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Cloning of sea urchin actin gene sequences for use in studying the regulation of actin gene transcription

(recombinant plasmid/positive hybridization-translation/restriction mapping/Southern hybridization/heteroduplex analysis)

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ABSTRACT **In order to investigate the regulation of actin gene transcription during early sea urchin development, a specific hybridization probe for actin sequences is required. Such a probe was produced by cloning cDNA transcribed from a sea urchin poly(A)-containing mRNA preparation enriched for actin message. Double-stranded DNA was ligated into the *Bam*HI restriction site of plasmid pBR322, and the resulting hybrid molecules were used to transform the *Escherichia coli* strain ML100. After preliminary screening of bacterial colonies by antibiotic sensitivity and hybridization back to the original cDNA, clones containing sea urchin DNA were further characterized by a positive translation assay in which total sea urchin mRNA was hybridized to plasmid, and the hybridized message then was eluted and translated in a reticulocyte cell-free protein-synthesizing system. In this way, one clone (pSA38) was found to hybridize selectively to sea urchin mRNA coding for a protein of 43,000 daltons. This protein was identified as actin by three criteria: electrophoretic migration in two-dimensional polyacrylamide gels, affinity for DNase I, and peptide mapping. Restriction endonuclease and heteroduplex mapping of pSA38 indicate that it contains a 1.5-kilobase-pair insert and is therefore likely to contain a large portion of the actin coding sequence. By using pSA38 as a hybridization probe, it has been found that the level of actin-specific RNA sequences increases dramatically during early sea urchin development.**

It has become clear in recent years that actin is a major constituent of nonmuscle cells, in which it plays an important role in governing cell shape and motility (1–3). This is especially true in the developing embryo, where differentiating cell types are continually reorienting themselves during the process of morphogenesis. Therefore, an important problem in developmental biology concerns the role of actin in early embryogenesis, particularly gastrulation.

A dramatic change in the rate of actin synthesis has been observed during early sea urchin development, when it increases from an almost undetectable level at morula to one of the most prominently synthesized proteins by blastula (4). A concomitant increase in translatable actin mRNA is observed over the same time period (4). In order to assess whether this large increase in actin mRNA is due to an alteration in actin gene transcription, a hybridization probe containing actin gene sequences is required. Such a probe can be constructed by taking advantage of recent advances in recombinant DNA technology, which allow one to generate a purified probe without the requirement for a purified mRNA as starting material (5, 6). The present paper reports the successful cloning and identification of actin gene sequences and the use of this cloned material as a hybridization probe to demonstrate a marked increase in actin RNA sequences during early sea urchin development.

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MATERIALS AND METHODS

Isolation of Sea Urchin mRNA. Polysomes prepared from blastulae of sea urchins (*Strongylocentrotus purpuratus*) (7) were disrupted in 10 mM Tris·HCl, pH 7.5/10 mM EDTA/0.5% $NaDodSO_4$/0.5 M NaCl and subjected to oligo(dT) affinity chromatography to isolate poly(A)-containing RNA (8). The material eluted from the column was treated with dimethyl sulfoxide to disrupt aggregates between the mRNA and any contaminating ribosomal RNA and again subjected to oligo(dT) affinity chromatography (9).

