Yuh et al., 1994) and EN16ATGRC (5′-GGACC-CGGGCGTCTCAAAAATTAGCAATA), and inserted at the same *Sma*I site. For injection, *Endo16-GL* and *Endo16-CAT* were linearized at the *Pst*I and *SacI* sites, respectively (see Fig. 1).

The *CyIIa-GL* construct contains a 1.7 kb *CyIIa* fragment, extending from −1032 to +682, including 37 bp downstream of the ATG start codon, which is able to drive spatially and temporally accurate transcription of other *CyIIa* expression constructs (M. I. and E. H. D., unpublished data). To create this plasmid, an *Apa*I-*Bam*HI *CyIIa* fragment was cloned into the *Sma*I and *Bgl*II sites of the pCAT-3 Basic Vector (Promega) plasmid, in which the CAT cDNA sequence was swapped for the Green Lantern-1 (Gibco BRL) cDNA (GL). This placed the *CyIIa* ATG start codon in frame with the GL start codon. For injection *CyIIa-GL* was linearized at the *Kpn*I site (Fig. 1).

The *H2b-GL* plasmid was constructed by cloning into the *Kpn*I site of pGreen Lantern-1 (Gibco BRL), a *Kpn*I fragment containing a downstream regulatory region, which extends from +936 to +967 of the sequence. This fragment had been linked by a pBR322 0.5 kb *Hin*fI spacer to the upstream regulatory region extending from −491 to +28 of the *S. purpuratus* L1 H2b gene (Zhao et al., 1990). The combined regulatory fragment was obtained by PCR of the act(+936+967) reverse mutant of the L1 H2b gene (Zhao et al., 1990), which was kindly provided by Professor Rob Maxson (USC Medical School), using oligos H2bL15 (5′-CACGGTACCTCTCAAAATAT-GATTGGCA) and H2bL13 (5′-CACGGTACCGATGATTGT-GATTCTCACGA). The resulting plasmid was linearized for injection with *Pst*I.

Microscopy

GFP-expressing cells were visualized on an Olympus epifluorescence microscope using ×10, ×20 and ×40 objectives. For fast screening of live specimens, embryos were allowed to grow in agarose tunnels submerged in sea water in a 50 mm Petri dish and directly observed with a long working distance ×40 objective. A fluorescein filter set was used. Data were recorded onto a video optical disc recorder (OMDR, Panasonic 3038) using a light-intensifying camera (Hamamatsu SIT) and image processor (Imaging Technology 151), operating with the VidIm software package (by permission of G. R. Belford, J. Stollberg, S. E. Fraser). Depending on which confocal microscope was used, the embryos were either placed on glass slides and covered with glass coverslips or placed at the bottom of a 35 mm Petri dish containing a large volume of sea water. The embryos on glass slides were imaged with a Bio-Rad MRC-600 laser scanning confocal system attached to a Zeiss Axiovert 35 microscope using an LD Achroplan ×40 objective, and the CoMOS version 6.01 software. Those embryos in Petri dishes were imaged with a Bio-Rad MRC-500 confocal system attached to an upright Zeiss Universal microscope, using a Zeiss ×40 water immersion objective and the same version of the CoMOS software. For fluorescent imaging, the BHS cube was used with both systems to provide excitation at the wavelength for FITC, and the MRC-600 used the 488DF10 line of the krypton-argon laser (Ion Laser Technology). Confocal images of a through-focus set of optical sections (z-series) were stored on optical discs. Z-series

were collected for a given embryo under bright-field as well as fluorescent illumination. This allowed us to overlay the corresponding bright-field and fluorescent sections of the collected z-series to determine the exact cells that were expressing the fluorescent protein. Z-series of fluorescent images were projected from within CoMOS using the maximum projection method so that all the fluorescent cells could be seen in one frame. All images collected using the VidIm software package and the confocal microscope were processed using Adobe Photoshop (versions 2.5 and 3.0).

RESULTS

Since there had been no prior systematic application of the GFP reporter in sea urchin embryos, the initial objective was to determine whether GFP would be expressed appropriately in all territories of the embryo and to establish the sensitivity with which this reporter could be detected, compared to our standard CAT whole-mount in situ hybridization (WMISH). In the following sections, we describe the expression of the GFP reporter driven by three different, previously characterized, *cis*-regulatory systems. The activity of these *cis*-regulatory elements, considered through embryonic time, includes most of the territories and lineage elements of the embryo.

Expression of *SM50-GL* in the cellular and syncytial skeletogenic mesenchyme

The *SM50* gene encodes a spicule matrix protein that is expressed exclusively in the skeletogenic mesenchyme lineages (Benson et al., 1987; Killian and Wilt, 1989; Sucov et al., 1988; Katoh-Fukui et al., 1992). The *cis*-regulatory region of this gene extends from –440 bp to about +120 with respect to the transcription start site and this DNA element confers accurate skeletogenic expression when associated with the CAT reporter (Sucov et al., 1988; Makabe et al., 1995). The entire sequence was included in the *SM50-GL* construct shown in Fig. 1. Zygotes were injected with 1500 copies of the linearized plasmid and embryos were collected at different stages, and observed by fluorescence microscopy. Examples of video image recordings of *SM50-GL* expression patterns are shown in Fig. 2.

At the mesenchyme blastula stage, the skeletogenic precursors ingress into the blastocoel. GFP can be detected in an average of 3.8 of the 32 skeletogenic cells per embryo at this stage, as measured in a sample of more than one hundred embryos expressing the *SM50-GL* construct. This value is about the same as the 3-5 skeletogenic mesenchyme cells per embryo that expressed *SM50-CAT* in the previous studies of Sucov et al. (1985) and Makabe et al. (1995). The implication is that, in these lineages, stable incorporation of the transgene has occurred on the average in one mesenchymal precursor per embryo no later than the 6th cleavage. There are then a total of eight cells in the skeletogenic clones, i.e., the eight daughters of the four large micromeres which found the skeletogenic lineages at 5th cleavage. Fig. 2A,B shows an example of a mesenchyme blastula-stage embryo containing several cells expressing *SM50-GL*, of which five can be discerned in the plane of focus shown.

