EVOLUTION OF THE SEA URCHIN SPERM PROTEIN BINDIN

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Abstract

The sperm protein bindin is responsible for the species-specific adhesion of the sperm to the egg. The role of bindin in the establishment of reproductive isolation in the species *Strongylocentrotus franciscanus* and *S.purpuratus* is considered. Evolutionary changes in the bindin molecule are described from an analysis of new cDNA sequences obtained from the species *Strongylocentrotus franciscanus* and *Lytechinus variegatus*. These sequences are compared to the previously obtained bindin sequence from *S.purpuratus*. The middle third of the mature bindin sequence is highly conserved in all three species, and the flanking sequences share short repeated sequences that vary in number between the species. This sequence comparison identified the regions of bindin that differ between the species, and that are therefore likely to be responsible for the species-specific properties of bindin.

The regions of the bindin molecule responsible for forming the contact between the sperm and the egg were investigated by assaying the ability of bindin-derived peptides to inhibit fertilization. Twenty-four peptides were studied: seven based on the *Strongylocentrotus purpuratus* bindin sequence, eleven based on the *S.franciscanus* bindin sequence, and six control peptides. Values for IC_{50}, the

concentration of peptide required to inhibit 50% of the productive sperm contacts, were extracted from experimental measurements of the extent of fertilization in the presence of various concentrations of these peptides. The IC_{50} value averaged 220 μM for the control peptides. Five subregions of bindin are represented by peptides that had IC_{50} values less than 20 μM; the most potent peptide (SfO) had an IC_{50} value of 2.2 μM. Peptide SfR, derived from a region of the *S.franciscanus* bindin that differs from the *S.purpuratus* bindin, inhibited fertilization species-specifically. The peptides inhibit fertilization with a steep dose-response relationship, which probably reflects a requirement for the engagement of multiple bindin monomers in the initiation of the sperm-egg bond. These results demonstrate that a few specific regions of the bindin molecule are involved in the sperm-egg contact, and that these regions mediate the species-specificity of the interaction.

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Chapter 1

Introduction

This thesis is an investigation of the basis of the species-specificity of bindin mediated sperm-egg adhesion. This introductory chapter explores the reasons why changes in bindin and its receptor may have played a role in the establishment of reproductive isolation between the species *Strongylocentrotus franciscanus* and *S.purpuratus*.

Speciation and its role in evolution have long been an interesting problem in biology, including for scientists at Caltech. Pioneering work on "the genetics of natural populations" was initiated at Caltech as a collaboration between Th. Dobzhansky, A.H. Sturtevant, and C.C. Tan (Provine, 1981). The general requirement for speciation in evolution was recognized by both Sturtevant and Dobzhansky. Sitting in Kerckhoff in 1938, Sturtevant wrote:

> The problem of the origin of interspecific sterility has long presented the greatest difficulty in the way of constructing a complete theory of the evolution of species.

Dobzhansky, also writing from Kerckhoff (in 1939), was more explicit:

> Two principal components may be recognized in the process of evolution. First, during the course of

> evolution the diversity of organisms is increased. Second, evolution involves the development of discontinuity in the living world, since the organisms become segregated into discrete arrays termed races, species, genera, etc. The increase in diversity is accomplished through the production of new genetic variants, mutations in the broad sense of this word, and is counteracted by heredity which tends to preserve the similarity between succeeding generations. The discontinuity is produced by isolation which hampers or prevents the interbreeding of the members of different discrete arrays. On the race level, the geographical isolation is the most important; on the species and higher levels, various physiological isolating mechanisms become increasingly more and more effective. Isolation has its antithesis in the mechanism of sexual reproduction and Mendelian recombination which tends to reunite the discrete groups into a single variable mass. (Dobzhansky, 1940)

Both Sturtevant and Dobzhansky recognized that at some level speciation requires gene pool isolation. In our current understanding, the concepts of evolution and speciation are so intertwined that they cannot be considered separately. One outcome of the process of evolution is the production of many species starting with only one. It was apparent to biologists studying the morphology of closely

related species that the nested distribution of shared characteristics among such species was best explained by the progressive derivation of species from previously existing species (e.g., Darwin, 1859). Biologists utilizing morphological data were able to construct phylogenetic "trees" that reported the order that the species diverged from one another. Many of these branching patterns have been subsequently confirmed by studies of sequence divergence (both DNA and protein) between the species.

An example of one such branching order, or "cladogram," is given in Figure 1. This cladogram was chosen since it covers the species that are discussed in this thesis. It is based on a cladistic analysis (Hennig, 1966) of morphological data (Smith, 1981, 1984, 1988); some parts of the scheme have been confirmed with molecular data (Hall et al., 1980; Britten et al., 1991). Note that for each species that is in existence today, at some point in the past the progenitors of that species had to "split" from a stock that was then in existence; each such "split" is a speciation event.

Biological species concept

Our concept of what constitutes a "species" has changed as a result of research and thinking about how species are formed and are maintained. Originally, "species" were

viewed just as distinct varieties, usually defined from a description of type specimens. This view extends back at least to Aristotle, who viewed species as "natural kinds." Darwin (1859) followed this view of species and defined species as "a set of individuals closely resembling each other." When a comparison of these "types" led to theories about how the species came to be, a new view of species changed from one of static entities to one of evolving populations of interbreeding individuals. This definition of species came to be known as the "biological species concept": "Species are groups of interbreeding natural populations that are reproductively isolated from other such groups" (Mayr, 1942, 1984). The "biological species concept" has been difficult to apply to some groups of organisms, such as those that breed parthenogenetically, but this is not a problem for the species studied in this thesis. The biological species definition has been useful for understanding species relationships for many animals, including sea urchins. It addresses a key problem raised by the "type" definition of species: How much difference between "types" of organisms should constitute a "species" difference? The biological species definition attempts to remove human bias from the judgment by attempting to ascertain whether the differences accumulated between two groups of animals are sufficient to prevent them from interbreeding. If the differences are too small, interbreeding between the groups will "average" the

differences and separate species will not form. If the differences prevent interbreeding, the groups will remain separate and continue to diverge.

Speciation and evolution

While it is a corollary of the biological species concept that speciation is required for evolution to occur, the biological species concept does not address the issue of to what degree the differences between species are *caused* by speciation. Opinions range from none (e.g., proponents of phyletic gradualism) to all (e.g., Goldschmidt; proponents of punctuated equilibrium).

The proponents of phyletic gradualism develop their views as a result of studies of the currently ongoing action of natural selection on populations. Frequently, a slow shift in morphology is apparent with time (or more commonly, with differences in the environment). This shift can be observed to produce groups of variants, or "races" within the species (cf. quote by Dobzhansky above). Proponents of phyletic gradualism perceive the differences between races and species to be merely matters of degree, and they expect that with time the races will accumulate enough differences to become separate species. Dobzhansky's view (1940) was that "the evidence for continuity between races and species is overwhelming." In this view speciation is merely the end

product of ongoing divergence, and therefore speciation in itself has nothing to do with the differences that accumulate between species. In the view of phyletic gradualism, the role of speciation in evolution is limited to providing a barrier that maintains separate species.

