Genetic Engineering

Principles and Methods

Volume 9

Edited by

Jane K. Setlow

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PREFACE

This series was conceived by Alexander Hollaender, who died December 6, 1986, at the age of 88. In his wisdom he saw the importance of such a series almost ten years ago, and proceeded to talk his coeditor into taking part. It has been difficult to do without him, personally as well as professionally, and to say, "Goodbye, Alex".

Jane K. Setlow

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GENE TRANSFER IN THE SEA URCHIN

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INTRODUCTION

Sea urchins began to be used over a century ago for studies of fertilization and development, and they are now among the best understood experimental models for early embryogenesis. The use of sea urchin embryos can be attributed in part to practical considerations. The adult animals are abundant, widely distributed, and easy to collect. Gametes are available in relatively large quantities during long breeding seasons, and under appropriate laboratory regimens they can be obtained all year round (1,2). Embryonic development is rapid and synchronous, occurs reliably under laboratory conditions, and in most species is complete within a few days. Unlike most invertebrates commonly utilized for research, echinoderms belong to the same great branch of the Animal Kingdom as do the vertebrates, i.e., they are deuterostomes. Thus to the advantage of experimental accessibility may be added the attraction of a developmental system that shares with the chordates a common if remote evolutionary ancestry. Definitive homologies in the morphogenesis of chordate and echinoderm embryos were noted by classical observers [reviewed in (3)], and in recent years specific molecular homologies have been reported as well. An example is the presence of introns in exactly the same positions in the actin genes of sea urchins, birds, and mammals, while in all protostome and other invertebrate groups the introns of those actin genes so far sequenced occur at a totally non-overlapping set of locations [e.g., (4-7)].

Most of the data summarized in this chapter have been obtained on embryos of Strongylocentrotus purpuratus, the purple sea urchin common on the West Coast of the U.S. and Canada. Embryogenesis in this species requires about three days. The

fully developed pluteus-stage embryo contains about 1800 cells and is equipped to undertake a free-living pelagic larval existence. Thus it has various differentiated structures, the most prominent of which are the skeletal apparatus, a functionally competent digestive tract, including mouth, muscular esophagus, stomach and intestine, and a regionally diversified ectoderm on which is mounted innervated bands of cilia, the coordinate motion of which is required for feeding and locomotion. In addition, the embryo contains yet undifferentiated bilateral coelomic pouches that together with several other larval structures will develop into the imaginal sea urchin rudiment. A pluteus stage S. purpuratus embryo is shown in Figure 1a. The morphological processes of sea urchin embryogenesis have often been reviewed [the interested reader is referred to references (8-11)]. A canonical sea urchin embryo cell lineage drawn from literature sources has recently been assembled (12). Current experiments in which a fluoresceinated dextran lineage marker was injected into each of the blastomeres of the eight cell-stage S. purpuratus embryo confirm the major features of this lineage, and in addition demonstrate the origins of the various regions of the embryonic ectoderm (13). Both axes of the sea urchin embryo are clearly specified by the 8-cell stage (10,13), though when isolated, placed in ectopic positions or in chimeric recombinations the blastomeres are found to retain developmental plasticity far into cleavage [reviewed in (8,9)].

In considering the potentialities of gene transfer studies carried out on sea urchins it is important to note that at least some species can be cultured from egg to egg with reasonable ease and efficiency (14-16). In the laboratory larvae of S. purpuratus require 5-6 weeks of feeding to achieve maturity. At this stage (Figure 1b) they contain about 5×10^4 cells, a majority of which are included in the imaginal rudiment. At metamorphosis the juvenile sea urchin emerges from the collapsed structures of the larva (Figure 1c). Within about a week a new mouth and anus have formed, the digestive system has been reorganized, and growth is resumed. A very young juvenile sea urchin is shown in Figure 1d. Sexual maturity in this species requires a further 8 to 10 months, though in warmer water species this interval may be much reduced.