From the onset of the gastrula stage, the pattern of expression of *SM50-GL* changes very dramatically, as illustrated in Fig. 2C-F. The number of expressing cells is now consistently 32 per embryo, equal to the total population of skeletogenic mesenchyme cells in this species at both gastrula and pluteus stages (Harkey et al., 1992). During gastrulation the skeletogenic cells establish filopodial contacts with one another and then fuse to form syncytial cables, within which the skeletal matrix is deposited (Gustafson and Wolpert, 1963; Decker and Lennarz, 1988; Brown et al., 1995). GFP produced by the minor fraction of skeletogenic cells expressing the construct, i.e., about one-eighth, has diffused through these cables to illuminate all the mesenchyme cells, thus generating striking images such as shown in Fig. 2C-F. This behavior is not seen with CAT mRNA, which by WMISH remains confined to the individual cells expressing *SM50-CAT* (Sucov et al., 1988; Makabe et al., 1995; Kirchhamer et al., 1996a,b); and though some diffusion in the skeletogenic syncytium is seen with CAT protein (Zeller et al., 1992), it is not complete, so that there are always a number of cells lacking the CAT marker, in contrast to the results obtained with GFP. GFP is thus an unexpectedly useful reporter for studies of the syncytial skeletogenic mesenchyme in sea urchin embryos. Its diffusibility amplifies the mosaic spatial signal generated by the transgene and so reveals every skeletogenic cell in the embryo. Furthermore, as indicated in the first line of Table 1, no significant ectopic fluorescence is produced by *SM50-GL*; the

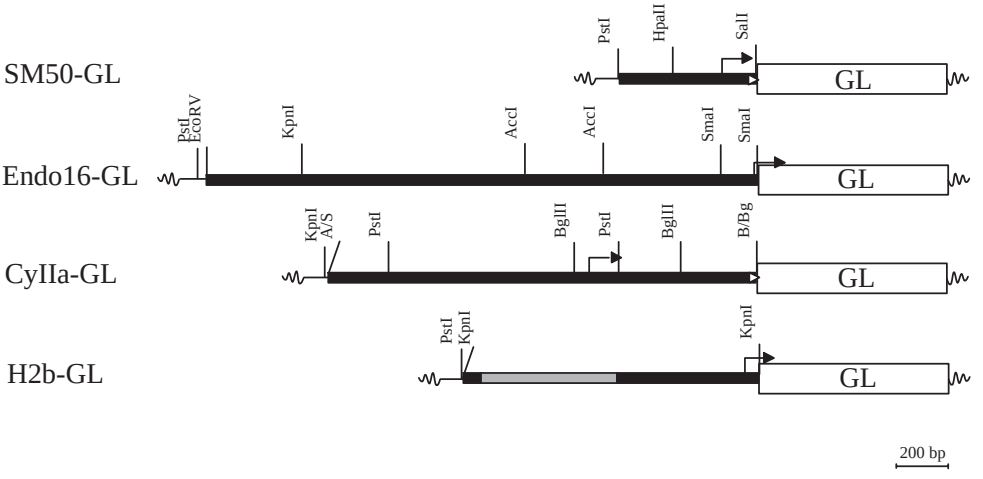

Fig. 1. Restriction maps of the plasmids used in this study. Filled boxes represent the upstream regulatory DNA sequence and the open box represents the Green Lantern reporter sequence (GL). The hatched box stands for a pBR322 *Hin*fI fragment. Bent arrows denote the transcription start site and open arrowheads in frame ATG start codons originally from sea urchin sequences. ATG start codons from the GFP ORF are not shown, which were present in every context. Plasmid vector sequences are depicted as wavy segments. B, Bg; *Bam*HI, *Bgl*II fusion site; A/S, *Apa*I/*Sma*I fusion site.

Table 1. Quantitative analysis of GFP expression observed at the late gastrula stage* for *SM50-GL*, *Endo16-GL* and *CyIIa-GL* constructs

Construct	Scored embryos†	Positive:negative‡ (% expression)§	% Gut expression¶	% PMC expression¶	% SMC expression¶	% Ectoderm expression¶
SM50-GL	122	62:60 (50.8)	1.6	98.4	1.6	4.8
Endo16-GL	115	72:43 (62.6)	100	4.2	2.8	5.5
CyIIa-GL	134	89:45 (66.4)	2.2	67.4	73.0	5.6

*Gastrulae were collected at 48 to 54 hours postfertilization.
†Values represent data for at least three separate experiments carried out on independent batches of eggs.
‡Embryos with ≥2 fluorescent cells were scored as positive.
§Expression = [∑ positive embryos/∑ scored embryos] × 100.
¶Territorial expression = [∑ embryos positive in a given territory/∑ positive embryos] × 100.
Abbreviations: PMC, primary mesenchyme cell; SMC, secondary mesenchyme cell.

small fractions of embryos displaying ectopic expression are the same as observed by WMISH for *SM50-CAT* (Makabe et al., 1995; Kirchhamer et al., 1996b).

Expression of *Endo16-GL* in vegetal plate and archenteron: GFP turnover

Endo16 is activated at the early blastula stage in the vegetal plate and it is expressed throughout the archenteron during gastrulation (Nocente-McGrath, 1989; Ransick et al., 1993). At the prism stage, *Endo16* transcripts disappear from the foregut, and towards the end of embryonic development from the hindgut, remaining confined to the stomach of the larva. The *Endo16-GL* construct used in this study includes a 2300 bp upstream sequence that suffices to faithfully recreate this pattern of expression when fused to a CAT reporter gene (Yuh et al., 1994; Yuh and Davidson, 1996). Fig. 3 shows examples of embryos expressing *Endo16-GL* at different stages. Large patches of fluorescent cells are observed within the vegetal plate of the two blastulae in Fig. 3A,B. Fluorescence is observed in clones of cells along the archenteron of the two gastrulae shown in Fig. 3C,D, and in the midgut of the early pluteus shown in Fig. 3E,F. Table 1 indicates only a few percent of ectopic expression in mesenchyme or ectoderm cells of gastrulae expressing *Endo16-GL*, again the same results as seen earlier for *Endo16-CAT* (Yuh and Davidson, 1996). Endogenous *Endo16* expression is extinguished in the foregut at about 40 hours and is never observed in any of the endomesodermal derivatives of the vegetal plate, i.e., secondary mesenchyme or coelomic pouches. The same pattern is followed by CAT mRNA distribution in embryos bearing *Endo16-CAT* (Yuh et al., 1994; Yuh and Davidson, 1996), due to the relatively short half life of CAT mRNA (Flytzanis et al., 1987). This phenomenon provides the opportunity to investigate the

