Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 5683–5687, October 1980
Biochemistry

Organization of actin gene sequences in the sea urchin: Molecular cloning of an intron-containing DNA sequence coding for a cytoplasmic actin

(recombinant DNA/message selection/restriction mapping/multigene family/intervening sequence)

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Communicated by Paul C. Zamecnik, June 16, 1980

ABSTRACT **Southern transfer and solution hybridization experiments, using as probe a DNA fragment that encodes for *Drosophila* actin, demonstrate cross hybridization to DNA from the sea urchin *Strongylocentrotus purpuratus*. Recombinant DNA clones that contained sea urchin genomic DNA fragments were constructed and screened for the presence of actin-encoding DNA sequences by colony hybridization with the *Drosophila* actin sequence. Two different putative actin-encoding clones were identified and were shown to specifically hybridize actin-encoding mRNA from a complex mRNA population. Southern blot hybridization experiments with both the *Drosophila* actin sequence and one of the cloned sea urchin sequences, in conjunction with solution hybridization data, suggest an actin gene copy number of 5–20 per haploid genome. DNA sequence analysis of one of the cloned sequences indicates that this fragment codes for a cytoplasmic form of actin and contains an intervening sequence of at least 200 nucleotides beginning immediately after amino acid 121 in the protein sequence.**

Actin is common to all eukaryotes thus far examined, is highly conserved across phylogenetic lines, and has been identified in diverse cell types (1). In addition to its role in muscle contraction, actin has been implicated in various cellular activities including mitosis, cytoskeletal structure, and cell motility (2). This multiplicity of roles within the cell suggests that a number of different proteins, encoded by a family of related genes, could be performing these functions. At the protein level, three major actin variants—usually designated α, β, and γ—have been identified in several vertebrates (3–6). Actin variants of identical molecular weight and similar isoelectric points have also been observed in *Drosophila* (7–9), *Dictyostelium* (10), and the sea urchin *Strongylocentrotus purpuratus* (unpublished data). The amino acid sequence of several of the vertebrate actin electrophoretic variants shows that they are encoded by different but related genes (11, 12). Further support for a multiple gene family derives from the observation that mammalian cell α, β, and γ actin mRNAs differ in molecular weight and poly(A) content (13, 14).

By using recombinant DNA technology, it is now possible to assay directly the frequency with which actin mRNA-complementary sequences are represented in genomic DNA. There are approximately 17 actin-encoding sequences in the genome of *Dictyostelium discoideum* (15) and 6 in the genome of *Drosophila melanogaster* (16, 17). Although it is not yet clear what fraction of these sequences actually represent functional units that are transcribed, these results strongly suggest the presence of an actin multigene family within these two organisms.

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An especially interesting feature of this family of genes is that they are expressed differently in several cell types, tissues, and developmental stages. This differential expression has been demonstrated both at the level of protein (3, 5, 18) and at the level of mRNA (4, 7). The actin genes thus constitute a multigene family whose coding sequences must be quite similar but whose different members may be associated with different regulatory regions or sequences in the genome. Detailed examination of this family of genes and their surrounding DNA sequences should ultimately provide information on the role of DNA organization in the regulation of actin gene expression.

In this paper we report the construction and identification of two *S. purpuratus* genomic DNA clones that have sequence homology with actin mRNA and demonstrate that actin-encoding sequences exist in multiple copies within the genome of this organism. We further show that one of these recombinant plasmids contains sequences encoding a cytoplasmic form of actin, that the coding region on this plasmid is not contiguous, and that the intervening sequence shows similarities to intervening sequences observed in vertebrate genes.

MATERIALS AND METHODS

Construction and Screening of Clones Containing Recombinant DNA. Total *Hin*dIII-digested sea urchin DNA from several animals was loaded onto 16–43% isokinetic neutral sucrose gradients. Fractions containing DNA fragments larger than 2.8 kilobases (kb) were pooled and aliquots of this size-fractionated sea urchin DNA were ligated into the bacterial plasmid vector pBR322.

Recombinant colonies that had been transformed with this DNA preparation were screened for the presence of actin-encoding sequences by a modification of the procedure of Grunstein and Hogness (19). The hybridization probe was a 1.8-kb *D. melanogaster* DNA fragment containing approximately 1.1 kb of actin-encoding sequence and 0.7 kb of non-actin-encoding sequence, which is present in the chimeric plasmid pDmA2.