Molecular aspects of sea urchin embryogenesis have been intensively investigated over the past two decades. This is indeed the best characterized of all embryonic systems with respect to areas of knowledge such as the nature and function of maternal mRNAs, control of the embryonic translational apparatus, the complexities, transcription rates and stage specificities of nuclear and messenger RNAs, and the characteristics of many specific genes and gene products that function early in development [see reviews in (12,17)]. The embryonic expression of the early and late histone genes [reviewed in (12,18)], the metallothionein genes (19,20), the tubulin genes (21), the actin genes, and many others, has been studied intensively. Of

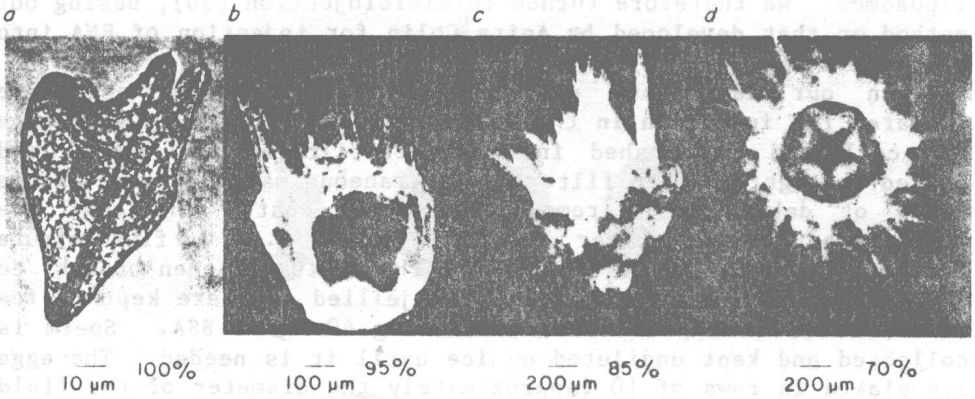

Figure 1. Stages of postembryonic development in S. purpuratus. (a) 70 hr pluteus stage embryo. (b) 5 week mature larva. (c) Metamorphosing larva. (d) Juvenile sea urchin, two months after metamorphosis. Relative survival in the laboratory after the pluteus stage is shown below each figure. These particular values are derived from two large batches of larvae that were carried through metamorphosis to sexual maturity (from Flytzanis et al.,Dev. Biol. 108, 431-442, 1985) (16).

particular interest in the present context are genes that function differentially, in certain regions or structures of the embryo, in particular cell lineages. A number of sea urchin genes that are thus spatially and temporally regulated, and that contribute in known ways to embryonic morphogenesis, has been identified, and this number is increasing rapidly. Examples include five different cytoskeletal actin genes, each of which is expressed only in a specific embryonic cell lineage or set of lineages, and on a particular temporal schedule (22-24), the muscle actin gene (ibid, 25), the Spec genes, which code for Ca^{2+} binding proteins (26,27), and a spicule matrix protein gene utilized in skeletal morphogenesis (28,29). To proceed beyond descriptive molecular characterization a test system is required in which cloned gene constructs can be introduced into eggs in such a manner that at least approximately normal ontogenic regulation will ensue. In the following we describe such a system.

METHODS OF INTRODUCING CLONED DNA INTO THE SEA URCHIN EGG

In preliminary experiments we found that the calcium phosphate precipitation method of DNA introduction could not be adapted to sea urchin eggs, at least in a simple fashion. Nor was it found possible to introduce cloned DNA into sperm by means of

liposomes. We therefore turned to microinjection (30), basing our method on that developed by Anita Colin for injection of RNA into sea urchin eggs (31,32).

In our laboratory unfertilized S. purpuratus eggs are prepared for injection in the following way. The newly shed eggs are collected and washed in Millipore-filtered sea water, and poured through gauze to filter out extraneous material and various kinds of debris. To remove the jelly coats, the eggs are resuspended in sea water titrated to pH 5. A five minute treatment is usually sufficient, and the medium is then brought to pH 8 and normal sea water added. Dejellied eggs are kept on ice in sea water or in sea water containing 40 mg/ml BSA. Sperm is collected and kept undiluted on ice until it is needed. The eggs are placed in rows of 10 (approximately the diameter of the field of view on the inverted microscope at 100X magnification) in a 60 mm Falcon petri dish lid containing 10 ml of filtered sea water. We pretreat the plastic lids for 1 minute with a 1% solution of protamine sulfate, and if the jelly coats have been removed the eggs will stick to the lid by electrostatic attraction. The unfertilized eggs are moved about in silanized pipettes. Eggs that have been put in rows in treated dishes can be fertilized in situ and remain attached to the dish by their vitelline envelopes until they hatch.