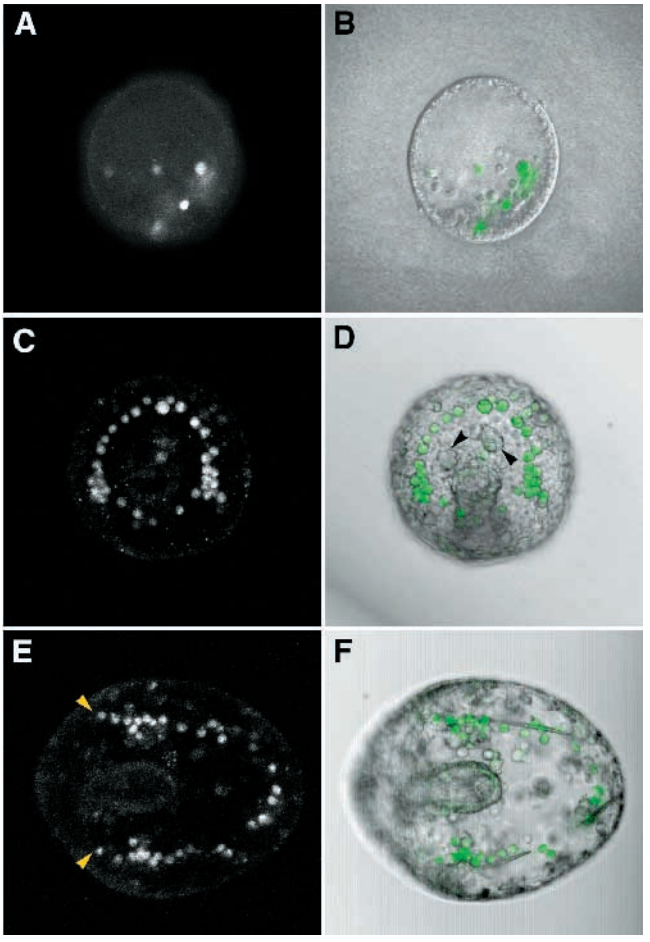

Fig. 2. Expression of *SM50-GL* in skeletogenic mesenchyme, observed at three different embryonic stages. (A,B) The images were obtained by epifluorescence microscopy. (C-F) Confocal images displayed as projections of a through-focus set of optical sections (z-series) obtained under fluorescent illumination (C,E), or as overlays of the corresponding bright-field (gray) and fluorescent (green) exposures of single optical sections (D,F). (A,B) Mesenchyme blastula showing expression in five primary mesenchyme cells; the same embryo is imaged in the left and right panels, in which the fluorescence image and the composite video images of bright-field and fluorescence exposure (in green) are respectively shown. (C,D) Mid-gastrula stage. The embryo is viewed along the vegetal-animal axis from the animal pole, with the future oral ectoderm facing down. GFP fluorescence is observed in all of the 32 skeletogenic cells, which at this stage are arranged as a ring around the gut. The cells concentrate in clusters on either side of the embryo, where spiculogenesis will begin. (D) A single optical section and includes almost all of these cells in the same focal plane. Also in focus in the same section are two secondary mesenchyme cells (arrowheads in D) which do not express GFP. (E,F) Early pluteus oriented with the oral face to the left and viewed from the anal surface. The composite video image in F shows the two anal spicules and the skeletogenic cells (green) located along them. The confocal fluorescence images projected in E show two additional expressing cells (arrowheads) belonging to the growing oral rods, which are not visible in the single optical section reproduced in F.

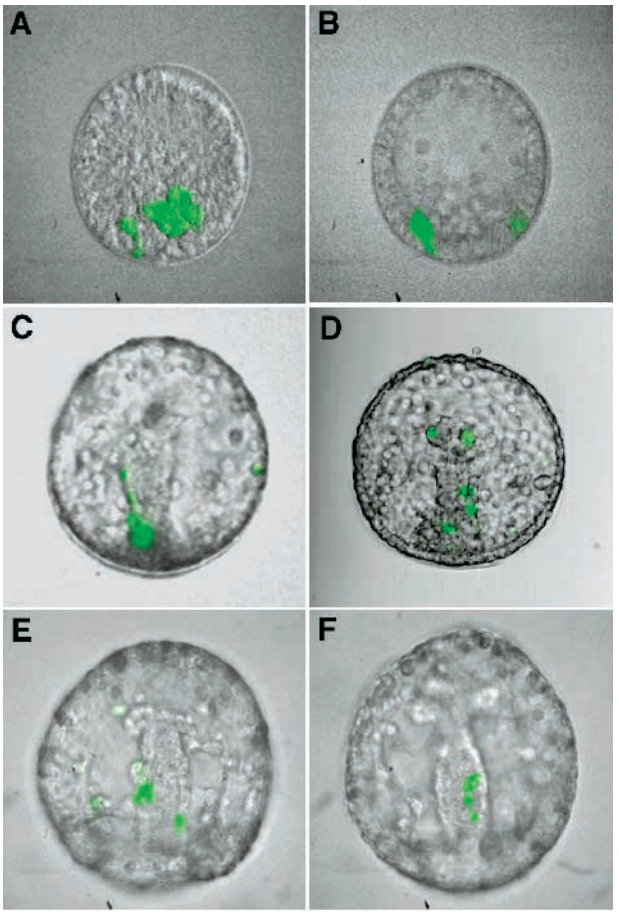

Fig. 3. Expression of *Endo16-GL*. Composite false color video images of bright-field (gray) and fluorescent (green) exposures are shown superimposed. The image in D was obtained on a confocal microscope; the others were generated using conventional fluorescence microscopy. Six different embryos, at three different stages are shown. (A,B) Mesenchyme blastulae showing *Endo16-GL* expression restricted to the vegetal plate. The embryo in A is oriented with the vegetal pole at the bottom and has been rotated with respect to the animal-vegetal axis in order to visualize the absence of expression in the skeletogenic precursors located at the center of the vegetal pole. The optical section of the embryo illustrated in B shows that expression is also excluded from the ingressed primary mesenchyme cells. (C,D) Two different mid-gastrular embryos showing expression in clones represented along the entire archenteron length. (E,F) Early pluteus stage embryos in which the tripartite gut and the coelomic pouches are visible. The embryo in E shows two clones of fluorescent cells in the midgut and in the hindgut. The embryo shown in F has a single large clone of expressing cells restricted to the midgut.