Preparation and Cloning of cDNA. cDNA was synthesized in a reaction mixture containing 50 mM Tris·HCl at pH 8.3, 6 mM $MgCl_2$, 10 mM dithiothreitol, 25 mM KCl, 0.1 mM EDTA, mRNA at 100 μg/ml, unlabeled deoxynucleotides at 800 μM each, 300 μM [α-^{32}P]dATP (60 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) or 250 μM [^{3}H]dATP (3.8 mCi/ml), $(dT)_{12-18}$, at 25 μg/ml, and 200 units of reverse transcriptase (generously supplied by J. W. Beard, Life Sciences) per ml. Incubations were at 42°C for 17 min. Single-stranded cDNA was made double-stranded by using reverse transcriptase, and the resulting molecules were digested with S1 nuclease to remove hairpin loops and create blunt ends as described by Ullrich *et al.* (5). These blunt-ended molecules were joined to *Bam*HI-specific decanucleotide linkers§ (Collaborative Research, Waltham, MA) in a reaction mixture containing 66 mM Tris·HCl at pH 7.6, 6.6 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, 3 μM decamer, double-stranded DNA at 40 μg/ml, and 10 units of T4 ligase (Miles) per ml at 14°C for 1 hr. The mixture was then brought to 2 mM in 2-mercaptoethanol and 0.1 mM in EDTA and digested at 37°C for 2 hr with *Bam*HI at 320 units/ml. Unligated linkers were separated from double-stranded DNAs by polyacrylamide gel electrophoresis, and the DNA was then recovered by electrophoretic elution.

The plasmid pBR322, used as the cloning vehicle, was purified by the procedure of El-Gewely and Helling (10). It was then digested with *Bam*HI, and treated with *Escherichia coli* alkaline phosphatase (Worthington) to prevent intramolecular rejoining (5). [The commercial alkaline phosphatase had been further purified according to Weiss *et al.* (11).] Plasmid and double-stranded DNAs were then ligated at 16°C for 1 hr in a reaction mixture containing 60 mM Tris·HCl at pH 8.0, 8 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, cDNA at 12.5 μg/ml, pBR322 at 125 μg/ml, and 110 units of T4 ligase per ml. The resulting hybrid molecules were used to transform *E. coli* according to the procedure of Lederberg and Cohen (12). The *E.*

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§ $\begin{pmatrix}\text{C-C-G-G-A-T-C-C-G-G}\\ \text{G-G-C-C-T-A-G-G-C-C}\end{pmatrix}$

coli host used in all experiments was ML100, a thymine-less derivative of RH201 provided by M. Lomax (13). All manipulations involving cells containing recombinant DNA molecules were carried out under P2 conditions as described in the *National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.*

Screening of Recombinant Plasmids. Plasmid DNA from ampicillin-resistant, tetracycline-sensitive transformants was isolated (10, 14) and tested for the presence of foreign DNA sequences by hybridization to sea urchin [^{3}H]cDNA by the procedure of Jeffreys and Flavell (15).

Clones meeting the above criteria were subsequently characterized by a "positive hybridization/translation" assay in which 20 μg of sea urchin mRNA was hybridized to 35 μg of cloned plasmid DNA immobilized on nitrocellulose filters. RNA that hybridized to the filter was washed and eluted according to Harpold *et al.* (16), except that all filters were further washed with 0.1 M Tris·HCl, pH 7.6/8 mM EDTA/0.5% $NaDodSO_4$ at 37°C for 90 min just prior to the elution of the bound mRNA. The bound mRNA was then translated in the reticulocyte lysate cell-free protein-synthesizing system (17) to identify protein encoded by plasmid. Reaction mixtures (25–50 μl) supplemented with 60 μg of mRNA and 0.1 mCi of [^{35}S]methionine (1000 Ci/mmol) per ml were incubated at 30°C for 1 hr. *In vitro*-synthesized products were subjected to electrophoresis on a 9% $NaDodSO_4$/polyacrylamide gel and visualized by autoradiographic techniques (7). Two-dimensional gel electrophoresis was carried out according to the procedure of O'Farrell (18).

Restriction Endonuclease Digestion and Southern Hybridization. Plasmid DNA was digested by restriction endonucleases as described by New England BioLabs for 1–2 hr at 37°C, and the resulting fragments were separated on 1% agarose in 50 mM Tris·HCl, pH 8.5/20 mM Na acetate/2 mM EDTA/18 mM NaCl (19). Electrophoresis was at 70–90 V in a vertical slab gel (14 cm × 1.5 mm × 16 cm) for ≈4 hr. DNA was visualized with ethidium bromide (0.1 μg/ml) and transferred to nitrocellulose filters according to Southern (20), except that the agarose gel was immersed for 5 min in 3.0 M NaCl/0.3 M Na citrate (pH 7.0) just before transfer. The filter was preincubated, hybridized, and washed according to Mears *et al.* (21). Hybridization was at 66°C for 12–24 hr with 10 ng of [^{32}P]cDNA (0.7 μCi/ml) per ml.