A DNA solution is prepared for injection by centrifugation to remove any suspended particles which could plug up the needle, and mixed with glycerol (to 40% glycerol) that has been similarly treated. Needles are pulled from 1 mm (O.D.) borosilicate glass capillaries on a DKl 70 or a Model P-77B Brown-Flaming micropipette puller. Capillaries are pre-cleaned in boiling 35% nitric acid and rinsed exhaustively in 0.2 μm filtered distilled water. The tips are closed when the needles are pulled, and the DNA solution is introduced from the open end with a drawn-out pipette. Needles are then inserted into an oil-filled micro-injection apparatus. The tips of the needles are broken just before injection begins, and flow continuously under pressure. The apparatus is assembled so that the plastic dish lids can be placed on the stage of an inverted microscope (Smith optics) and the eggs injected in groups of ten. Most of the studies reviewed below have been carried out on eggs injected into the cytoplasm. The flowing needle is inserted into the cytoplasm and removed as soon as it becomes clear that it did in fact penetrate the cell membrane. After the eggs are injected they are fertilized, and the embryos allowed to develop at 15°C in the same dishes (Figure 2). If embryos are to be raised for longer than two or three days they are transferred to gently shaking Erlenmeyer flasks at 15°C.

Sea urchin eggs have completed meiosis when they are shed. Thus there is a haploid number of chromosomes in the nucleus [in Strongylocentrotus purpuratus n = 21 (33)]. The haploid pronucleus is much smaller than is the germinal vesicle of the oocyte. At the time of fertilization both egg and sperm nuclei

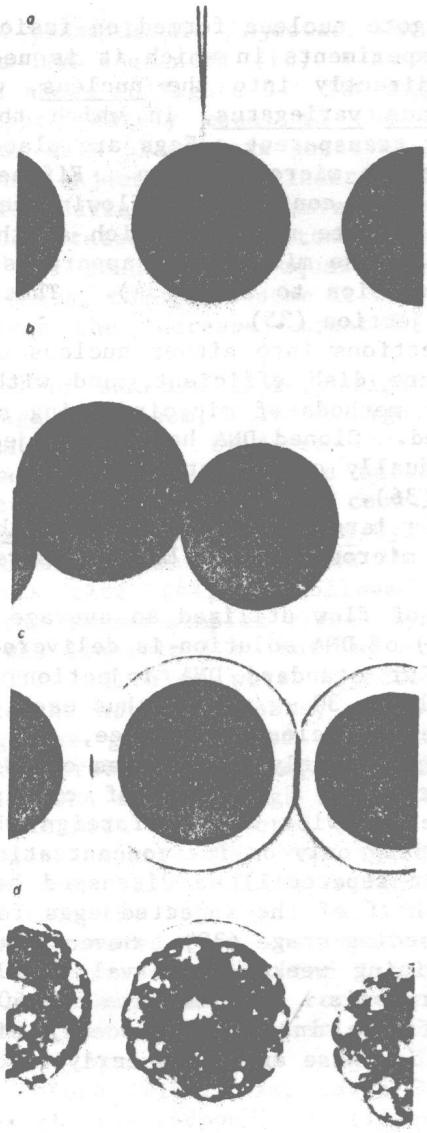

Figure 2. Microinjection of sea urchin eggs. (a) Unfertilized eggs (80 μm in diameter) fixed by electrostatic attraction to a tissue culture dish. A flowing injection needle can be seen above the row of eggs. (b) An egg penetrated by the flowing needle. A clear area of injected DNA solution appears in the cytoplasm at the tip of the needle. (c) Microinjected eggs fertilized in situ. Elevated fertilization membranes indicate successful fertilization. (d) Cleaving eggs still attached to the dish by their fertilization membranes (from McMahon et al., Dev. Biol. 108, 420-430, 1985).

swell and the zygote nucleus formed on fusion is about 15 μm in diameter. For experiments in which it is necessary to introduce the cloned DNA directly into the nucleus, we use a sea urchin species, Lytechinus variegatus, in which the egg cytoplasm is almost completely transparent. Eggs are placed in rows as above, but fertilized before microinjection. Fifteen to thirty minutes post-fertilization the continuously flowing needle can be inserted directly into the zygote nucleus, which at this stage is held in place in the egg by the microtubule apparatus that maneuvered the pronuclei into position to fuse (34). These eggs also develop normally after injection (35).