relative stability of the GFP reporter. Eggs were co-injected with *Endo16-CAT* and *Endo16-GFP* and, at late gastrula or early pluteus stages, the patterns of fluorescence were individually recorded. The same embryos were then fixed and individually studied by WMISH to determine their patterns of CAT mRNA distribution. A quantitative comparison of the disappearance of CAT mRNA and of GFP from foregut and coelomic pouches of these embryos is shown in Table 2. At the late gastrula stage (48 hours), the two constructs express almost the same way in every respect, except that there is about 30% greater retention of foregut expression when visualized as GFP fluorescence than is observed for CAT mRNA. At the pluteus stage, however, results with the two reporters are more divergent. No embryos display CAT mRNA in the coelomic pouches, while 9% of *Endo16-GL* embryos do, and only 23% as many embryos display any CAT mRNA in the foregut area at 60 hours as do at 48 hours, while the equivalent figure for GFP is 37%. We conclude that GFP is a little less than two-fold more stable than is CAT mRNA, at least in the archenteron, assuming that 'disappearance' of the respective reporter molecules indicates decline in concentration of these molecules below the threshold of detection. Given a certain decay rate for each reporter and an intensity of expression per cell that varies from embryo to embryo (as always appears to be the case), the decrease in the number of embryos above the expression threshold with time would measure these decay rates. On this basis, the data of Table 2 would indicate that, in the foregut, in the period sampled by the 48 and 60 hour mea-

Table 2. Restriction of *Endo16-GL* expression at late embryonic stages as compared to *Endo16-CAT* expression*

Construct	Stage	Positive:negative‡ (% expression)§	% Endoderm expression¶	% Foregut expression¶	% Coelomic pouch expression¶	% Ectopic expression‡‡	Fold restriction¶¶
Endo16-GL	gastrula	30:19 (61.2)	100	53.3**	–††	6.7	–
	prism	55:53 (50.9)	100	20.0	9.1	3.6	1.8
Endo16-CAT	gastrula	24:16 (60.0)	100	41.6**	–††	4.1	–
	prism	42:37 (53.2)	100	9.5	0	4.8	4.4

*Zygotes were co-injected with equal amounts of linearized *Endo16-GL* and *Endo16-CAT* plasmids, which contain identical *Endo16* regulatory sequences. Embryos were collected at different stages, scored by microscopic examination for GFP fluorescence, and then analyzed by WMISH to detect CAT RNA.

†Gastrulae and prism-stage embryos were collected at 48 and 60 hours postfertilization, respectively.

‡Embryos with ≥2 fluorescent cells (in the case of GFP detection) or with more than two stained cells (in the case of CAT RNA detection) were scored as positive.

§% expression = [Σ positive embryos/Σ scored embryos] × 100.

¶Territorial expression = [Σ embryos positive in a given territory/Σ positive embryos] × 100.

**Since at the gastrula stage the tripartite gut structure is not yet formed, the percentage reported for this stage refers to the number of embryos positive in the upper third of the archenteron.

††Coelomic pouches are not yet formed at the gastrula stage.

‡‡Ectopic expression = [Σ embryos positive in ectoderm + mesenchyme cells/Σ positive embryos] × 100.

¶¶Restriction, from gastrula to prism, was calculated as fold reduction of expression in the upper third of the archenteron. Restriction = % foregut expression at gastrula stage/[% foregut expression + % coelomic pouch expression] at prism stage.

surements, the half life is about 5.5 hours for CAT mRNA and 8.3 hours for GFP. The CAT mRNA decay rate may be higher in the coelomic pouch region than in the foregut, and it is ~8-fold higher in 24 hour blastula cells, as determined earlier (Flytzanis et al., 1987).

Transient expression of *CyIIa-GL*: slow turnover increases sensitivity of transgene assay

The *CyIIa* cytoskeletal actin gene displays perhaps the most complex pattern of expression of any of the five *S. purpuratus* cytoskeletal actin genes. This pattern is characterized by transient periods of expression in certain mesenchymal and endodermal tissues. Cox et al. (1986) noticed that at least some of the cells that express *CyIIa* are motile at the stage of expression, but not all motile cells seem to express the gene at any one moment. Briefly, the pattern of expression through developmental time is as follows (Cox et al., 1986; Miller et al., 1996; M. I. A. and E. H. D., unpublished results): at the blastula stage (24 hours), *CyIIa* is expressed in vegetal plate and in some but not all ingressed skeletogenic mesenchyme; during early gastrulation (30 hours), the gene is active only in some cells at the tip of the archenteron; at mid-gastrulation (36 hours), skeletogenic cells express *CyIIa* while archenteron cells do not; at late gastrula (48 hours) delaminating secondary mesenchyme as well as skeletogenic cells express *CyIIa*; in prism-stage embryos (60 hours), expression extends to coelomic pouches and the circumblastopore region; at 72 hours, expression is extinguished in mesenchymal cells but is active in midgut and hindgut. The transience of these expression domains, combined with the instability of CAT mRNA, result in rather weak WMISH signals in embryos bearing a *CyIIa-CAT* fusion gene controlled by a 1.7 kb DNA fragment that contains the complete embryonic *CyIIa cis*-regulatory domain (M. I. A. and E. H. D., unpublished results). Though this signal suffices to define the necessary *cis*-regulatory elements, we have found it inadequate for many partially active constructs that had been mutationally altered. However, when the same regulatory sequence was associated with the GFP reporter (*CyIIa-GL*; see Fig. 1), it generated a very strong signal. This is shown dramatically in Fig. 4. The images reproduced in Fig. 4A-D represent normal patterns of transgene expression for their respective stages: in Fig. 4A,B the blastula stage embryo expresses in both vegetal plate and ingressing skeletogenic mesenchyme and, in Fig. 4C,D, the late gastrula stage embryo expresses *CyIIa-GL* in both skeletogenic mesenchyme, identifiable by the ring-like syncytial arrangement and in some secondary mesenchyme cells arising from the tip of the archenteron. If compared, as noted above, to *Endo16-CAT*, *CyIIa-CAT* expression at these stages is relatively low while, in contrast, *CyIIa-GL* expresses more intensely than does *Endo16-GL* (cf., Table 1). The relative difference in intensity of the two markers is undoubtedly due to the lower turnover rate of GFP vis-a-vis CAT mRNA, resulting in higher steady state content and this difference is accentuated by the brief duration of each phase of *CyIIa* expression. In addition, the spread of the fluorescent protein through the skeletogenic syncytium increases the ease of visualization of this lineage remarkably. In Fig. 4E, F, which displays a pluteus stage embryo, the pattern of GFP expression can be seen to be the sum of current and earlier phases of expression. Thus pluteus stage embryos do not express *CyIIa* in skeletogenic cells, but only in midgut and hindgut, while the embryo shown here clearly displays not only a labeled skeletal syncytium but also labeled gut. A somewhat similar result was obtained by Zeller et al. (1992) by visualizing CAT protein immunologically in embryos bearing another *CyIIa-CAT* fusion construct. The half life of both of these reporter proteins is evidently >10 hours in the late skeletogenic mesenchyme, though it is to be stressed that GFP can be detected with much greater sensitivity than is afforded by immunofluorescent staining of CAT protein (Zeller et al., 1992).