Goldschmidt (1940) took a dissenting view, based on his belief that there is no bridge between the formation of races within a species and the large differences seen between species. Goldschmidt thought the minor changes accumulated between races of the same species were due to "microevolution." He thought the differences between species were qualitatively different and must be due to a fundamentally different process (which he termed "macroevolution"). In his view, speciation is a dramatic and rare event in evolution, and occurs through the mutation of a few key developmental control genes. Goldschmidt viewed the homeotic genes of *Drosophila* as candidates for these developmental control genes. Goldschmidt's "hopeful monster" hypothesis was widely criticized by the phyletic gradualists. Despite the criticism, continued research on homeotic genes has reaffirmed that major alterations in body plan can be produced by changes in a few key developmental genes (e.g., Lewis, 1978; Bender et al., 1983; Bowman et al., 1991).

The main proponents of punctuated equilibrium (Eldridge and Gould, 1972; Gould and Eldridge, 1977; Stanley, 1979;

Fortey, 1988) are paleontologists. Their view of the fossil record is that a "species" is an unchanging entity and that speciations are recorded by abrupt changes in morphology from one form to another. Other views similar to those of Goldschmidt and the proponents of punctuated equilibrium have been expressed (e.g., "saltatory evolution"). All of these proposals view speciations as dramatic events in the history of populations, and consider speciation to be intimately linked with the morphological differences between species.

Modes of speciation

Views on how speciation is accomplished are linked to the above mentioned views on the degree of divergence that accompanies speciation. The dominant view, at least for biologists studying land animals, is that most speciation occurs in allopatry - i.e., speciation occurs in geographically separated populations (Dobzhansky, 1940; Muller, 1940). Phyletic gradualism and allopatric speciation are usually linked concepts stating the same idea: speciation is the end result of a long period of natural selection acting differentially on geographically separated populations.

Modified versions of allopatric speciation have been proposed that would shorten the time required for the divergence of the species. Mayr's theory of genetic

revolutions (1963) assumes that speciation is usually initiated in a completely isolated population, but also assumes the process is aided by a temporary bottleneck in population size with its associated founder effects (Mayr calls this "peripatric" speciation). These founder effects are described as resulting from the statistical selection of an "incompatible" combination of gene alleles that would be rare in the parental population. The result would be a transition from "one well-integrated and stable condition through a highly unstable period to another period of balanced integration."

Various other schemes that involve varying degrees of isolation with varying degrees of reinforcement from genetic factors have been proposed by population geneticists. These vary from allopatric (two large isolated populations) to parapatric (partial contact between largely isolated populations) to sympatric (derivation of one species from within the range of the parent species, e.g., Smith, 1966). Several of these schemes are reviewed by Barton and Charlesworth (1984).

Sympatric speciation requires the rapid acquisition of reproductive isolation. In the absence of this isolation, the "incipient species" would rapidly be bred into oblivion by competition from the more numerous surrounding parental species ("genetic swamping," the sometimes successful method used to control the medfly). Several mechanisms that might

provide for rapid reproductive isolation have been discovered. The first was genetic incompatibility through polyploidization (for a review see Dobzhansky, 1951, 1970). Another was genetic incompatibility through polymorphisms in chromosome translocations and inversions (e.g., Sturtevant and Beadle, 1936; Sturtevant and Tan, 1937; Sturtevant, 1938; White, 1968). It has also been suggested that polymorphism for a transposable element capable of producing a high degree of hybrid dysgenesis, such as the *Drosophila* P-element, might be sufficient to induce reproductive isolation (Hagiwara et al., 1987; Kidwell et al., 1988; Kidwell, 1990). The general relevance of these schemes to strictly sympatric speciation is questionable, since these mechanisms do not prevent cross-mating. As is the case for the medfly program, the prevention of the development of fertile hybrids is insufficient to keep the smaller population from spending its gametic output in matings with the more numerous population (and therefore not making crosses that propagate the population). The conclusion reached by most is that sympatric speciation in the strict sense is very unlikely to occur.

Sea urchin reproductive ecology

Virtually all of the foregoing discussion has derived from observations of land species. The idea that this might give a biased view inappropriate to marine organisms has

been expressed by many, including Mayr (1954):

> The great importance of ecological factors in speciation is stressed by most recent students. Since the ecology of marine organisms is fundamentally different from that of such typical land animals as mammals, birds, land snails, and butterflies, one might expect modes of speciation in the oceans that differ completely from the typical geographic speciation of land animals. This possibility has been suggested repeatedly within recent years; yet a thorough study of speciation patterns in marine organisms has remained a desideratum.

The ecological differences referred to include the external fertilization and planktonic larvae utilized by many marine invertebrates. Most sea urchins release their gametes into open water where fertilization takes place, which eliminates most of the potential for courtship behaviors as a means of reproductively isolating species. Most sea urchins also have planktonic larvae (plutei) that swim for several weeks in the water column, which creates the potential for wide dispersal. Strathmann (1978) observed the time it took for the plutei of five species of sea urchins (one of which was *S.franciscanus*) to settle out of larval culture (measured from the time of fertilization). The minimum larval period (the time at which the first plutei settled) ranged from 51 to 141 days for the five

species (*S.franciscanus* = 62 days). The maximum larval period (the time at which the last plutei were observed to settle and undergo metamorphosis) averged over five months (173±35 days; *S.franciscanus* = 131 days). This lengthy larval period gives sea urchins a potential for wide dispersal, greatly reducing the probability of the formation of geographically isolated populations.

There are several indications that sea urchin species (and *S.purpuratus* in particular) are composed of large, well-mixed populations. The current population size of *S.purpuratus* is estimated to be 15 billion within the kelp beds alone (Palumbi and Wilson, 1990). The interindividual single copy DNA (scDNA) polymorphism is 4% for *S.purpuratus* (Britten et al., 1978). By comparison, the intraspecific scDNA polymorphism of both *Drosophila melanogaster* and *D.simulans* is less than 0.5% even when individuals from different continents are compared (Caccone et al., 1988). The high degree of sequence polymorphism in *S.purpuratus* is interpreted as the accumulation of many neutral (or nearly neutral) changes within a large population over a long time interval. The equally high polymorphism seen when comparing the two haploid genomes of a given individual to genomes separated by the 1,900 km between southern California and British Columbia (Britten et al., 1978) suggests that the sequence changes are mixed well over the entire population. The single copy DNA polymorphism of *S.franciscanus* is also

high at 3.2% (Grula et al., 1982). Palumbi and Wilson (1990) studied RFLPs of the mitochondrial DNA of *Strongylocentrotus droebachiensis* and *S.purpuratus* and were also unable to find any indication of population subdivision among the Pacific populations of both species (although there were differences between Atlantic and Pacific *S.droebachiensis*). This lack of population divergence was also seen in a study (A. Cameron and J. Minor, unpublished observation) of the RFLPs of bindin and actin genes from *S.purpuratus* individuals from Laguna Beach, Ca., Diablo Canyon, Ca., and Friday Harbor, Wa. This study also failed to detect any population subdivisions within *S.purpuratus*.