Actin Purification and Peptide Mapping. Rabbit muscle actin was prepared according to Spudich and Watt (22). Rat brain actin, and both *in vivo*- and *in vitro*-labeled sea urchin actins were purified by DNase I-affinity chromatography (23). Prior to chromatography of *in vitro*-synthesized actins, the reticulocyte reaction mixture was centrifuged at 100,000 × *g* for 1 hr (4°C) and then diluted 1:100 with 50 mM Tris·HCl, pH 7.5/1 mM $CaCl_2$. Purified proteins were then digested and the resulting fragments were mapped according to Cleveland *et al.* (24). Protein (about 1 mg/ml) was cleaved by V8 protease or chymotrypsin (130 μg/ml) for 30–45 min at 37°C. Labeled and unlabeled peptide fragments were separated on a 15% $NaDodSO_4$/polyacrylamide gel with the stacking buffer system described elsewhere (7) and visualized by staining and fluorography (7).

Electron Microscopy. Heteroduplexes were formed and mounted for electron microscopy by the formamide technique of Davis *et al.* (25) using 60% formamide in the spreading solution and 30% formamide in the hypophase. Heteroduplex molecules were measured by using a Numonics digitizer interfaced with a Hewlett-Packard 9825A computer.

RESULTS AND DISCUSSION

Identification of a Clone Containing Actin Gene Sequences. The mRNA used as template for making the DNA cloned in these experiments was prepared from sea urchin blastulae. Upon translation of this RNA in wheat germ or reticulocyte cell-free protein-synthesizing systems, actin was found to represent at least 10% of the total polypeptide products. Initial screening of the clones was based on the fact that pBR322 contains two antibiotic-resistance markers, ampicillin and tetracycline. Because the *Bam*HI site in which the foreign DNA was inserted is located within the tetracycline gene, transformant clones could be identified by screening for resistance to ampicillin and sensitivity to tetracycline. Such transformants were further screened for the presence of sea urchin gene sequences by hybridization of plasmid DNA back to the original single-stranded radioactive cDNA.

Clones identified in this way as containing sea urchin DNA were further screened by a "positive hybridization/translation" assay (16). In this procedure, blastula mRNA was hybridized to cloned DNA immobilized on nitrocellulose filters, and the RNA that hybridized to the filter was eluted and translated in a cell-free protein-synthesizing system. One clone, designated pSA38, was found to hybridize selectively to mRNA that translated into protein comigrating with rabbit muscle actin in $NaDodSO_4$/polyacrylamide gels (Fig. 1).

Three criteria were used to confirm the identity of this pSA38-encoded protein as actin. The first was its behavior during two-dimensional electrophoresis. For comparison, pu-