Imaging of GFP expression constructs in the feeding larva

Though of very great biological interest, the feeding larva has so far not been accessible to transgenic analysis of gene expression. Conventional whole-mount procedures are difficult to apply for a number of reasons. By 5 weeks, the larva has about 1.5×10^5 cells, ~100 times more cells than does the complete embryo per se (Cameron et al., 1989). The external larval structures are delicate, as they remain only a single cell layer thick, while the highly cellularized rudiment has a multilayered and convoluted organization that can be particularly difficult to interpret when sectioned at unspecified angles. Cameron et al. (1989) had shown that feeding larvae continue to express the *CyIIa* gene throughout their development, and given their nearly transparent structure (except for the algal contents of the stomach), the use of a fluorescent reporter provided the opportunity to apply image processing to microscopic analysis of larval gene expression. The possibilities that arise from this approach are evident at once in Fig. 5. Larvae descendant from eggs injected with *CyIIa-GL*, of 1 and 2 weeks of age, are shown in Fig. 5A and B, respectively. Large clones of gut cells expressing GFP can be seen in both larvae, in Fig. 5A in both stomach and intestine; and in Fig. 5B along the right side of the stomach epithelium. From the size and distribution of these clones in the growing larval digestive tract, it is clear that they include the products of larval cell division. Furthermore, the images shown were taken on living larvae, which were apparently not harmed by the experience, since they continued to develop normally thereafter. Expression was observed in the same relative patterns at 4 weeks of age (not shown); i.e., in larvae almost ready for metamorphosis.

The gut is the terminal locus of *CyIIa* expression during embryogenesis per se, and these observations show that this expression continues throughout larval life. However, in adult *S. purpuratus*, *CyIIa* is also expressed, though at relatively low levels, in coelomocytes, tubefeet and lantern muscle (all tissues of mesodermal origin), as well as continuing to be expressed in the intestine and perhaps elsewhere in the digestive tract. We did not observe GFP fluorescence in 2- to 4-week-old larvae in the rudiment per se, where at least the forming tubefeet and oral assemblage including lantern muscle might have been expected to display expression. One possible explanation is that the *CyIIa cis*-regulatory element included in the *CyIIa-GL* construct lacks the regulatory module(s) that control(s) expression in the adult mesodermal domains, though it suffices to reproduce the complete embryonic pattern of expression as well as embryonic, larval

Table 3. Quantitation of gene expression in embryos bearing both *H2B-GL* and *Endo16-CAT* constructs*

Localization of *H2b-GL* expression†			*Endo16-CAT* expression††
			CAT molecules/embryo $\times 10^{-5}$¶¶
GFP expression pattern	Number of embryos	% of total	0 1 2 3 4
Gut‡	105	30	
Not in gut§	123	34	
No expression	128	36	
Embryos not screened for GFP expression**	200	–	

*Zygotes were co-injected with *H2b-GL* and *Endo16-CAT* constructs and grown for 48 hours (late gastrula stage).

†Embryos were sorted into three different pools according to the localization of GFP fluorescence.

‡This pool includes all embryos which show GFP expression in ≥2 or more gut cells, regardless of other patterns of expression.

§This pool includes all embryos which show GFP expression in ectodermal and/or mesenchyme cells, but not in the gut.

¶This pool includes all embryos in which GFP expression was not detectable.

**Embryos from the same co-injection experiment which were not screened for GFP expression. The CAT activity per embryo was measured in two pools of 100 embryos each. This value is to be considered the average value, taken over *Endo16-CAT* expressing and non-expressing embryos. The percent of embryos expressing *CAT* (calculated as [$\sum$ positive embryos/$\sum$ scored embryos] × 100) was determined by WMISH in a sample of ~100 embryos collected from this pool to be about 50%.

††CAT emzyme activity was measured in lysates of 100 embryos collected from each screened pool or from embryos that were not screened, essentially as reported by McMahon et al. (1984).

¶¶The quantity of CAT enzyme molecules per embryo was determined by comparison of the activity of the samples to standards of known CAT concentration.

and adult gut expression (these observations; and M. I. A. and E. H. D., unpublished data).

GFP expression as a mosaic co-transformation marker

Many new applications would be potentiated could the clone of embryonic cells in which a transgene is incorporated be identified a priori. In the following experiments, eggs were injected with a mixture consisting of a territory-specific transgene, *Endo16-CAT*, carrier DNA and the *H2b-GL* construct. *H2b-GL* contains *cis*-regulatory elements from the *L1 H2b* gene (Zhao et al., 1990; see Fig. 1), and at certain stages expresses GFP in all regions of the embryo. GFP expression patterns were recorded for individual living embryos and CAT mRNA expression in these same embryos was then determined by WMISH or by CAT enzyme assay. The specific object was to assess the reliability with which the GFP expression pattern could be used as an index of the locus of incorporation of the *Endo16-CAT* transgene, without disturbing the tissue-specific accuracy of expression of the latter. The rationale is provided by earlier studies which demonstrated, as briefly reviewed in Introduction, that exogenous linear DNA molecules are rapidly ligated together into one or a few concatenates when introduced into sea urchin egg cytoplasm (Flytzanis et al., 1985; McMahon et al., 1985; Livant et al., 1991). Thus the *H2b-GL* marker construct and the territory-specific *Endo16-CAT* construct might be expected to be incorporated together in the same blastomere nuclei, and the pattern of GFP expression should thus indicate the exact locus in the embryo of the transgenic clone(s) of cells at subsequent stages. A caveat to these experiments is that the *L1 H2b* regulatory system begins to be expressed only at mid-blastula stage (Zhao et al., 1990), and thus *H2b-GL* could only be used for detection of clonal incorporation patterns after this point in development. Furthermore, the *L1 H2b* gene is not actively expressed in the gut later in development, since these cells cease to divide by the time of archenteron formation (Ruffins and Ettensohn, 1996).