The population variation of sea urchins has also been studied by allozyme variation (Marcus, 1977; Lessios, 1979, 1981; Stickle et al., 1990). Marcus (1977) detected differences in allozyme frequency between populations of *Arbacia punctulata* from Woods Hole, Mass. and Beaufort, North Carolina; but failed to detect any significant population difference between *A.punctulata* from Beaufort and Panama City, Florida (in the Gulf of Mexico). The allozyme population difference in *A.punctulata* is exceptional for sea urchins. Lessios (1981) reported no significant intraspecific populational differences in the allozymes of seven species of echinoids (four species from the Pacific, three species from the Caribbean). Stickle et al. (1990) reported no population differences for *S.droebachiensis* from

Washington and Alaska (700 miles apart). The general result from studies of single copy DNA, mitochondrial DNA RFLPs, nuclear DNA RFLPs, and allozymes is that population subdivision among populations of sea urchins is rare. These results are in contrast to the situation in land animals, where the lower degrees of polymorphism are usually structured as a graded distribution, or "cline," across the entire range. In land animals, animals from the same locality have fewer polymorphisms than ones from the ends of the range (Avise et al., 1987; Moritz et al., 1987). This difference is an indication that speciation by geographic isolation of populations of sea urchins is much less likely than for land animals.

The species *S.purpuratus* and *S.franciscanus* are also well mixed with each other. The ranges of these two species overlap from Alaska to Baja California (Boolootian, 1966; Harrold and Pearse, 1987). Within this area, the species are intermixed both intertidally and subtidally (Tegner and Dayton, 1981). Tegner and Dayton (1981) even suggest that the survival of small post-metamorphic *S.purpuratus* is enhanced when they are able to shelter underneath *S.franciscanus* individuals.

The reproductive seasons of both species overlap extensively as well (Boolootian, 1966). In addition to this apparent lack of geographical or temporal isolation among breeding populations of *S.purpuratus* and *S.franciscanus*,

these species also appear to lack courtship behaviors that could provide an alternative means of reproductive isolation. Sea urchin fertilization is external, and there is no evidence that these two species segregate before spawning (Reese, 1966; Pennington, 1985; see also Dix, 1969). While both *S.purpuratus* and *S.franciscanus* do occasionally migrate, the observed migrations are in search of food and consist of mixtures of both species (Boolootian, 1966; Reese, 1966; Harrold and Pearse, 1987). There is evidence that sea urchins contain substances that induce spawning (e.g., Fox, 1924; Lewis, 1958; Cochran and Engelmann, 1976). While there is the possibility that sea urchins could spawn in response to species-specific signals from the the opposite sex, the induction of spawning by extracts from tissues (e.g., gonadal extracts) is not species-specific (Palmer, 1937). For the species *S.purpuratus* and *S.franciscanus*, the spawning response has been reported as either not species-specific or even not to occur at all (reviewed in Pennington, 1985). In southern California, *S.purpuratus* and *S.franciscanus* appear to spawn simultaneously in response to winter storms (P. Leahy, personal communication).

The production of interspecies hybrids of *S.purpuratus* and *S.franciscanus* has a long history (e.g., Hagedorn, 1909; Loeb et al., 1910; Loeb, 1915; Lillie, 1921; Newman, 1923; Harvey, 1942; Moore, 1943; Chaffee and Mazia, 1963). In

most of these studies, a low yield of hybrids can be obtained by adding excess sperm (see data in Lillie, 1921; and in chapter 3). We (A. Cameron and J. Minor, unpublished observation) have repeated this observation, confirmed that the offspring were hybrids by using an M-actin RFLP polymorphism, and have grown the hybrids through metamorphosis to the feeding juvenile sea urchin stage. There are also reports of naturally occurring interspecies hybrids (Swan, 1953), but these are rare and have not been confirmed with molecular markers. The standard chromosomal karyotypes of *S.purpuratus* and *S.franciscanus* are both 2n=42 (Gerhart, 1983).

Thus it is clear that the evolution of reproductive isolation in sea urchins is unlikely to occur as a result of the factors that usually produce the isolation of incipient species founder groups in land animals. The mixing in the water column of the gametes of many externally fertilizing species of marine invertebrates is a general occurrence (Pearse et al., 1988). A notable example is the synchronous spawning of 103 species of scleractinian coral on the Great Barrier Reef (Babcock et al., 1986). The prime candidate that remains for the establishment of reproductive isolation for the *Strongylocentrotus* species is the species-specificity of gamete interaction.

Successive events in sea urchin fertilization

The structure of the gametes and the steps required for fertilization in sea urchins have been reviewed extensively (e.g., Epel, 1977, 1978; Vacquier, 1980, 1986; Rossignol et al., 1984a) and are briefly summarized here.

Sperm. All echinoid sperm have a conical head that in the *Strongylocentrotus* species measures 5 μM by 2 μM (Chia et al., 1975). The sperm head (from posterior to anterior) contains the mitochondrion, the nucleus, the apical nuclear indentation (a G-actin filled pit on the anterior end of the nucleus), and the acrosomal vesicle.

Eggs. The eggs of *S.purpuratus* and *S.franciscanus* are also very similar in composition, but the *S.franciscanus* egg is larger (egg diameter from plasma membrane to plasma membrane: 80 μm for the *S.purpuratus* egg, about 140 μm for the *S.franciscanus* egg). At the surface of both eggs are several layers, which are (from the inside out): the cortical layer (which contains many vesicles located just under the plasma membrane), the egg plasma membrane, the vitelline layer, and the egg jelly layer. The vitelline layer is a complex of least 25 proteins and glycoproteins that coats the outer surface of the egg plasma membrane (Niman et al., 1984). In electron micrographs, it appears to rest on the egg membrane like a layer of straw (Kidd, 1978; Kidd and Mazia, 1980). The egg jelly layer is a diffuse, hydrated layer composed of protein, fucose sulfate,

and sialic acid (SeGall and Lennarz, 1979; Mikami-Takei, 1991). The jelly layer varies in size, but typically extends about 40 μM out from the plasma membrane.

Sperm activation. Sea urchin sperm are stored in a quiescent and immotile state in the testes. Upon spawning, the sperm are diluted into seawater and become motile due to the lower CO_2 concentration (and higher pH) of seawater (reviewed in Trimmer and Vacquier, 1986; Brokaw, 1990).