Positive Hybridization-Selection and Cell-Free Translation. Linearized plasmid DNA was loaded onto nitrocellulose filters (0.7 μg/mm^2) by the protocol of Kafatos *et al.* (20). Fifteen to 20 μg of total tube foot RNA was hybridized to five filters (4 μg of DNA) at successively lower temperatures (5°C increments; 30 min at each temperature, beginning at 65°C and ending at 45°C). After this 2.5-hr incubation, the hybridization solution (containing the nonhybridized RNA) was removed for translation. The filters were then extensively washed and the RNA was eluted in water at 100°C for 1.5 min. *In vitro* trans-

Abbreviation: kb, kilobase(s).

lation of the hybridization-selected and nonhybridized RNA was performed by using the mRNA-dependent reticulocyte lysate translation system described by Pelham and Jackson (21).

DNA Sequencing. pSpG17 plasmid DNA was digested with *Eco*RI and the resulting fragments were labeled at their 5′ ends with ^{32}P after treatment with bacterial alkaline phosphatase. Subsequent digestion with *Hin*cII yielded four terminally labeled fragments. The *Eco*RI/*Hin*cII fragment (approximately 0.90 kb) that was labeled at one end was isolated and its sequence was determined (22). The 1.6 kb *Eco*RI fragment was additionally digested with *Bam*HI and the sequence of the resulting 0.45 kb singly labeled fragment was determined (22).

RESULTS AND DISCUSSION

Drosophila Actin-Encoding DNA Hybridizes with Sea Urchin DNA. In order to begin an examination of the structure and organization of the actin genes in the sea urchin, we attempted to hybridize an actin-encoding DNA fragment from *D. melanogaster* to sea urchin DNA. We reasoned that actin-encoding sequences from *Drosophila* might be similar enough to cross-hybridize with actin-encoding sequences in sea urchin DNA, because actin is known to be highly conserved between species, showing as little as 6% amino acid substitution between *Acanthamoeba* actin and vertebrate muscle actins (12). Sea urchin genomic DNA was digested with several restriction enzymes, subjected to electrophoresis on agarose gels, transferred to nitrocellulose filter paper (23), and hybridized with a ^{32}P-labeled actin-encoding DNA fragment from *Drosophila*. This cloned *Drosophila* genomic DNA fragment contained approximately 1.1 kb of actin-encoding sequence and an additional 2.5 kb of non-actin-encoding DNA, which is single-copy sequence in the in the *Drosophila* genome (17). Crossreaction occurred with sea urchin DNA fragments of several sizes at the moderately high criterion of 0.5 M NaCl at 70°C (Fig. 1).

Construction, Screening, and Characterization of Recombinant DNA Clones. *S. purpuratus* genomic DNA was digested to completion with *Hin*dIII and all fragments larger than 2.8 kb were selected for cloning from a neutral sucrose gradient. This size fractionation removed 50–60% of the sea urchin genomic DNA fragments (data not shown) but only 20% of the actin crosshybridizing fragments (Fig. 1) and thus resulted in an enrichment. Approximately 8000 transformants were screened by using a ^{32}P-labeled *Drosophila* actin-encoding fragment as a probe. Six of the 8000 colonies showed significant reaction with the *Drosophila* probe.

Digestion of each of the six plasmid DNAs with *Hin*dIII indicated that the inserted sea urchin DNA fragments were of two size classes. Three contained a 3.6-kb sea urchin DNA insert, and the other three contained a 7.0-kb insert. Additionally, digestion of these DNAs with other enzymes showed that, within the two size classes, each 3.6-kb fragment and each 7.0-kb fragment had common restriction sites, suggesting that these six clones represent only two different sea urchin DNA fragments. Southern transfer and hybridization to all six sea urchin fragments confirmed that each hybridized with the *Drosophila* probe that had been used for screening.