Two experiments were carried out, one qualitative, which is illustrated in Fig. 6, and one quantitative, results of which are given in Table 3. In the experiment of Fig. 6, late gastrula-stage embryos (48 hours) were selected that displayed GFP fluorescence in endodermal clones, and these embryos were then put through the WMISH procedure to detect the locus of *Endo16-CAT* expression. Five illustrative embryos are shown in Fig. 6A-H. Here it can be seen that within the domain of normal *Endo16* expression at this stage, i.e., hindgut and midgut, the exact same cells that express GFP (Fig. 6A,C,E,G,I) also express CAT mRNA (Fig. 6B,D,F,H,J). This experiment demonstrates, within the resolution of the images, a perfect coincidence of incorporation of the two co-injected constructs. In addition, exactly as expected, GFP is seen in regions where CAT is not, e.g., in mesenchymal cells (Fig. 6A,B and G,H) or foregut (Fig. 6C,D and E,F). These territories should contain the exogenous DNA in some cases if this is also present in endoderm cells, since they descend from common early lineage precursors (Cameron et al., 1987, 1991), but neither mesenchyme nor foregut cells are expected to express *Endo16-CAT* (Yuh and Davidson, 1996). Similarly, an embryo selected for ectodermal GFP expression, shown in Fig. 6K, does not express *Endo16-CAT* at all, as shown in Fig. 6L.

In the experiment shown in Table 3, gastrulae grown from eggs co-injected with *H2b-GL* and *Endo16-CAT* were sorted into pools according to the localization of GFP fluorescence and CAT enzyme activity was then determined in samples of 100 embryos from these pools. To provide a standard of comparison, *Endo16-CAT* expression was also measured in a blind sample of 100 embryos that had not been assessed for GFP expression. About 50% of the individual embryos in the blind sample expressed *Endo16-CAT*, as determined by WMISH (Table 3), slightly lower than observed in earlier studies on embryos receiving somewhat larger amounts of *Endo16-CAT* by itself (i.e., 60-80%; Yuh and Davidson, 1996). Table 3 shows that the level of CAT activity in this

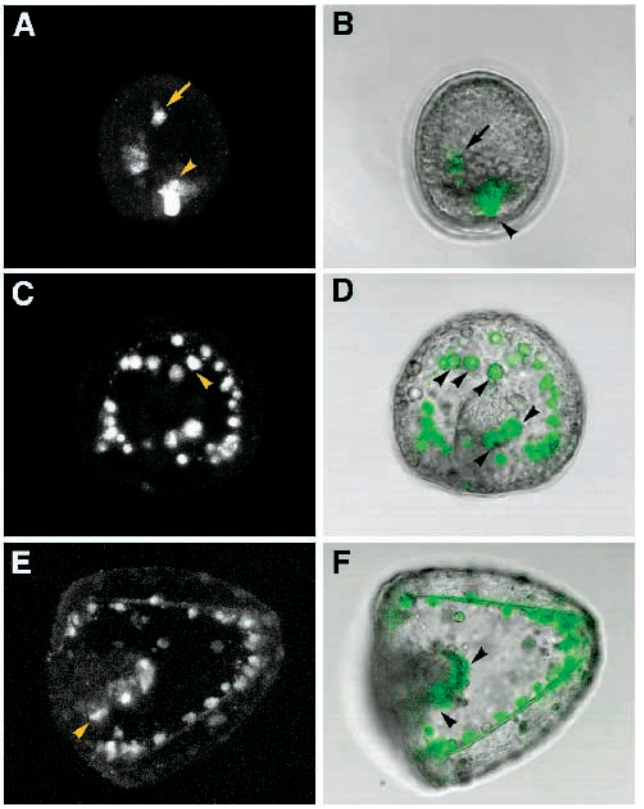

Fig. 4. Expression of *CyIIa-GL*. Fluorescent confocal images of the three embryos expressing GFP shown in the left panels (A,C,E) were each obtained by projecting 10 to 20 optical sections (z-series) in a single frame. The images in the right panels (B,D,F) are each composite false color video images consisting of the bright-field image of a single optical section (gray) overlain on the fluorescent exposure (green) of the same section. (A,B) A mesenchyme blastula expressing *CyIIa-GL*, with the vegetal pole facing down. Arrows in A and B point to mesenchyme cells, not all of which are visible in the single optical section shown in B. The projection of fluorescent z-series shown in A allowed us to visualize in the same frame all the fluorescent cells. In this embryo, strong expression is observed in the vegetal plate (arrowheads A and B) and in the mesenchyme cells ingressing into the blastocoel (arrows). (C,D) A late gastrula showing expression in both skeletogenic and secondary mesenchyme cells. The embryo is viewed from the animal pole, with the oral surface facing down. In D, five secondary mesenchyme cells are indicated by arrowheads over the tip of the archenteron. In C, the same five cells plus another (yellow arrowhead), located more distally from the blastopore, are visualized in the projection of the fluorescent z-series. (E,F) Anal view of an embryo at early pluteus stage oriented so that the oral surface faces left. Arrowheads in F show two patches of endoderm expression in the hindgut and foregut (right to left, respectively). The arrowhead in E points to expression in a coelomic pouch, which is not included in the optical section shown in F. Expression can also be seen in the skeletogenic cells adherent to the anal skeletal rods; all but one of the skeletogenic cells visible in the plane of the optical section contain GFP.

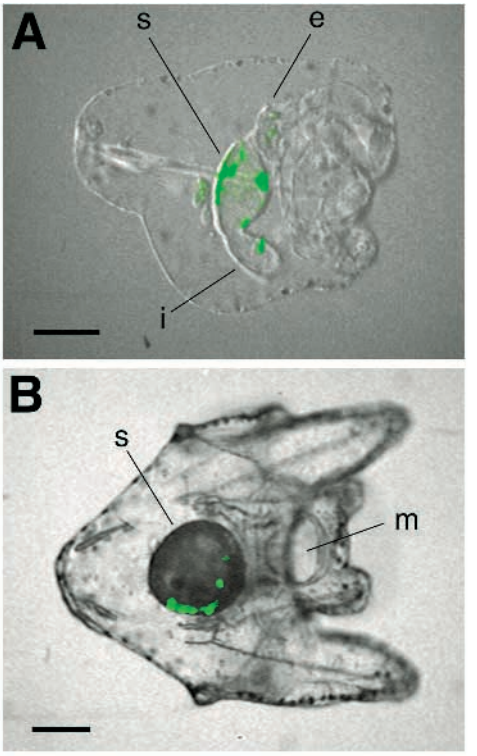

Fig. 5. Visualization of *CyIIa-GL* expression in larvae. (A) Lateral view of 1-week-old pluteus oriented with the oral face to the right and the anus facing down. The larva is slightly flattened to facilitate imaging. GFP expression is observed in two major patches of cells in the stomach and two smaller clusters at the stomach-intestine junction and in the wall of the intestine, respectively. (B) Pluteus at 2 weeks of age viewed from in front, oral surface to the right. Cells expressing *CyIIa-GL* are observed along the right side of the stomach epithelium. Abbreviations: e, esophagus; i, intestine; m, mouth, s, stomach. Scale bars equal 100 μm.