Sperm chemotaxis. When the sperm are in the vicinity of the egg, they may come into contact with small peptides that diffuse out from the egg jelly layer. These peptides are bound by specific receptors on the sperm (reviewed in Garbers, 1989; see also Schulz et al., 1991). There are many forms of these peptides, and they seem to further activate the respiration and swimming of sperm. One of these peptides, *resact*, is derived from *Arbacia punctulata* eggs and is a chemoattractant for *A.punctulata* sperm (Ward et al., 1985). It is possible that the sperm swim up the concentration gradient of such peptides to locate the egg.

Acrosome reaction. When the sperm come into contact with the egg jelly layer, a fucose sulfate glycoconjugate induces the sperm to undergo the acrosome reaction (SeGall and Lennarz, 1979; Mikami-Takei et al., 1991). The acrosomal vesicle undergoes exocytosis, exposing its contents: the acrosomal granule (or "globule," Afzelius and Murray, 1957).

The granule is pushed forward by the extension of the acrosomal process, which is caused by the polymerization of the G-actin stored in the apical nuclear indentation.

Sperm binding. The major constituent of the acrosomal granule is the protein *bindin* (Vacquier and Moy, 1977). An early study of spermatozoa (Popa, 1927) hinted at the function of bindin by saying that the acrosome reaction caused the sperm tip to become sticky: "Fresh preparations of spermatozoa of *Arbacia* in egg-water also show quite clearly the elimination of a substance through the pointed apex of the spermatozoon. This appears as a small granule on the points of almost all spermatozoa. The adhesion of the spermatozoa to one another or to other objects is made by means of this granule." The bindin exposed by the acrosome reaction is responsible for the adherence of sperm to the egg vitelline layer, where bindin interacts with its receptor. The bindin receptor of *S.purpuratus* is a large glycoprotein complex located in the egg vitelline layer-plasma membrane complex (reviewed in Ruiz-Bravo and Lennarz, 1989; see also chapter 2). The species-specificity of the receptor requires both carbohydrate and protein. Bindin is reviewed in depth in chapter 2.

Egg activation. About 3 seconds after sperm attachment, a change in the membrane potential of the egg is induced (Epel, 1977; Schatten and Hulser, 1983). Five seconds after the potential change, a wave of increased free Ca^{+2} spreads

around the egg from the site of sperm attachment. The sperm becomes immotile and begins to be incorporated 16 seconds after attachment. There is circumstantial evidence that suggests that the sperm-egg membrane fusion might also be mediated by bindin (Glabe, 1985ab; Kennedy et al., 1989). Sixteen seconds after the beginning of the calcium wave, the calcium induces the exocytosis of the cortical granules, which in turn causes the elevation of the fertilization membrane (reviewed in Vacquier, 1981). The fertilization membrane is formed from the elevation and cross linking of the vitelline layer by enzymes released from the cortical granules (Shapiro, 1991), and the elevation is completed by one minute after sperm attachment. The fertilized eggs then proceed with cleavage and hatch at the swimming blastula stage 18 hours later.

Species-specificity of fertilization

The cause of the species-specificity of fertilization in sea urchins depends on the species that are compared. In this section the possible causes of species-specificity in sea urchin fertilization are reviewed, with particular reference to the species *S.purpuratus* and *S.franciscanus*. All of the species-specificities observed are due to a failure in at least one of three steps. These steps are, in order of occurrence, sperm chemotaxis to the egg, induction of the acrosome reaction, and adhesion of the sperm to the

egg vitelline layer. It is this last step that is mediated by bindin. In a study of cross fertilization between four sympatric species of sea urchins from Bermuda, Summers and Hylander (1975) concluded that in the majority of cases (9 out of 11) the failure of cross fertilization was due to a failure of sperm to adhere to the egg vitelline layer. The remaining two of eleven cases were due to a failure of the acrosome reaction. It is also possible that species differences could exist in egg chemotactic molecules which would result in species-specificities in fertilization.

Egg-derived peptides have been described as "species-specific" activators of sperm respiration and chemotaxis. *Resact*, a peptide derived from the egg jelly of *Arbacia punctulata*, is a chemoattractant for *A.punctulata* sperm (Ward et al., 1985). Resact is not a chemoattractant for *S.purpuratus* sperm, but chemotaxis has not been demonstrated for *S.purpuratus* sperm. The most prevalent peptide from *S.purpuratus* egg jelly, *speract*, fails to act as a chemoattractant for either *S.purpuratus* or *A.punctulata* sperm (Ward et al., 1985). The ability of egg peptides to activate respiration demonstrates more reciprocity: speract activates the respiration of *S.purpuratus* sperm (and not *A.punctulata* sperm) and resact activates the respiration of *A.punctulata* sperm (and not *S.purpuratus* sperm). However, this activation can only be demonstrated at non-physiological pH.

These data should not be interpreted to mean that egg chemotactic peptides are a general cause of species-specificities in fertilization. There are a number of reasons why this is the case, especially for the species *S.purpuratus* and *S.franciscanus*. Egg peptides and their effects tend to be conserved among closely related species. The one difference in the effect of an egg peptide that is observed is between the distantly related species *A.punctulata* and *S.purpuratus* (divergence time: ~200 Mya compared with ~35 Mya for the *Strongylocentrotus-Lytechinus* divergence and ~10 Mya for the *S.purpuratus-S.franciscanus* divergence; see figure 1). Hence, speract (the prevalent peptide from *S.purpuratus* eggs) activates the respiration of both *S.purpuratus* and *Lytechinus pictus* sperm (Bentley and Garbers, 1986). Speract and related peptides are shared among many species, including *S.purpuratus*, *S.nudus*, *Hemicentrotus pulcherimus*, and *Tripneustes gratilla* (Suzuki et al., 1988; Yoshino et al., 1989). Yoshino et al. (1989) showed that 28 speract-related peptides from five different species (from three different families) showed no species preference since all activated the respiration of *Hemicentrotus pulcherimus* sperm. This lack of species-specificity in the activation of sperm respiration is not limited to purified peptides: egg extracts and egg jelly extracts from five species (from three different families) also all activated the respiration of *Hemicentrotus pulcherimus* sperm (Suzuki et al., 1982).

Due to the general conservation of speract-related egg peptides and the general lack of specificity of their effects, it is unlikely that egg peptides play a role in the species-specificity of fertilization between the species *S.purpuratus* and *S.franciscanus*. This conclusion is in agreement with the experimental results of Loeb (1915): the eggs (or egg conditioned waters) of both species activate the motility of the sperm of the other species. In the present work the possibility that the species-specificity of fertilization was due to a failure of chemotaxis resulting in differing numbers of sperm reaching the eggs was excluded by counting the number of sperm reaching the eggs in fertilizations and cross fertilizations (see chapter 3). There were no significant differences in the numbers of sperm per egg, which indicates no cross-species incompatibilities in the overall ability of sperm to locate the eggs.

As mentioned above, the work of Summers and Hylander (1975) showed that the acrosome reaction is usually nonspecific and rarely accounts for the species-specificity of fertilization. The acrosome reaction of *S.purpuratus* and *S.franciscanus* sperm has been studied in the laboratories of V. Vacquier and C. Glabe. Both laboratories found no species-specificity in the induction of the acrosome reaction between the species *S.purpuratus* and *S.franciscanus* (B. Brandriff and V. Vacquier, personal communication; A.