We find injections into either nucleus or cytoplasm of eggs fixed to a culture dish efficient, and with practice not very difficult. Other methods of microinjecting sea urchin eggs have also been reported. Cloned DNA has been injected into sea urchin eggs held individually on a suction pipette, the technique used with mouse eggs (36). Successful injections have also been done using the slightly larger eggs of S. droebachiensis on the stage of a dissecting microscope [R. Maxson, personal communication, (31,32)].

At the rate of flow utilized an average of 2 pL (about 1.5% of the egg volume) of DNA solution is delivered into the cytoplasm of each egg. Our standard DNA injection solutions are at a concentration of 10 to 30 μg/ml and thus each egg receives several thousand molecules of cloned sequence, of ~10 kb in length. Injection of a significantly larger mass of DNA is toxic (30). In preliminary experiments a variety of clones and plasmids was injected, and the behavior of the foreign DNA was the same in every case, depending only on its concentration and form (linear, relaxed circle, or supercoil) as discussed below. In a typical experiment about half of the injected eggs fertilize and develop normally to the feeding stage (30). Seventy percent of the plutei survive the remaining weeks of larval development and undergo successful metamorphosis. We attribute the 50% primary mortality to the trauma of the injection process, since development is typically blocked in these eggs very early in cleavage.

FATE OF EXOGENOUS DNA IN THE DEVELOPING SEA URCHIN

In initial studies we used a large number of different recombinant DNA clones. Plasmid DNA containing no eukaryotic sequences was also injected. Inclusion of Drosophila melanogaster P-factor elements (37,38), sea urchin repetitive sequence elements (39), or a sea urchin transposon-like element (40) neither enhanced nor reduced the number of 5-week larvae in which the injected sequences were found. As long as the initial DNA concentration is not too high, we find only one parameter which affects the amount of foreign DNA that can be detected in larvae developing from injected eggs. This is the physical form of the

DNA. If supercoiled plasmids are injected, the larvae retain no detectable exogenous DNA sequences (16). A typical experiment illustrating this is shown in Figure 3. The signal obtained from an amount of DNA approximately equal to the amount originally injected is shown for each plasmid in position 1-D. It is clear that those plasmids injected in linear form were usually replicated many-fold during early development. When such experiments were quantitated by densitometry, the increase in exogenous sequences was found to be equal to 4 to 9 rounds of replication in most cases, though in some it was even greater, and in 4 to 5% of larvae the increase amounted to 10^4 (about 15 replications)(16).

Linear molecules of DNA are rapidly ligated together after injection into the egg cytoplasm, forming high molecular weight, end-to-end concatenates (30). Evidence for this is shown in Figure 4. For this experiment a plasmid called pISA (shown in Figure 4a) was injected. This is a 5.1 kb construct containing 5' sequences from the sea urchin S. purpuratus CyI cytoplasmic actin gene (43) ligated to the Tn5 aminoglycoside 3' phosphotransferase (neomycin resistance) gene (44,45) followed by the poly(A) addition site from the herpes simplex virus thymidine kinase gene (46). The plasmid was linearized by cutting at its unique BamHI site, and about 9000 molecules were injected into each egg. Blastulae were collected after 24 hours, and DNA was extracted, run on an 0.8% agarose gel, blotted onto nitrocellulose, and hybridized with a Tn5 probe. pISA sequences from 150 blastulae (lane 4) migrate with DNA of very high molecular weight relative to the size of the plasmids as injected (lanes 1 and 2). It is obvious that the concatenated DNA has replicated, since the standard lane 2 of the gel contains slightly more DNA than originally injected into the 150 eggs, yet gives a much smaller signal than the DNA recovered from the embryos. The experiment shown in Figure 5 demonstrates that the injected molecules are ligated end-to-end in a random manner, regardless of orientation. Here eggs similarly injected with linearized pISA were harvested after 48 hours of development (prism stage, late gastrula) and either analyzed as before (Figure 5a, lanes 3 and 4), or after digestion with a restriction endonuclease (lanes 5-8). Figure 5b shows diagrammatically the fragments to be expected after digestion with BglII of random concatenates of BamHI linearized pISA molecules. The 1.4 kb fragment is not seen on the gel since the probe hybridizes to Tn5 sequences (open bar) only. Lanes 4 and 7 contain DNA of embryos that developed from eggs injected with plasmids linearized with HincII rather than BamHI. This part of the experiment shows that random concatenates form from "blunt-ended" (HincII digested) as well as "sticky-ended" (BamHI digested) DNA molecules. The same phenomenon has been shown previously to occur in transformed tissue culture cells (47-49) and amphibian eggs (50,51). The unfertilized sea urchin egg contains a ligase (52) as well as DNA polymerase activity. In a