Selective Hybridization of Actin mRNA by Two Cloned Sea Urchin Sequences. In order to verify that the cloned DNAs encode actin, single representatives of each size class of cloned sea urchin sequence [designated pSpG17 (3.6 kb) and pSpG28 (7.0 kb)] were used in message-selection experiments with total RNA from sea urchin tube foot tissue. In these experiments, cloned plasmid DNA was immobilized in a "dot" on a nitrocellulose filter (20) and the RNA was then hybridized to the filter-bound DNA. The RNA molecules that hybridized to the DNA were eluted from the filters by heating, and both the hybridized and unhybridized fractions were translated in a rabbit reticulocyte *in vitro* protein synthesizing system (21). The RNA that hybridized to the cloned *S. purpuratus* fragment directed the translation of a 43,000-dalton protein with no other exogenous-RNA-dependent products present at detectable levels (Fig. 2). In addition, when the selection was performed with pBR322 DNA alone, no exogenous-RNA-dependent products were seen (see Fig. 3*F*). This is in marked contrast to the translation of total tube foot RNA, which produced not only the 43,000-dalton product in high abundance but also many other polypeptides. In Fig. 2*B*, the protein band appears to run as a doublet, a phenomenon observed occasionally but not always when tube foot RNA was translated. The source of this variation is unknown.

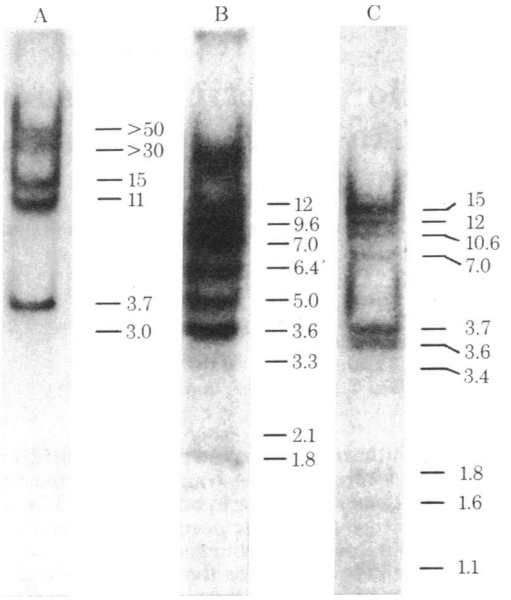

FIG. 1. Southern blot hybridization demonstrating sequence homology between genomic *S. purpuratus* DNA and a *Drosophila* actin-encoding DNA fragment. *S. purpuratus* genomic DNA, prepared from a single animal, was cleaved with *Bam*HI (*A*), *Hin*dIII (*B*), and *Eco*RI (*C*), electrophoresed on 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized with the entire *Drosophila* actin-encoding plasmid pDmA2 which had been nick-translated with [^{32}P]dCTP. The size of each fragment, in kb, is indicated.

In order to confirm that the 43,000-dalton product being translated from the hybridized RNA is actin, and to explore the possibility that the cloned sequences might hybridize to more than one actin variant mRNA, the selected products were resolved by two-dimensional gel electrophoresis (26, 27). The 43,000-dalton product of translation of total tube foot RNA separated into two spots in the isoelectric focusing dimension of the gel; one spot comigrated with mouse β actin and the other was slightly more basic than mouse γ actin (Fig. 3 *A* and *B*). The comigration of the 43,000-dalton sea urchin-RNA-dependent products with mouse actin variants in two-dimensions provides further evidence that the product is actin and clearly suggests the possibility that two actin variants exist in tube foot tissue. The 43,000-dalton product translated from RNA that specifically hybridized to pSpG17 also separated into two spots in the isoelectric focusing dimension (Fig. 3 *C* and *D*). These spots corresponded, in mobility and relative concentration, to those from total tube foot RNA. A similar result was seen when pSpG28 DNA-selected-RNA translation products were similarly