Lopez and C. Glabe, personal communication).

In contrast to the data that fail to show any species-specificity in chemotaxis or the acrosome reaction between the species *S.purpuratus* and *S.franciscanus*, species-specificity has been demonstrated for both bindin and its receptor for these species. Glabe and Vacquier (1977) demonstrated the specificity of bindin using an egg agglutination test, in which bindin is mixed with eggs and the degree to which bindin causes the eggs to stick together is assayed (see chapter 2). *S.purpuratus* bindin agglutinates *S.purpuratus* eggs and *S.franciscanus* bindin agglutinates *S.franciscanus* eggs, while neither bindin agglutinates the eggs of the other species. This shows the specificity of bindin and implies that there is a specific receptor for it on the egg. Glabe and Vacquier (1978) demonstrated the presence of a species-specific *S.purpuratus* bindin receptor by showing that an excess of a *S.purpuratus* receptor preparation inhibited the interaction of *S.purpuratus* bindin with the *S.purpuratus* receptor preparation (the *S.franciscanus* preparation was ineffective in inhibiting the interaction). Recently, Foltz and Lennarz (1990) have shown that a 70 kDa proteolytic fragment of the *S.purpuratus* bindin receptor retains the ability both to species-specifically interact with bindin and to species-specifically inhibit fertilization.

The foregoing discussion indicates that the species-

specificity of fertilization between the species *S.purpuratus* and *S.franciscanus* is due to a failure of the bindin of one species to recognize the receptor of the other species. The specificity of both bindin and its receptor has been demonstrated directly. In addition, the other fertilization steps that cause species-specificities in other sea urchin species are known to be compatible in crosses between *S.purpuratus* and *S.franciscanus*.

Organization of thesis

This chapter (chapter 1) discusses the importance of speciation in evolution and the importance of reproductive isolation for speciation. The aspects of the biology of *S.purpuratus* and *S.franciscanus* that indicate that the specificity of gamete interaction is likely to be the main factor in the reproductive isolation in these species is discussed. The steps leading to fertilization are discussed, and the evidence that the specificity of fertilization is due to bindin and its receptor is reviewed.

Chapter 2 is a review of what was known about bindin prior to the start of this thesis work. Chapter 2 has been published: Minor et al., 1989.

Chapter 3 reports bindin sequences for the species *Strongylocentrotus franciscanus* and *Lytechinus variegatus*. It compares them to the previously characterized

S.purpuratus sequence, and determines the degree of fertilization specificity between these species. Chapter 3 is in press: Minor et al., 1991a.

Chapter 4 describes the localization of the regions of the bindin molecule involved in the sperm-egg contact, and in the species-specificity of this contact. Chapter 4 is being prepared for submission: Minor et al., 1991b.

Chapter 5 summarizes what is now known about bindin, both from my own work and from the work of others done during the course of this thesis. The relevance of these data to our understanding of speciation is discussed, and directions for future research are considered.

The appendices include other published work on sea urchin genomes and on bindin molecular biology in which I have participated:

Appendix A describes a study of sea urchin actin gene linkage utilizing genomic RFLP polymorphisms. Appendix A has been published: Minor et al., 1987.

Appendix B is a study of the accumulation of bindin transcripts in developing sea urchin testes. Appendix B has been published: Cameron et al., 1990.

Appendix C is a study of the transcriptional and translational control of bindin by in situ hybridization to adult testes. Appendix C has been published: Nishioka et

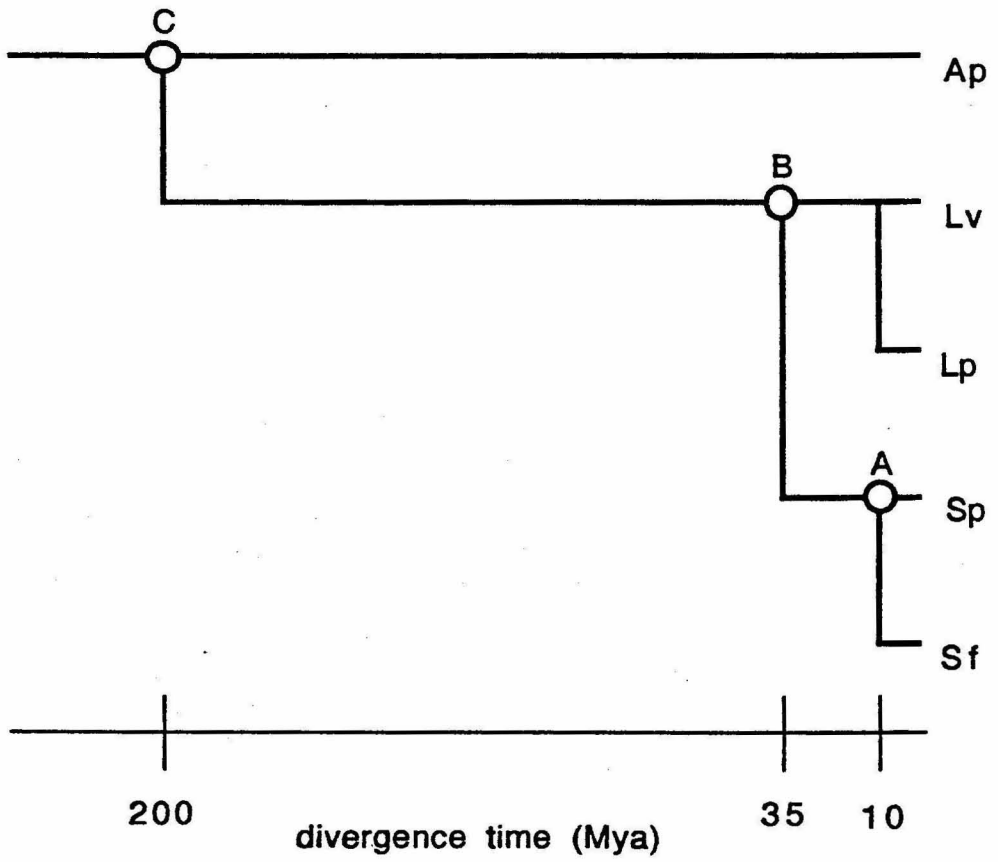
al., 1990.

REFERENCES

References for chapter 1 are included with the references at the end of chapter 5.