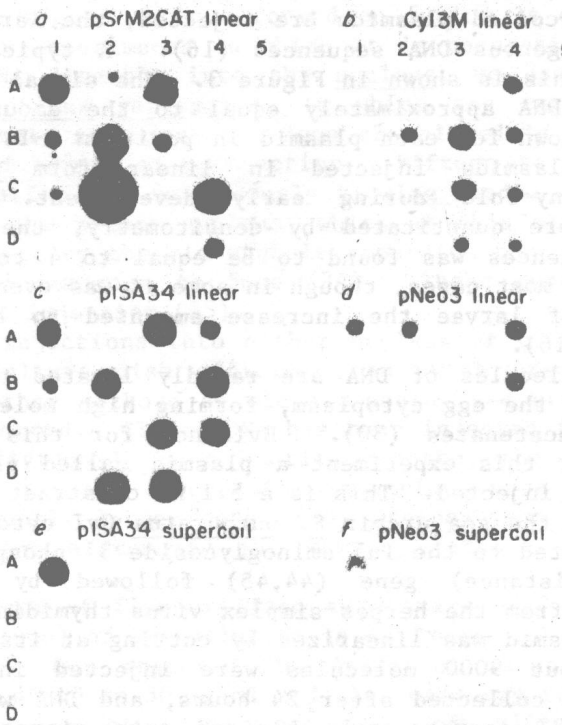

Figure 3. Exogenous DNA sequences in 5-week, 50,000-cell, cultured larvae. The DNA was introduced by injection of the indicated plasmids into the cytoplasm of unfertilized eggs. (a) pSrM2CAT contains the 77 nt tandem repeat from the Moloney murine sarcoma virus, the SV40 early promoter, t splice junction, and poly(A) addition site and the gene coding for the enzyme chloramphenicol acetyl transferase (41). (b) CyI3M consists of a 1.0 kb fragment containing the 3' noncoding and flanking sequences of the sea urchin actin gene, M, isolated from plasmid pSp28, inserted in the 3' noncoding sequence of the actin gene CyI (42). (c) and (e) pISA34 contains a sea urchin repetitive sequence inserted between the EcoRI sites of pISA, replacing some CyI actin promoter sequences (pISA is shown in Figure 4). (d) and (f) pNeo3 contains the Tn5 aminoglycoside 3' phosphotransferase gene (Neo) linked to a promoter and poly(A) addition site from the herpes virus thymidine kinase gene (tk) cloned into pBR322 (B. Wold, personal communication). Column 1 of each panel contains standard quantities of the injected plasmids, which have been linearized and spotted on the nitrocellulose filters: positions A, 1.25×10^6 molecules; B, 2.5×10^5 molecules; C, 5×10^4 molecules; D, 1×10^4 molecules. Columns 2, 3, and 4 contain the DNA of individual larvae grown from injected eggs, and column 5 the DNA of larvae grown from uninjected eggs. The filters were hybridized to appropriate probes, washed, and autoradiographed (from Flytzanis et al., Dev. Biol. 108, 431-442, 1985).