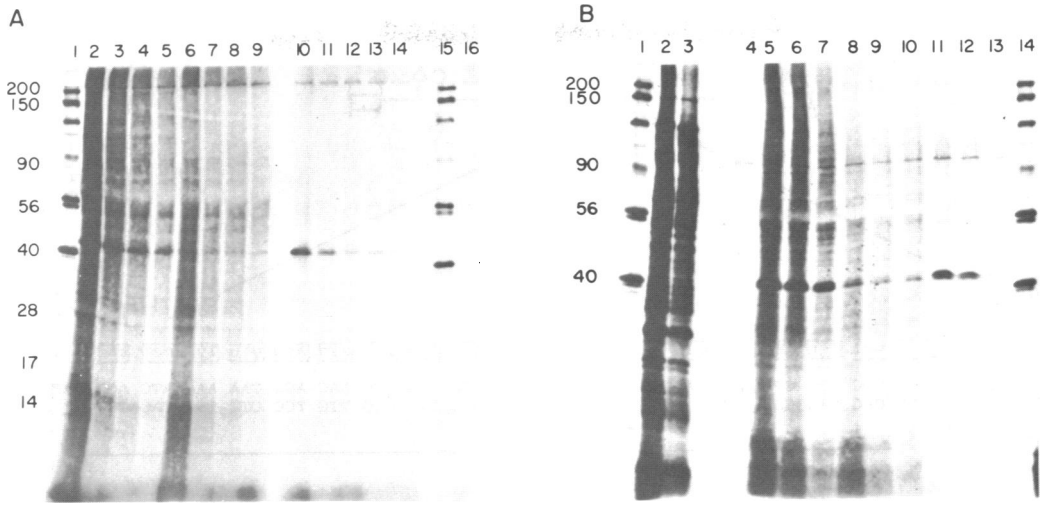

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of message-selection translation products. Plasmid DNA from pSpG17 and pSpG28 was linearized, denatured, and immobilized on a nitrocellulose filter and then hybridized with total tube foot RNA. Both the nonhybridized and the hybridized fractions were translated in a reticulocyte lysate *in vitro* protein-synthesizing system. The [^{35}S]methionine-labeled products were subjected to electrophoresis on polyacrylamide gels (24) followed by fluorography (25). (*A*) Translation products from a message-selection experiment performed with pSpG17 (3.6-kb sea urchin insert). Lanes: 1 and 15, marker proteins whose molecular weights are indicated ($\times 10^{-3}$) on the ordinate; 16, no exogenous RNA added; 2–5, total tube foot RNA, each lane containing half the amount of added RNA as in the previous lane; 6–9, unhybridized tube foot RNA after 2.5-hr incubation with pSpG17 DNA; each lane containing half the amount of RNA as in the previous lane; 10–14, RNA hybridized to and eluted from the pSpG17 DNA-containing nitrocellulose filters, each lane containing half the amount of RNA as in the previous lane. (*B*) Translation products from a message selection experiment performed with pSpG28 (7.0-kb sea urchin insert). Serial 1:2 dilutions of RNA were used in total tube foot RNA (lanes 5–7), unhybridized RNA (lanes 8–10), and hybridized RNA (lanes 11–13). Other lanes: 1 and 14, markers; 2 and 3, tobacco mosaic virus RNA; 4, no added RNA.

subjected to electrophoresis (data not shown). Fig. 3*F*, which is similar to the fluorogram when no RNA was added to the translation system (data not shown), demonstrates the absence of these products from RNA selected by pBR322 DNA.

The presence of the two actin variants as translation products of the selected RNA suggests that each cloned fragment might hybridize two actin variant mRNAs. However, when total tube foot RNA was separated on an agarose gel, transferred to diazobenzyloxymethyl-paper (28), and hybridized with either of the two sea urchin actin clones, RNA of only one size class (2.1 kb) was detectable (not shown). It is not yet clear whether the two actin variants from tube foot RNA result from translation of two different mRNAs of similar size or from modifications, by the reticulocyte system, of a single gene product (29).