Figure 1. Evolutionary relationships among some sea urchin species. The species diagrammed are: Ap, *Arbacia punctulata*; Lv, *Lytechinus variegatus*; Lp, *L.pictus*; Sp, *Strongylocentrotus purpuratus*; and Sf, *S.franciscanus*. The phylogenetic relationships are from Smith (1981, 1984, 1988). Smith's analysis is based on a combination of a cladistic analysis of the morphology of extant species and an examination of the fossil record. The divergences marked on the horizontal axis (A, B, and C) are drawn proportional to divergence time, the separations on the vertical axis are arbitrary. The divergence times (relative to *S.purpuratus*) are: A, 3.5-20 million years ago (Mya); B, 30-40 Mya; and C, ~200 Mya. Total single copy DNA divergences (median values from Hall et al., 1980 and Britten et al., 1991) are: A, 19%; and B, 31% (between *S.purpuratus* and *Lytechinus pictus*).

Figure 1



Chapter 2

4

The Molecular Biology of Bindin

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I. INTRODUCTION

Fertilization includes a complex cell recognition event. In sea urchins, in which fertilization is external, successful fertilization depends on the sperm and egg recognizing one another among all of the other cells present in the seawater and avoiding the eggs and sperm of closely related species. It is not surprising, therefore, that there are many steps involved in this recognition phenomenon. The sea urchin fertilization pathway is a well-studied system and many of the steps have been identified and characterized at the molecular level.

Both the male and female sea urchins release their gametes in open seawater. Dilution of the sperm into seawater raises the intracellular pH of the sperm, resulting in an activation of respiration and motility (reviewed in Trimmer and Vacquier, 1986). When the swimming sperm approach the egg, they come

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into contact with a hydrated extracellular matrix known as the jelly layer. Small peptides from this layer induce increases in respiration and motility (see Chapter 1 of this volume). Resact, the egg jelly layer peptide from the sea urchin *Arbacia*, seems to also be an egg chemoattractant for sperm (Ward *et al.*, 1985). These peptides are bound by receptors on the surface of sperm (Farrell *et al.*, 1978). Another egg jelly constituent, a fucose-sulfate-rich glycoconjugate, induces the acrosome reaction (SeGall and Lennarz, 1979). The acrosome reaction is the exocytosis of the acrosome vesicle and the extension of the acrosome process. The major constituent of the acrosome vesicle is the acrosome granule, a large particle composed mainly of the protein bindin. The fingerlike acrosome process is extended from the sperm head by the polymerization of actin. After the exocytosis of the acrosomal vesicle, bindin coats the surface of the extending acrosomal process. It is this bindin on the acrosomal process that is responsible for the species-specific adhesion of the sperm to the egg. This chapter reviews the characterization and molecular cloning of bindin, the first protein involved in cell recognition at fertilization to be cloned at the molecular level (Gao *et al.*, 1986).

Bindin was originally isolated as the particulate contents ("granule") of the sea urchin sperm acrosome (Vacquier and Moy, 1977). The isolation involved disrupting sea urchin *(Strongylocentrotus purpuratus)* sperm in a detergent buffer, and then isolating the detached acrosomal vesicles by filtering the supernatant from a low-speed centrifugation through a glass fiber column. The column passed the acrosomal granules (about 0.2 μm in diameter) but trapped sperm heads and tails. The resulting preparation was greater than 95% pure bindin. An even purer preparation of bindin can be obtained by solubilizing the acrosomal granule protein in urea, and chromatography of the solution on DEAE-cellulose. Bindin elutes in 4 *M* urea and 650 m*M* sodium phosphate, pH 6.6 (Vacquier, 1983).

Several reviews on bindin have been published (e.g., Vacquier and Moy, 1978; Vacquier, 1980; Glabe *et al.*, 1982a; Rossignol *et al.*, 1984a) so only some of the more recent experiments are reviewed in any detail here. The apparent molecular weight of the purified bindin by SDS–polyacrylamide gel electrophoresis is 30,500 (Vacquier and Moy, 1977), but HPLC measurements indicate a size of 25,000 (T. Sasagawa and K. Walsh, personal communication). Bindin appears to be 100% protein and does not stain for carbohydrate by either phenol–sulfuric acid or anthrone (Vacquier and Moy, 1977). The evidence that bindin is the mediator of sperm–egg adhesion comes from two lines of evidence. First, bindin is localized to the site of sperm–egg attachment by antibindin antibodies (Moy and Vacquier, 1979; see Fig. 1). Second, purified bindin agglutinates sea urchin eggs in a species-specific manner (Glabe and Vacquier, 1977; Glabe and Lennarz, 1979). This interaction is reciprocal: *S. purpuratus* bindin agglutinates only *S. purpuratus* eggs (and not *Strongylo-*

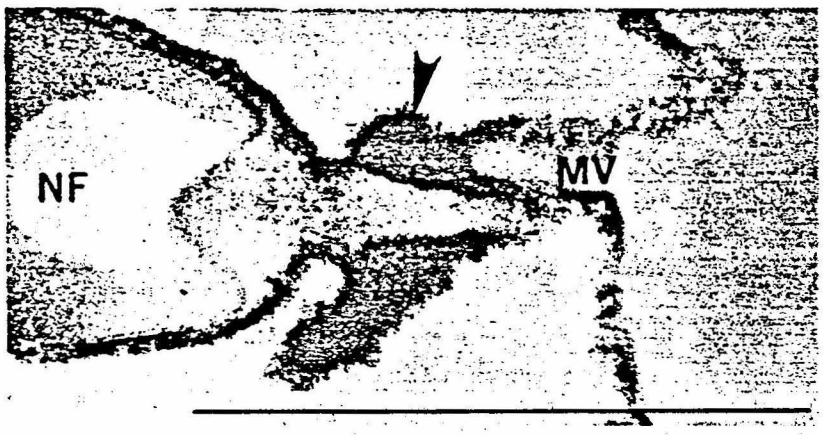
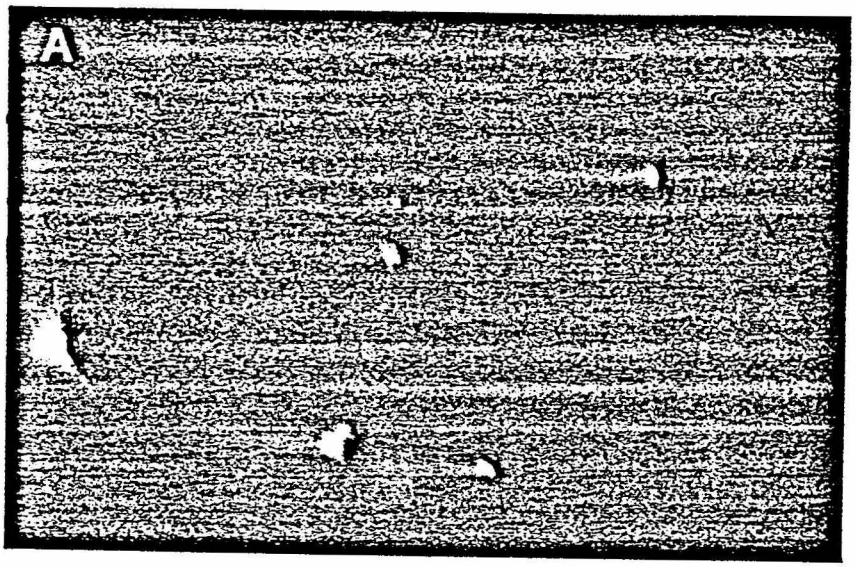
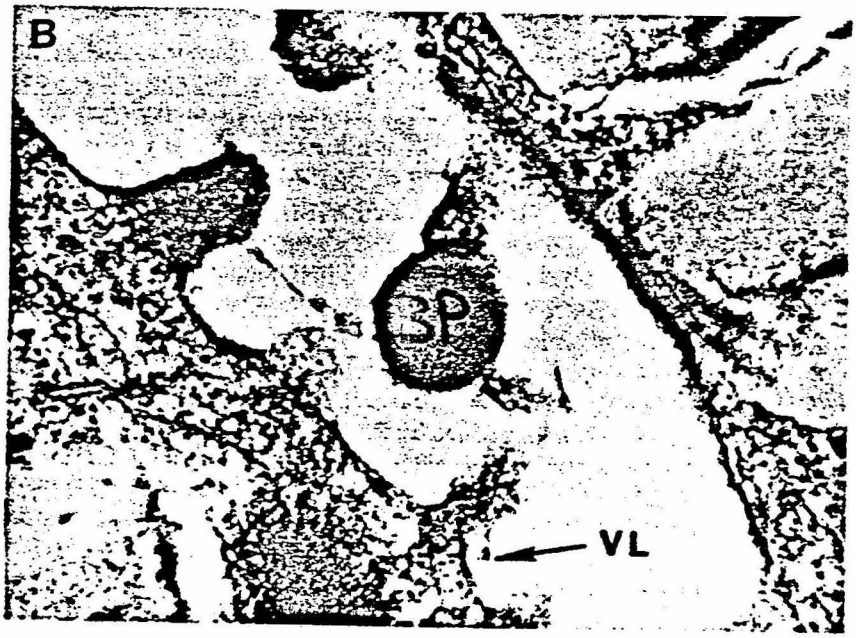