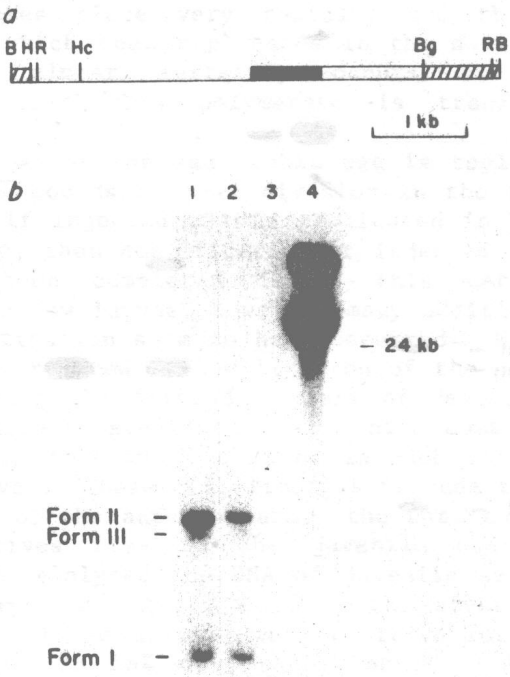

Figure 4. Gel blot analysis of the fate of injected linear DNA molecules during sea urchin embryogenesis. (a) Restriction map of the injected plasmid pISA. Hatched bar, promoter sequences from sea urchin CyI actin gene; open bar, aminoglycoside 3' phosphotransferase gene from the Tn5 transposable element (5' to 3'; R to L); solid bar, poly(A) addition sequences from herpes simplex virus thymidine kinase gene; line, pBR322 sequences. H = HindIII, B = BamHI, Bg = BglII, R = EcoRI, Hc = HincII. (b) DNA gel blot of embryos that developed from eggs injected with 9000 molecules each of the BamHI linearized pISA plasmid. An aminoglycoside phosphotransferase probe was used. Lanes 1 and 2, DNA migration standards representing 3×10^6 and 1.9×10^6 molecules, respectively, of pISA in three conformational states: form 1 (supercoiled), form II (relaxed circular), form III (linear). Lane 3, DNA extracted from 150 uninjected blastulae (24 hr post-fertilization). Lane 4, DNA from 150 injected blastulae (from McMahon et al., Dev. Biol. 108, 420-430, 1985).