non-sorted pool was almost exactly half of that in the pool that had been selected to contain only embryos expressing GFP in the gut. This result independently confirms the conclusion that *H2b-GL* and *Endo16-CAT* were incorporated in the same cells, so that embryos displaying endodermal GFP fluorescence also display CAT mRNA. Some embryos that display GFP expression in territories outside of the gut, however, also express *Endo16-CAT*. This is shown in the second line of Table 3, where the CAT activity is about a third of that obtained in the pool selected for gut or GFP expression. The explanation is that by 48 hours *H2b-GL* expression in the gut has been lost from some embryos because the *L1 H2b* gene regulatory system is down-regulated in gut cells by the late gastrula stage; thus only 30% of embryos now display GFP in the gut (Table 3) rather than the 50% that display *Endo16-CAT* expression (Table 3), or the ≥60% that actually contain the exogenous DNA in endoderm cells (Tables 1, 2; Yuh and Davidson, 1996).

The experiments in Fig. 6 and Table 3 clearly demonstrate co-integration of the GFP construct with a second construct expressed in a territory-specific manner. This then opens the way to the use of GFP expression patterns in living embryos for the detection and selection of given spatial patterns of clonal incorporation. Although the *L1 H2b cis*-regulatory system that we applied is indeed widely expressed, the method could be even further improved by use of a more ubiquitously expressed GFP construct.

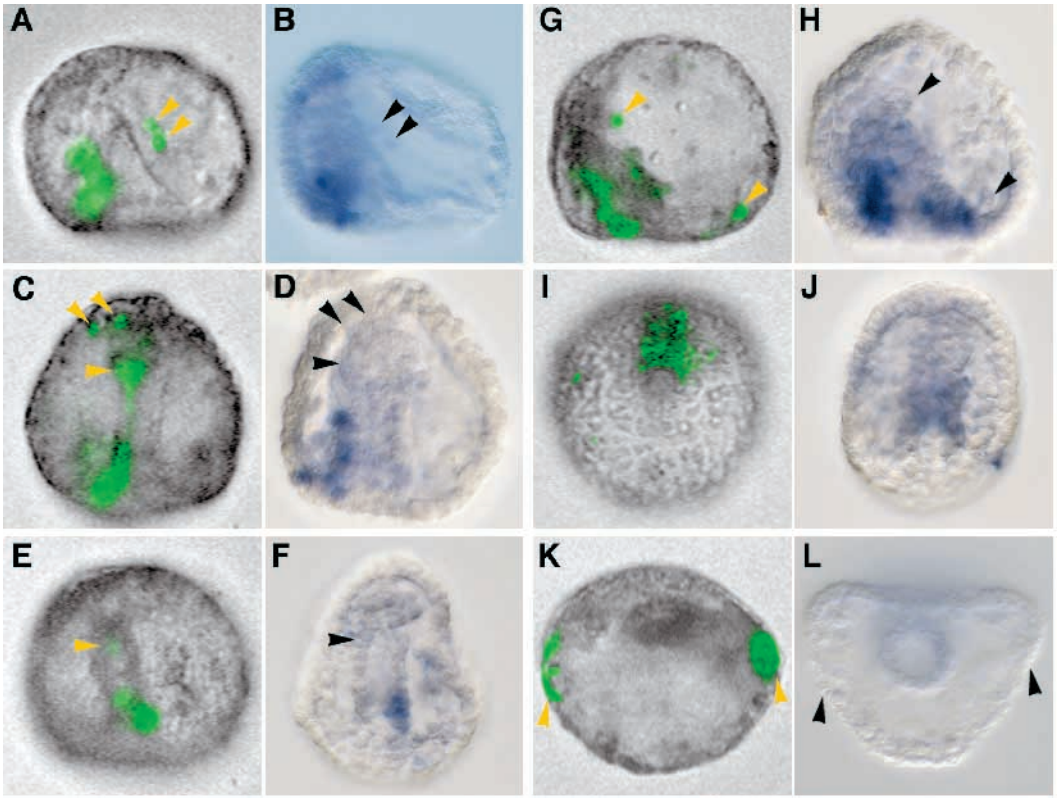

Fig. 6. Expression of co-injected *H2b-GL* and *Endo16-CAT*. Zygotes were co-injected with *H2b-GL* (Fig. 1), in which GFP is expressed under the control of the *L1 H2b cis*-regulatory system, and *Endo16-CAT* (Yuh et al., 1994), in which expression is under the control of the complete *cis*-regulatory system of the gut-specific *Endo16* gene. Embryos were mounted on a glass slide in sea water and were screened for GFP expression at 48 hours (late gastrula). Single embryos showing fluorescence in clones of cells belonging to the endodermal territory were imaged, fixed and individually processed for in situ hybridization in order to localize CAT RNA expression. Two images are shown for each individual gastrula: (A,C,E,G,I,K) composite false color images of bright-field (gray) and fluorescence (green) are shown superimposed; (B,D,F,H,J,L) the spatial distribution of CAT transcripts in each embryo is indicated by the blue staining obtained by WMISH using a digoxigenin-labeled antisense CAT RNA probe. Yellow arrowheads in A,C,E,G and K indicate cells that express GFP but do not express CAT mRNA. Black arrowheads in B,D,F,H and L indicate the same regions in these embryos after WMISH. (A,B) Lateral view of a gastrula showing a clone of cells on the oral side of the gut expressing both green fluorescent protein and CAT RNA. Only GFP expression is observed in two mesenchyme cells on the aboral side of the embryo. (C,D and E,F) Gastrulae shown in frontal view, with the gut in the center. Note in D and E, the absence of CAT mRNA staining in foregut and in secondary mesenchyme cells (arrowheads), territories excluded from the normal pattern of *Endo16* expression at late gastrula. (G,H) An embryo displayed in lateral view, with the oral side to the left. Two large clones of cells in the hindgut region express both GFP and CAT, and some mesenchyme cells express only GFP (arrowheads in H). (I,J and K,L) Optical sections through the animal-vegetal axis with the oral ectoderm at the top and the aboral ectoderm at the bottom. (I,J) A gastrula, slightly rotated with respect to the animal-vegetal axis, in order to visualize clones of cells extending into the archenteron from the blastopore rim that express both GFP and CAT RNA. (K,L) An embryo displaying GFP expression only in ectodermal cells (K) displays no CAT staining (L).