Fig. 1. Immunocytological localization of bindin at the sperm-to-egg bond. The preparation was reacted with a polyclonal antibody against bindin, and then with a second antibody against the first coupled to horseradish peroxidase. The arrow points to the dark precipitate formed by the horseradish peroxidase enzyme activity. Bindin is localized to the area between the sperm acrosomal process and the egg microvillus (MV). NF, Nuclear fossa, the area in the sperm head emptied by the exocytosis of the acrosomal vesicle. Bar, 1 μm. [From Moy, G. W., and Vacquier, V. D. (1979). *Topics Dev. Biol.* 13, 31–44.]

centrotus franciscanus eggs), whereas *S. franciscanus* bindin agglutinates *S. franciscanus* eggs but not *S. purpuratus* eggs. Furthermore, the contacts between eggs in these species-specific agglutinates invariably contain bindin (Glabe and Lennarz, 1979; see Fig. 2).

The species specificity of bindin implies that there is a receptor for it on the surface of the egg. Crude preparations of this receptor show species-specific binding to bindin (Glabe and Vacquier, 1978; Rossignol *et al.*, 1981). In contrast to bindin, the bindin receptor appears to be a high-molecular-weight proteoglycan-like molecule (Glabe and Vacquier, 1978; Glabe and Lennarz, 1981). Extensive proteolytic digestion of receptor preparations release carbohydrate-rich fragments that inhibit fertilization, but not species specifically (Rossignol *et al.*, 1984b). This suggests that the species specificity of the bindin–receptor interaction is due to structure provided by the protein, and/or by the interaction of many bindin–receptor units arranged in an oriented fashion on the surfaces of the gametes (Glabe, 1978). Further attempts to isolate species-specific bindin receptors have been made by chromatography of the products of limited proteolysis of the egg surface (Ruiz-Bravo and Lennarz, 1986), and by raising antibodies to elevated vitelline envelopes isolated in the presence of soybean trypsin inhibitor (Acevedo-Duncan and Carroll, 1986). The bindin receptor is discussed in Chapter 2 of this volume.


A


B
3P
VL

Studies on purified bindin have focused on its ability to bind sugars and on its interaction with membranes. Bindin reacts strongly with fucans (polymeric fucose sulfate esters) from egg jelly in salt concentrations equivalent to seawater. Although it binds other fucose sulfate esters (e.g., fucoidan) with high affinity as well, bindin has lower affinity for other sulfated polysaccharides (e.g., lambda carrageenan, a sulfated galactan). Despite the lectin-like properties of bindin (Glabe *et al.*, 1982b), the lower affinity of bindin for desulfated fucans and high affinity for polyvinyl sulfate indicate the sulfates are as or more important than the saccharide backbone for binding (DeAngelis and Glabe, 1987). The affinity of bindin for sulfated fucans drops dramatically as the fucan chain length decreases from 60 to 20 saccharide residues. Since polysaccharide chains in this size range are longer than the diameter of a globular protein of bindin's molecular weight, it is possible this result indicates that bindin functions better in multimeric forms. Bindin has also been shown to integrate into phospholipid vesicles in the gel phase (Glabe, 1985a) and to induce membrane fusion of mixed-phase vesicles (Glabe, 1985b). These studies suggest that bindin may participate in membrane fusion as well as in sperm–egg adhesion.

Polyclonal antibodies to bindin have been used to judge the phylogenetic conservation of bindin. Although these antibodies react with the acrosome-exposed sperm of echinoids (sea urchins and sand dollars), they do not react with other members of the phylum Echinodermata (starfish) or with several other phyla (Annelida, Mollusca, or Chordata) (Moy and Vacquier, 1979). Fragments of the bindin protein from two species of sea urchins (*S. purpuratus* and *S. franciscanus*) have been sequenced (Vacquier and Moy, 1978; T. Sasagawa and K. A. Walsh, personal communication). Analysis of the N-terminal sequences (see Fig. 3) indicates that these sequences are similar up to amino acid 40, after which the *S. franciscanus* bindin contains two more repeats of amino acids 31–40, whereas the *S. purpuratus* bindin does not.

Fig. 2. (A) Fluorescence micrograph of *Strongylocentrotus purpuratus* eggs agglutinated by fluorescein-labeled bindin particles. Purified bindin was conjugated to fluorescein *in vitro* and mixed with unfertilized eggs. The agglutinated eggs were viewed with a fluorescence microscope. Bindin is invariably found at the egg–egg contacts. Photo courtesy of Charles Glabe. (B) Electron micrograph of a bindin particle at an egg–egg bond. Purified bindin was added to unfertilized eggs and the resulting preparations were viewed in the electron microscope. A bindin particle (BP) is seen between two eggs (VL, vitelline layer of lower egg). Several egg microvilli can be seen in contact with the bindin particle. [From Glabe, C. G., Lennarz, W. J., and Vacquier, V.D. (1982a). Sperm surface components involved in sea urchin fertilization. *In* "Cellular Recognition" (W. A. Frazier, L. Glaser, and D. I. Gottlieb, eds.), pp. 821–832. Liss, New York.]