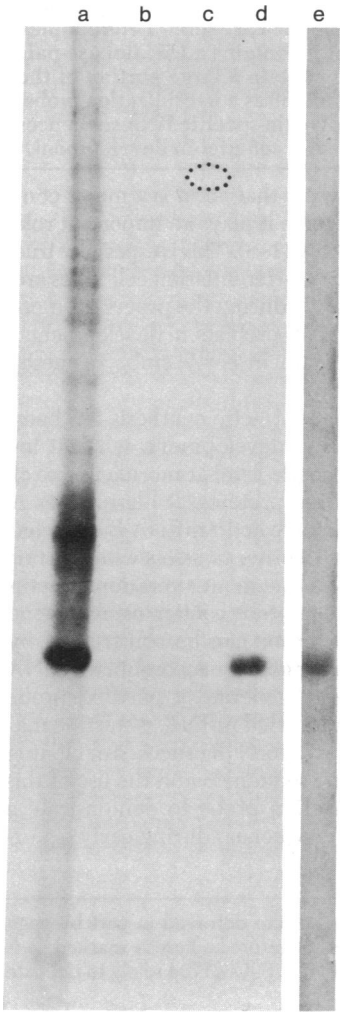

FIG. 1. Screening of sea urchin clones by positive hybridization-translation. Total poly(A)-containing RNA was translated either before or after hybridization and elution from plasmid DNA. Translation products were separated on a 9% $NaDodSO_4$ polyacrylamide gel and visualized by fluorography. Lanes: a, translation of total sea urchin poly(A)-containing RNA; b, translation of reticulocyte lysate endogenous mRNA; c, translation of mRNA eluted from pSA30 (the location of the minor protein of high molecular weight which appears upon extensive overexposure is indicated by the circle); d, translation of mRNA hybridized to plasmid pSA38; e, stained rabbit muscle actin.

rified sea urchin, rabbit muscle, and rat brain actins were used as standards. Labeled sea urchin actin was found to separate into three types: a major species (type I) which comigrated with β-actin prepared from rat brain; and two minor species, one slightly more basic (type II) and the other more acidic (type III) than the major species (Fig. 2). When the translation products of the mRNA purified by hybridization to plasmid pSA38 DNA were analyzed in this two-dimensional gel system, the same actin spots were seen (Fig. 3*B*). There are several possible explanations for the occurrence of multiple spots: the minor spots may be incompletely processed forms of actin; they may represent species of actin chemically modified during purification, resulting in charge artifacts; or the messages coding for heterogeneous forms of actin may be so similar in base sequence that they all hybridize to the same plasmid DNA. The possibility that the multiple spots are an electrophoretic artifact seems unlikely because they always appear in the same relative ratio, regardless of the sample concentration applied.