Coding Region of pSpG17 Is Noncontiguous and Represents a Cytoplasmic Actin. Both actin-encoding clones, pSpG17 and pSpG28, were mapped by digestion with several restriction enzymes. The fragments that contained actin-encoding sequence were identified by Southern blot hybridization with the *Drosophila* actin-encoding DNA fragment. The results of this mapping of pSpG17 are depicted in Fig. 4*A*. The 0.45-kb *Bam*HI/*Eco*RI fragment and the 0.65-kb *Hin*cII fragment hybridized with the *Drosophila* actin-encoding probe. The region between these two fragments (composed of a 0.3-kb *Eco*RI/*Pst* I fragment and a 0.65-kb *Pst* I/*Hin*cII fragment) was probed in association with adjacent fragments; these experiments gave hybridization intensities suggesting that the actin-encoding sequence also exists within this region. The greatest distance between the ends of known coding fragments in this plasmid (indicated by arrows in Fig. 4*A*) is about 2 kb. Because only 1.1 kb is necessary to code for actin, it is possible that the entire coding region lies within the plasmid. Also, in light of the sizes observed for sea urchin actin messenger RNAs (1.7 and 2.1 kb) (unpublished data) and in conjunction with the sequence data reported below, it is unlikely that pSpG17 could contain information for more than a single actin gene. The pattern of restriction enzyme sites for pSpG28 is entirely different from that for pSpG17 (not shown), indicating that they represent separate regions of the genome and possibly are derived from separate genes.

The nucleotide sequence surrounding the central *Eco*RI site in the pSpG17 insert was determined as described by Maxam and Gilbert (22) and is shown in Fig. 4*B*. Several important

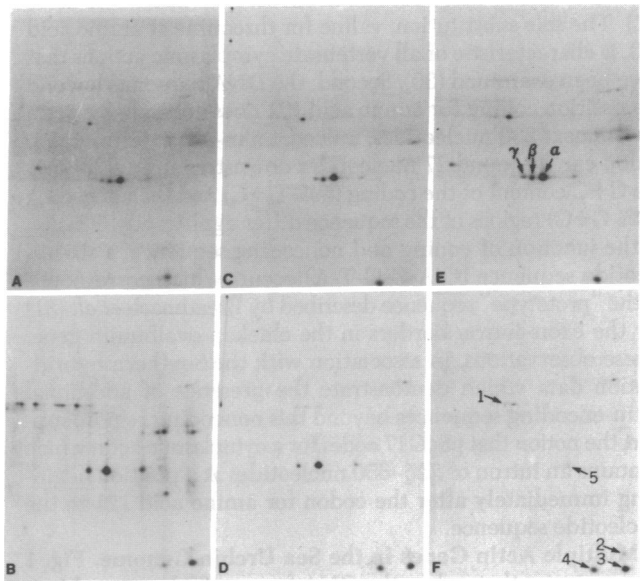

FIG. 3. Two-dimensional gel electrophoresis of message-selection translation products. Unlabeled α, β, and γ actins were added (labeled in *E*) to each sample as markers and the translation products were then subjected to two-dimensional gel electrophoresis (26), stained (*A*, *C*, and *E*), and fluorographed (*B*, *D*, and *F*). In each panel, the left is the more basic side. (*A* and *B*) Stained gel and fluorogram for translation of total tube foot RNA. (*C* and *D*) Translation of tube foot RNA that had hybridized to pSpG17 DNA. (*E* and *F*) Translation of tube foot RNA that had hybridized to pBR322 DNA. In *F*, five abundant endogenous lysate proteins are labeled for orientation.