DISCUSSION

GFP as a general marker for transgene expression in sea urchin embryos

These observations provide a firm base for the general use of GFP as a transgene reporter in sea urchin embryos. We show that (1) GFP can be used in every major territory of the embryo, i.e., skeletogenic mesenchyme (Figs 2, 4), secondary mesenchyme (Fig. 4), vegetal plate and archenteron (Figs 3, 4), and ectoderm (Fig. 6), (2) the sensitivity with which expression of GFP transgenes can be detected equals or exceeds that afforded by WMISH against CAT reporter mRNA, as demonstrated by comparisons of several constructs in which given *cis*-regulatory systems were associated with either GFP or CAT reporter sequences and assayed in the same batch of embryos (Table 1), and (3) the stability of the protein product exceeds that of CAT mRNA, at least in some regions of the embryo.

The relatively high stability of expressed GFP is of course a two-edged sword. This is a particularly useful feature for genes such as *CyIIa*, which is expressed transiently in many different regions of the embryo. The GFP product of the *CyIIa-GL* construct persists significantly longer and therefore accumulates to a greater extent, than does the CAT mRNA produced from the corresponding *CyIIa-CAT* construct. In practice, this makes a lot of difference in terms of sensitivity, and *CyIIa* expression is far more difficult to study using the CAT reporter and WMISH than using the GFP reporter. However, GFP stability can obscure the developmental extinction of expression, a major feature of the process by which early expression patterns are spatially refined, and it thus generates a synthetic pattern of expression that is the sum of current and earlier patterns. Examples of this phenomenon were encountered with both the *Endo16-GL* and *CyIIa-GL* constructs. In embryos bearing *Endo16-CAT* constructs, the normal loss of foregut expression at midgastrula is faithfully revealed by the disappearance of CAT transcripts from this region at late gastrula stage (Yuh et al., 1994; Yuh and Davidson, 1996). In contrast, as Table 2 shows, many of the *Endo16-GL* embryos retain staining in the foregut long after the gene has shut off there; we calculated a half-life on the order of 8 hours for the GFP product in this tissue. The stability of GFP may be

even higher in skeletogenic mesenchyme. Thus in embryos bearing *CyIIa-GL*, staining persists in the skeletogenic mesenchyme late into development and can be observed at least 20 hours beyond the point at which expression is down-regulated in these cells (Cox et al., 1986; M. I. A. and E. H. D., unpublished data; Miller et al., 1996).

A second aspect in which GFP is inferior to CAT mRNA as a reporter is that CAT enzyme content is easily quantitated biochemically, by assay of enzyme activity. However, GFP emissions could be assessed by quantitative imaging procedures. Furthermore, the kinetics with which CAT enzyme is generated from given transgene variants can provide illuminating quantitative insights into *cis*-regulatory system function (e.g., Yuh et al., 1996).

Special advantages and new experimental approaches afforded by use of GFP transgenes in sea urchin embryos

For the sea urchin gene transfer system, GFP is not simply an easier-to-use reporter that can be visualized in living embryos, but also a means of new forms of experimental exploration. Three novel applications of GFP transgene technology that are specific to this system became evident in the course of this work. These are its display of the syncytial skeletogenic mesenchyme, the ease of detection of GFP-transgene expression in feeding larvae and its use as a clonal marker for co-introduced expression constructs.

Display of the complete skeletogenic syncytium by GFP expression is particularly useful in transgenic studies, since under normal conditions only about 2-5 skeletogenic cells per embryo receive and express the exogenous DNA (Makabe et al., 1995). Depending on the stage, these cells are not always distinguished by position from other kinds of mesenchyme cells. This is particularly problematical because WMISH protocols result in dissolution of the skeleton per se. Now, however, as illustrated dramatically in Figs 2 and 4, the complete skeletogenic syncytium and every skeletogenic cell, or almost every cell, can be illuminated in the context of the native skeletal structure, in consequence of the activity of the small clone of cells that actually express the transgene. This advance changes skeletogenic transgene expression from one of the more difficult to perhaps the easiest of any to detect.

The potential importance of the observations illustrated in Fig. 5 needs little comment, as for the first time they open the way to investigation of gene regulation during the larval phase of development, when the adult body plan is formed. Almost nothing is known of gene regulation in this postembryonic process, and yet it is of enormous interest, from developmental and evolutionary viewpoints (Raff, 1987; Wray and Bely, 1994; Davidson et al., 1995; Popodi et al., 1996; Peterson et al., 1997). The use of fluorescent markers, in living larvae, invites the application of confocal imaging and image processing, and this is indeed what is likely to be required for resolution of the locus of transgene expression within the complex structure of the larval rudiment.

The experiments of Fig. 6 and Table 3 indicate conclusively that GFP expression can be used as an accurate indicator of the clonal location of an exogenous transgene, in embryos of any stages beyond cleavage. We made use of our earlier demonstrations that exogenous DNA molecules are concatenated together prior to their stable incorporation into blastomere nuclei (McMahon et al., 1985; Flytzanis et al., 1985; Livant et al., 1991). When *H2b-GL* and *Endo16-CAT* were co-injected into eggs, the cells displaying GFP fluorescence coincided exactly with the cells displaying CAT mRNA, that is, within the endodermal domains where the *Endo16* gene is active. Fig. 6 also provides a striking, if by now superfluous, display of differential gene expression, as cells where the *Endo16-CAT* transgene is not active yet display GFP expression. Thus these cells contain both transgenes, but only express the *L1 H2b* regulatory system. We think that, in respect to future applications, the use of the GFP construct as a mosaic expression marker is likely to prove the most important result in this paper. A number of applications can be envisioned in which it would be necessary to sort embryos a priori, according to the locus of transgene incorporation. Primary among these is ectopic expression of genes encoding transcription factors or signaling molecules. Since relatively large numbers of eggs are easily injected, virtually any mosaic pattern of incorporation consistent with the known lineage of this embryo can be obtained and recognized. Thus the consequences of any particular clonal overexpression, in any desired domains or cell types of the embryo, are now directly testable. Mosaic incorporation of injected DNA is typical of most species of egg and this application is likely to be widely useful.

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