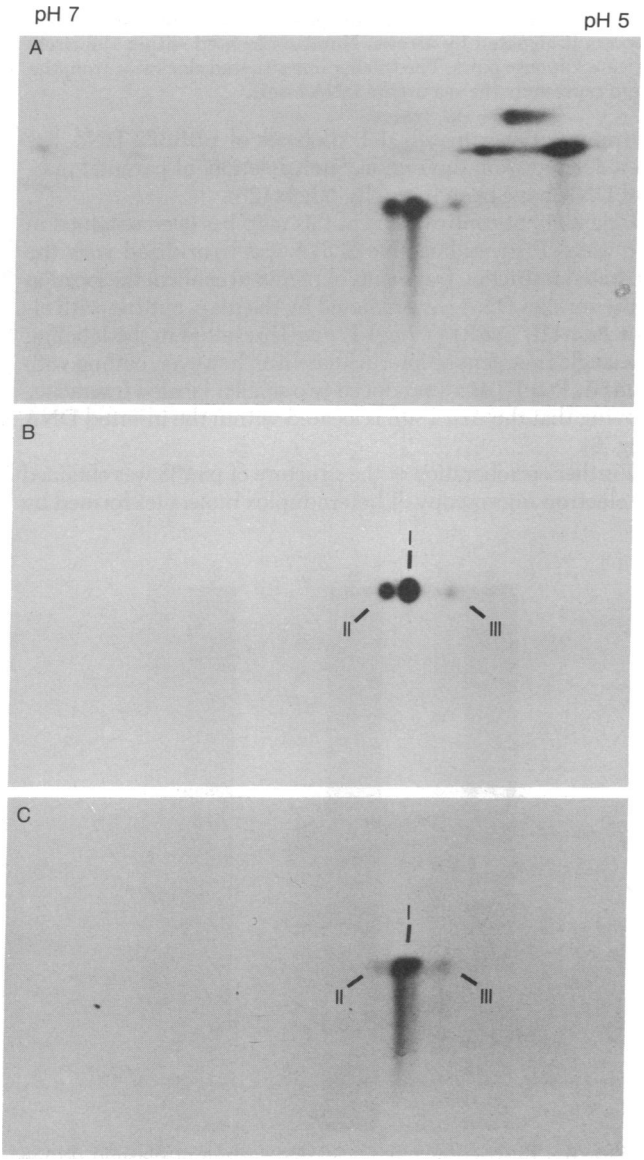

FIG. 2. Fluorograms of *in vitro*- and *in vivo*-labeled sea urchin proteins separated by two-dimensional gel electrophoresis. (*A*) *In vitro*-labeled proteins synthesized in reticulocyte lysate upon addition of total sea urchin poly(A)-containing RNA. (*B*) Similar preparation of *in vitro*-labeled proteins subjected to DNase I affinity chromatography prior to electrophoresis. (*C*) *In vivo*-synthesized sea urchin gastrula proteins subjected to DNase I affinity chromatography prior to electrophoresis. Of the three spots tentatively identified as actin, type I comigrated closely with rat brain β-actin.

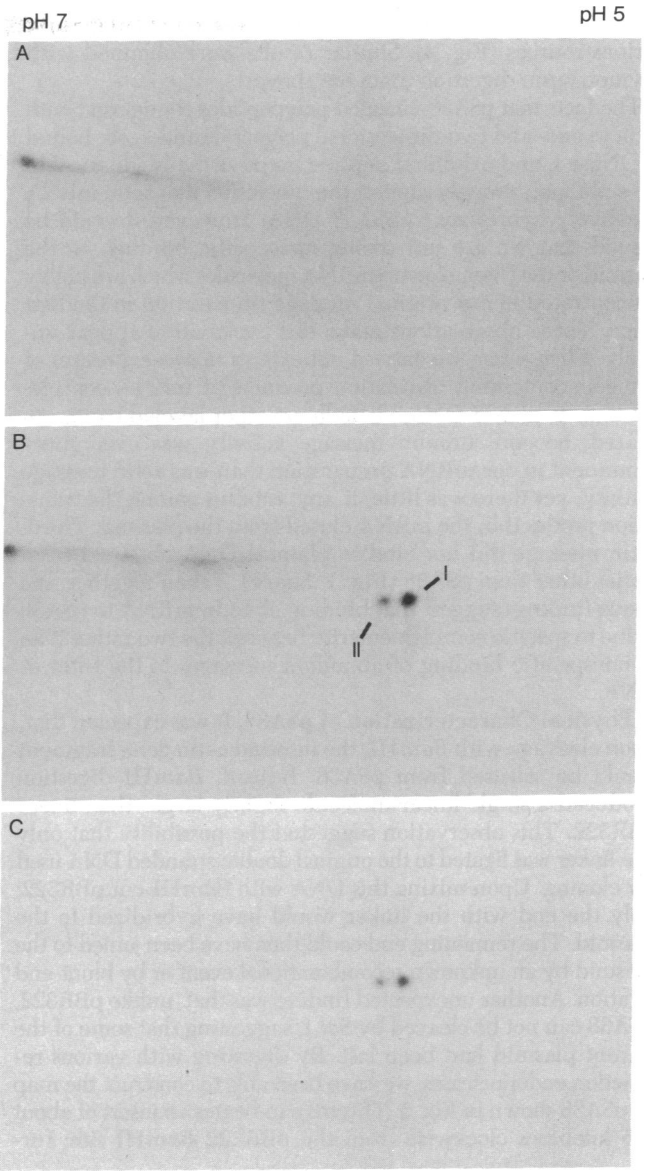

FIG. 3. Fluorograms of *in vitro*-labeled pSA38-encoded proteins separated by two-dimensional gel electrophoresis. (*A*) Translation of reticulocyte endogenous mRNA. (*B*) Translation of mRNA hybridized to pSA38 plasmid DNA. (*C*) Same as *B*, except that the *in vitro*-synthesized products were subjected to DNase I affinity chromatography prior to electrophoresis. Type III actin was not readily visible either because its mRNA does not hybridize to pSA38 DNA or because it represented a low percentage of the total *in vitro*-labeled actin (compare with Fig. 2*B*).