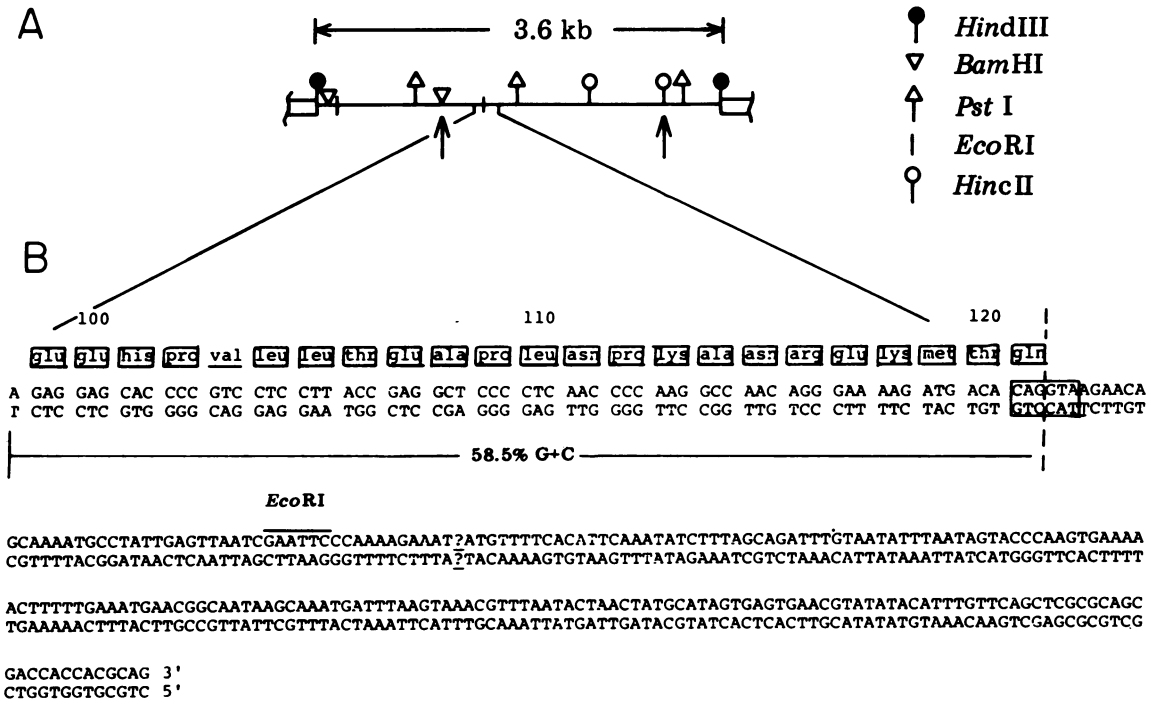

FIG. 4. Organization of actin-encoding plasmid pSpG17. (*A*) Restriction map of pSpG17. The most likely arrangement of the recognition sites for four enzymes is represented (*Kpn* I and *Sal* I did not cleave the sea urchin DNA). The pBR322 plasmid vector sequences are indicated by broken bars flanking the cloned sea urchin DNA insert. The fragments that contained actin-encoding sequence were identified by hybridization, after Southern transfer (23), to a nick-translated 1.8-kb pDmA2 *Hin*dIII fragment that contained a *Drosophila* actin-encoding sequence. Sequences complementary to the *Drosophila* probe have been localized to the region bounded by the vertical arrows. (*B*) Nucleotide sequence surrounding central *Eco*RI site of pSpG17 insert shown in *A*, with the amino acids it encodes. Those in boxes are matches with the rabbit skeletal muscle actin amino acid sequence. The broken vertical line represents the point beyond which no correspondence could be found (see text). The nucleotide sequence at the coding–noncoding junction which corresponds to the "prototype" sequence of Breathnach *et al.* (31) is enclosed in a box.

features relevant to actin gene organization are evident from the sequence in this region of the gene. First, the 5′ portion of the sequence codes for 23 amino acids which correspond almost exactly to amino acids 99–121 in rabbit skeletal muscle actin (12). The sole substitution, valine for threonine at amino acid 103, is characteristic of all vertebrate cytoplasmic actions that have been examined (30). Second, the DNA sequence beyond the position coding for amino acid 121 does not code for actin for the next 230 nucleotides; indeed, an in-phase termination codon can be found 27 nucleotides downstream. In addition, the G+C content of the coding (59% G+C) and the noncoding (34% G+C) regions of the sequence differ significantly. Finally, at the junction of coding and noncoding sequence, a six-nucleotide sequence (C-A-G-G-T-A) occurs which corresponds to the "prototype" sequence described by Breathnach *et al.* (31) for the exon–intron borders in the chicken ovalbumin gene. These observations, in association with the Southern hybridization data which demonstrate the presence of additional actin-encoding sequences beyond this noncoding region, support the notion that pSpG17 codes for a cytoplasmic actin which contains an intron of 225–650 nucleotides at a position beginning immediately after the codon for amino acid 121 in the nucleotide sequence.