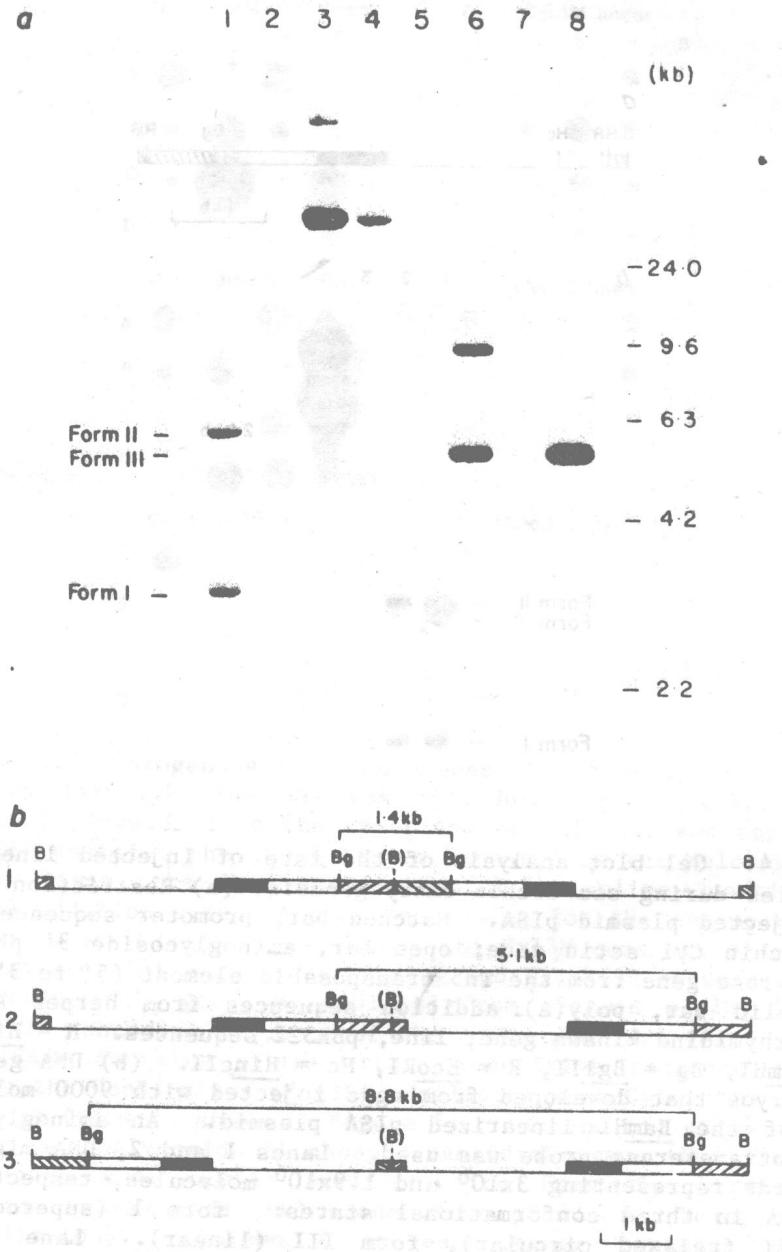

Figure 5. Concatenation of linear DNA molecules in the sea urchin egg. (a) About 9000 molecules of pISA linearized with BamHI or HincII as indicated were injected into eggs. Late gastrulae (48 hr post-injection) were collected and examined by gel blot analysis for the exogenous sequences. Lane 1, 3×10^6 molecules of pISA in the three indicated conformational states. Lane 2,

similar experiment eggs were harvested within 60 minutes of injection, before the first cleavage. When DNA was extracted, run on a gel, blotted, and hybridized, exogenous sequences were detected in a high molecular weight band (30). This indicates that ligation takes place very rapidly, and that it is the concatenated DNA which then replicates in the developing embryo. DNA replication almost certainly occurs in the nuclear compartment, to which DNA polymerase is transported during cleavage.

The genomic DNA of the sea urchin egg is replicated through approximately nine rounds of cell division in the first 24 hours of development. If injected DNA is replicated in synchrony with the nuclear genome, then amplification of injected sequences will have generally been completed during this early period of development. In a few larvae, however, many additional rounds of exogenous DNA replication seem to have occurred. We believe that this difference is related to localization of the concatenates of exogenous DNA during the first few hours of development. It is likely that DNA incorporated into cells which continue to divide after the cleavage stage will be found in much larger quantities in the 5-week larvae. These cell lineages include those that form the oral ectoderm of the anterior arms, the gut, and the imaginal rudiment which gives rise to the juvenile sea urchin after metamorphosis. We analyzed the DNA of juvenile sea urchins that developed from injected eggs, 2 to 3 months after metamorphosis (16). Only 12% of these animals were positive for the exogenous sequences, compared to 58% of 5-week larvae. Apparently the

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undigested DNA from 64 uninjected gastrulae. Lane 3, undigested DNA from 67 gastrulae injected with BamHI linearized pISA. The position of the labeled fragments is the same as that of the total high-molecular-weight embryo DNA observed by ethidium bromide staining (not shown). Lane 4, undigested DNA from 64 gastrulae injected with HincII linearized pISA. Lane 5, BglII digestion of DNA from 55 uninjected gastrulae. Lane 6, BglII digestion of DNA from 55 gastrulae injected with BamHI linearized pISA. Lane 7, BglII digestion of DNA from 55 gastrulae injected with HincII linearized pISA. Lane 8, BamHI digestion of DNA from 55 gastrulae injected with BamHI linearized pISA. Exposure of Kodak XAR5 film was for 72 hr with an intensifying screen. (b) Concatenated forms of pISA that would arise following random end-to-end ligation of molecules linearized at the BamHI sites. The size of BglII fragments expected from the various concatenated forms is shown. 1, head-to-head concatenate; 2, head-to-tail or tail-to-head concatenate; 3, tail-to-tail concatenate. Brackets indicate the size of the predicted fragments that would be released upon BglII digestion. Abbreviations are as noted in the legend to Figure 4a (from McMahon et al., Dev. Biol. 108, 420-430, 1985).