The second criterion used to identify the pSA38-encoded polypeptides as actin was their ability to bind to DNase I, a characteristic property of actin (23). mRNA hybridized to plasmid pSA38 was translated *in vitro* and the resulting polypeptides were subjected to DNase I affinity chromatography. When the material bound to DNase I was analyzed on two-dimensional gels, the same spots comigrating with purified actin were found (Fig. 3*C*).

The final criterion used to identify the pSA38 gene product as actin was peptide mapping. When V8 protease was used to digest the *in vitro* translation products of mRNA purified by hybridization to pSA38 DNA, the resulting peptide pattern was

almost identical to that obtained with purified actins from various sources (Fig. 4). Similar results were obtained with chymotrypsin digestion (data not shown).

The facts that pSA38-encoded polypeptides comigrated with actin in one- and two-dimensional polyacrylamide gels, bound to DNase I, and exhibited peptide maps virtually identical to the actin map strongly support the conclusion that actin mRNA selectively hybridizes to pSA38 DNA. However, it could be argued that we are just seeing nonspecific binding, to the plasmid or the filter, of actin mRNA molecules which are highly concentrated in our original message preparation in the first place. Three observations make this explanation appear unlikely. First, when we carried out extensive overexposures of our gels containing translation products of mRNA complementary to pSA38 DNA, virtually no other labeled bands appeared. Second, tubulin message activity was even more prominent in our mRNA preparation than was actin message activity, yet there was little, if any, tubulin among the translation products of the mRNA eluted from the plasmid. Third, actin message did not bind to plasmid DNA obtained from clones other than pSA38 (Fig. 1, lane c). Taken together, the above findings suggest that binding of actin mRNA to pSA38 is due to specific complementarity between the two rather than to nonspecific binding of abundant messages to the filter or DNA.

Physical Characterization of pSA38. It was expected that, upon cleavage with *Bam*HI, the inserted actin gene fragment would be released from pSA38. Instead, *Bam*HI digestion produced a single linear molecule slightly larger than linear pBR322. This observation suggested the possibility that only one linker was ligated to the original double-stranded DNA used for cloning. Upon mixing this DNA with *Bam*HI-cut pBR322, only the end with the linker would have hybridized to the plasmid. The remaining end could then have been joined to the plasmid by an unknown recombinational event or by blunt-end ligation. Another unexpected finding was that, unlike pBR322, pSA38 can not be cleaved by *Sal* I, suggesting that some of the parent plasmid had been lost. By digesting with various restriction endonucleases, we have been able to construct the map of pSA38 shown in Fig. 5. This map indicates an insert of about 1.5 kilobases clockwise from the pBR322 *Bam*HI site; furthermore, approximately 1.1 kilobases of pBR322 DNA has been deleted from this region. Such deletions of parental plasmid DNA have been found by others (26).

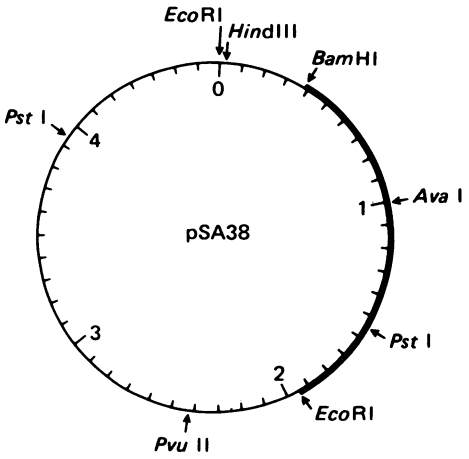

FIG. 5. Physical map of plasmid pSA38. Restriction endonuclease sites are designated by arrows. Numbers located within the circle indicate kilobase pairs. The thicker line situated clockwise from the origin represents the sea urchin DNA insert.