Multiple Actin Genes in the Sea Urchin Genome. Fig. 1 demonstrates that, when the DNA from a single sea urchin is digested with *Eco*RI, *Bam*HI, or *Hin*dIII, the *Drosophila* actin probe hybridizes with 6–10 sea urchin DNA fragments of different sizes. If each of these bands contains a single gene or a portion of a single gene, there can be no more than 10 coding sequences in the genome. These data, however, do not exclude the possibility that each band contains several closely linked genes or tandemly repeated restriction fragments containing actin-encoding sequences. In order to obtain a reasonable estimate of the number of actin genes in the sea urchin genome, two additional experiments were performed. First, the 1.8-kb *Hin*dIII fragment containing the *Drosophila* actin-encoding region was nick-translated, sheared to 300 nucleotides, and

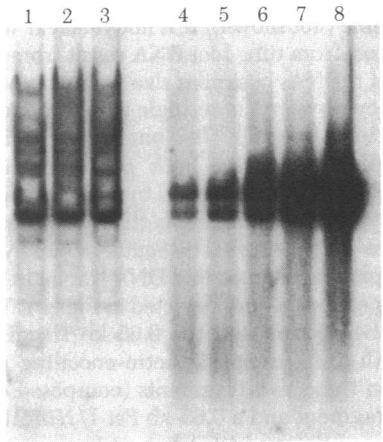

FIG. 5. Reconstruction experiment to determine actin gene copy number. *S. purpuratus* DNA (5 μg) isolated from three separate animals was cleaved with *Hin*dIII and run in parallel on a 0.8% agarose gel with lanes containing increasing amounts of *Hin*dIII-digested pSpG17 DNA to which 5 μg of *E. coli* DNA had been added prior to digestion. The DNA was then transferred to nitrocellulose paper and hybridized to nick-translated pSpG17 DNA. Lanes: (1–3) *S. purpuratus* DNA; 4–8, 0.5, 1, 2.5, 5, and 10 genome copy equivalents of the pSpG17 3.6 kb insert, respectively. The upper band (4.3 kb) in these lanes represents hybridization of pBR322 plasmid sequences present in the probe. The polymorphism of the *Hin*dIII sites relative to the actin-encoding fragments seen in these three animals will be discussed in a later publication.

reassociated in solution with an excess of sheared sea urchin genomic DNA (300 nucleotides). The least squares solution of the data (not shown) yielded a single kinetic component with a reassociation rate constant (k) of 5.2×10^{-3} M^{-1}·sec^{-1}. Under these conditions, the rate constant for the reassociation of sea urchin single copy sequences is 1×10^{-3} M^{-1}·sec^{-1} (32, 33). The ratio of these rate constants, 5.2, suggests a copy number of approximately 5 actin genes per haploid genome.

Because the kinetics of hybridization may be affected by sequence divergence (34) leading to a possible underestimate of the number of actin genes, a second estimate of gene copy number was determined by means of a reconstruction Southern blot experiment. Sperm DNA from three animals was cut with *Hin*dIII; for comparison, DNA quantities corresponding to 0.5, 1, 2.5, 5, and 10 genome copy equivalents of the pSpG17 3.6-kb insert were mixed with *Escherichia coli* DNA and similarly digested with *Hin*dIII. All samples were run in parallel on an 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized to nick-translated pSpG17 DNA. The intensity of the 3.6-kb band in the three animals assayed corresponded to a genome equivalent of one or two copies (Fig. 5). pSpG17 does not contain more than a single actin gene (see previous section) and it hybridized as intensely as any other band when an actin sequence specific probe was used (Fig. 1). Also, between 8 and 11 actin-encoding *Hin*dIII fragments were present in individual animals (Fig. 5). These observations suggest that there are probably no more than 20 actin genes per genome. We thus conclude that there are between 5 and 20 actin genes per haploid sea urchin genome.

We thank Ms. Emily Shen for technical assistance. We also thank Dr. Sam Wadsworth for helpful discussions, Dr. Tim Hunt for providing rabbit reticulocyte lysate, and Dr. Peter Lomedico for help in DNA sequence determination. We especially thank Drs. E. Fyrberg and N. Davidson for providing us with the *Drosophila* actin-encoding clone pDmA2. This research was supported by the following Grants: GM 24620, GM 25492, P30 CA12708, and RR05528 from the National Institutes of Health and by funds from the Mimi Aaron Greenberg Memorial Institute. This research was carried out during the tenure of postdoctoral fellowships (to D.S.D. and J.A.S.) from the Muscular Dystrophy Association.

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