Independent confirmation of this map has been obtained in two ways. First, radioactive cDNA was hybridized with the separated restriction fragments of pSA38 to confirm the location of the foreign DNA. As predicted by the map, cutting with either *Bam*HI/*Eco*RI or *Eco*RI/*Pvu* II resulted in the labeling of a single fragment of intermediate size; however, cutting with *Bam*HI/*Pvu* II/*Ava* I produced two smaller labeled fragments, proving that the *Ava* I site is located within the inserted DNA (Fig. 6).

Further corroboration of the structure of pSA38 was obtained by electron microscopy of heteroduplex molecules formed by

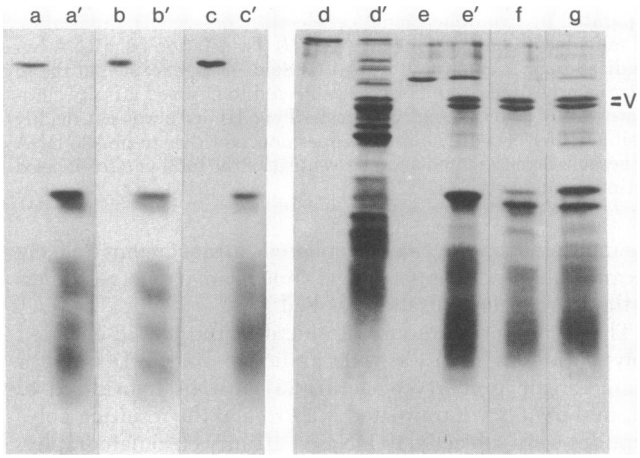

FIG. 4. Polyacrylamide gel electrophoresis of V8 protease-digested actins. Lanes: a and a', uncut and cut *in vivo*-labeled sea urchin actin; b and b', uncut and cut *in vitro*-labeled pSA38-encoded protein; c and c', uncut and cut *in vitro*-labeled sea urchin actin synthesized from total mRNA; d and d', uncut and cut stained bovine serum albumin; e and e', uncut and cut stained sea urchin gastrula actin; f, V8 protease-digested and stained rabbit muscle actin; g V8 protease-digested and stained rat brain actin. V, position of V8 protease.

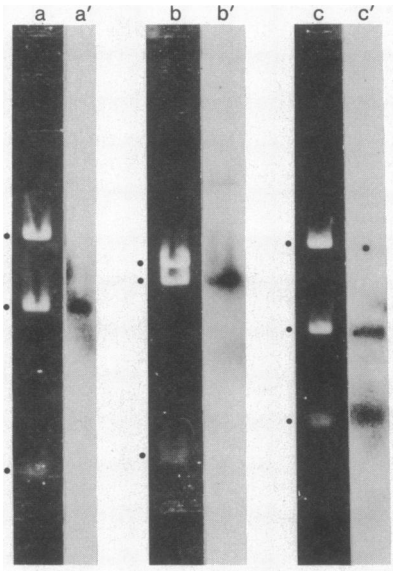

FIG. 6. Restriction endonuclease digestion of plasmid pSA38. Purified pSA38 DNA was cut by various combinations of enzymes, and the resulting fragments were electrophoretically separated on 1% agarose, stained (dots indicate fragment location), and transferred to a nitrocellulose filter (20). Fragments containing foreign DNA were identified by hybridization of this filter with [^{32}P]cDNA. Lanes: a and a', pSA38 digested with *Bam*HI/*Eco*RI; b and b', pSA38 digested with *Pvu* II/*Eco*RI; c and c', pSA38 digested with *Bam*HI/*Pvu* II/*Ava* I. For each pair, the left lane is ethidium bromide-stained and the right lane is an autoradiogram of the hybridized [^{32}P]cDNA.

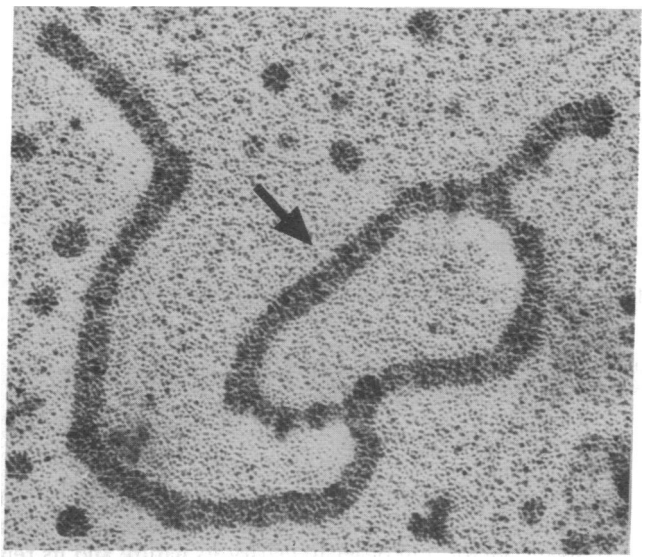

FIG. 7. Heteroduplex molecule formed by hybridization between single-stranded pSA38 and pBR322. Plasmids pSA38 and pBR322 were digested with *Pvu* II, denatured, and heteroduplexed. One single strand of the resulting insertion/deletion loop consists of sea urchin actin DNA sequences (arrow) and the other single strand represents the DNA deleted from pBR322 during the formation of pSA38 (×154,000.)

hybridizing single-stranded pBR322 to pSA38. From the map presented above, it can be predicted that there should be regions of homology between pBR322 and pSA38 flanking both sides of the insert. It was further predicted that the actin DNA insert in pSA38 and the DNA deleted from pBR322 during the formation of pSA38 would together contribute to an insertion/deletion loop in pSA38/pBR322 heteroduplexes. To test these predictions, pSA38 and pBR322 were cut with either *Hin*dIII or *Pvu* II and were then heteroduplexed. Fig. 7 shows an example of the heteroduplexes formed with *Pvu* II-cut molecules. Similar results were seen with the *Hin*dIII-cut preparations (data not shown). Analysis of these structures indicated that the insert loop accounted for 32% of the total length of plasmid pSA38, which is very close to results obtained from agarose gel electrophoresis of restriction fragments, in which the insert was found to account for about 33% of the total length of the plasmid.

Because the size and amino acid sequence of many of the actins are already known (27) it can be estimated that the actin mRNA coding region consists of about 1200 bases. Mammalian actin mRNAs have been sized at about 2000 bases (28), which indicates the presence of about 800 bases of untranslated material at the 5′ and 3′ ends. Given this data, it can be predicted that our insert of about 1500 base pairs probably contains a large portion of the actin structural gene sequences.

In order to determine whether there is a significant change in actin-specific RNA sequences during early sea urchin development, pSA38 DNA was nick-translated in the presence of ^{32}P-labeled deoxynucleotides (29) and hybridized to four-cell, blastula, and gastrula poly(A)-containing RNAs covalently bound to diazotized paper (30). Relative to RNA obtained from the four-cell stage (700 cpm hybridized), there was a 7.2-fold (5020 cpm hybridized) and a 12.8-fold (8950 cpm hybridized) enhancement in levels of actin-specific RNA in blastula and gastrula, respectively. A similar increase in actin sequences was found when pSA38 was hybridized to total sea urchin RNA. These data, which suggest that the stimulation of actin synthesis observed during early sea urchin development may be due to enhancement of the rate of actin gene transcription, will be reported in detail elsewhere.

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