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Sp 1   TyrValAsnThrMetGlyTyrProGlnAlaMetSerProGlnMetGlyGlyValAsnTyrGlyGlnProAlaGln
Sf      - Gln - GlnGlyAsn - - - - - - Asn - - Ser - - - - - - - - - - - - - -

Sp 26  GlnGlyTyrGlyAlaGlnGlyMetGlyGlyProValGlyGlyGlyProMetGlyGlyProProGlnPheGlyAla
Sf      - - - - - - [- - - - - - AlaPhe - - -][GlnGlyMet - GlyAlaVal * - Gly]

Sp 51  LeuProProGlyGlnAlaAspThrAspPheGlySerSerSerSerSerValAspGlyGlyAspThrThr
Sf     [GlnGlyMet - Gly - ValGlyGlyGly] * Phe * AlaPhe - ProGlyGluAlaGluAlaAsp
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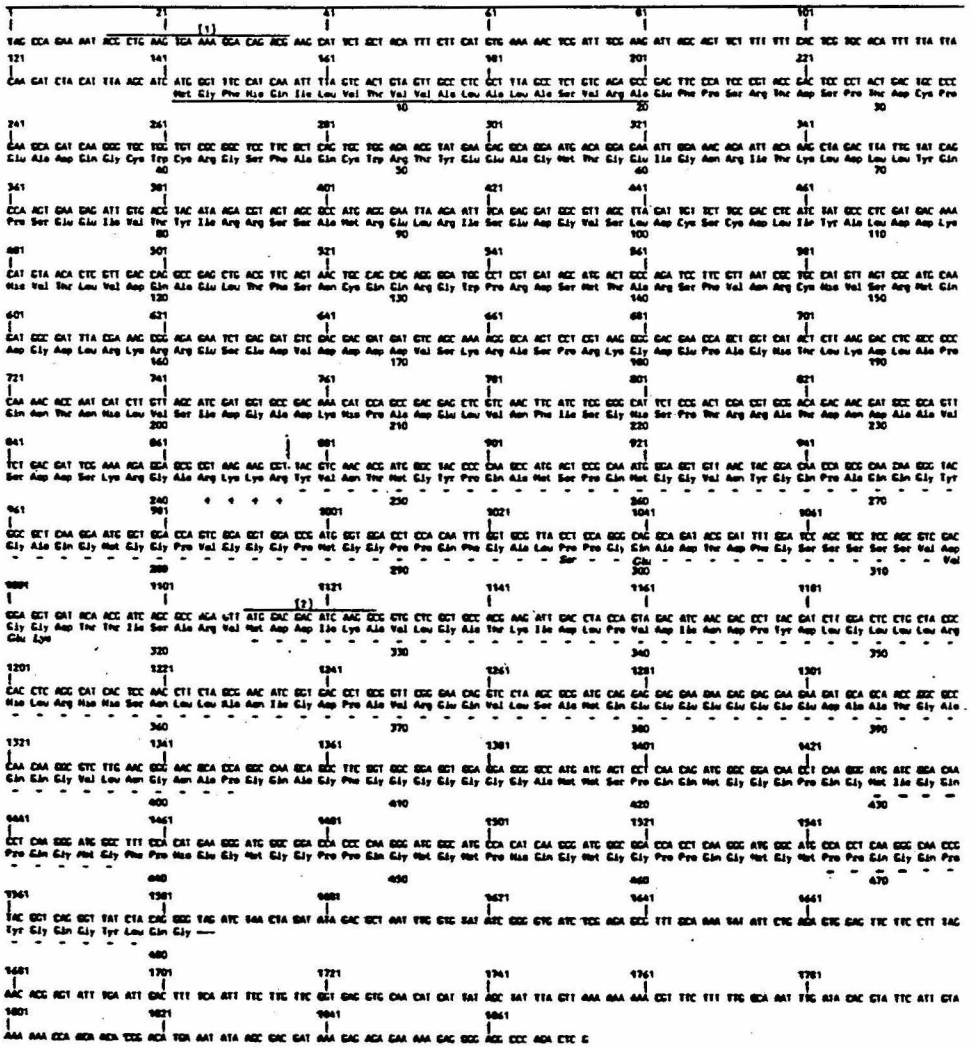
Fig. 3. Comparison of N-terminal protein sequences of mature bindins from *S. purpuratus* (Sp) and *S. franciscanus* (Sf). The bracket indicates the 10 amino acid motif that is repeated in the Sf sequence. Amino acids that are identical are marked with dashes, undetermined amino acids are marked with asterisks. [From Gao, B., Klein, L. E., Britten, R. J., and Davidson, E. H. (1986). *Proc. Natl. Acad. Sci. U.S.A.* 83, 8634–8638.]

II. BINDIN AND cDNA CLONING

As bindin is an abundant sperm acrosomal protein, it was not difficult to isolate the mRNA encoding it from whole sea urchin testis. Testes were taken from *S. purpuratus* at a time when the sea urchins were active in sperm production. The testes were chopped into small pieces and thoroughly washed to free them of mature sperm. The cells were then lysed in a buffer containing urea and proteinase K to free the RNA and to degrade the RNases. The resulting lysates were layered on a cesium chloride cushion and the RNA was pelleted by ultracentrifugation. The collected RNA was further purified by oligo(dT) cellulose chromatography, which retains polyadenylated [poly(A)$^+$] RNAs. The poly(A)$^+$ RNAs were translated *in vitro* in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The resulting labeled proteins were immunoprecipitated with bindin antibodies, and analyzed on an SDS–polyacrylamide gel. The result was a single band migrating at an apparent molecular

Fig. 4. Sequence of a bindin cDNA. The 1876-nt-long bindin cDNA was sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977; Messing, 1983). The nucleotide sequence is numbered from the 5′ end of the cDNA. The start of transcription is approximately 50 bases 5′ from nucleotide 1. The start of translation is at nucleotide 142, and the encoded amino acids are numbered from there. The mature bindin sequence begins at amino acid 246. Underneath the derived amino acid sequence is a comparison with bindin sequence previously obtained at the protein level (Vacquier and Moy, 1978; T. Sasagawa and K. A. Walsh, personal communication). Amino acids that are identical are marked with dashes, differences are noted in italics. Overline [1] is the sequence of the oligonucleotide used for the primer extension experiment. Overline [2] is the protein sequence used for the derivation of the sequences in the oligonucleotide mix. The underline is the putative leader polypeptide. Arrows mark the site of potential cleavage with trypsin-like enzymes. [From Gao, B., Klein, L. E., Britten, R. J., and Davidson, E. H. (1986). *Proc. Natl. Acad. Sci. U.S.A.* 83, 8634–8638.]

weight of about 60,000. Control experiments indicated that this protein shared a reactive epitope with bindin, despite its anomalous molecular weight. We interpreted these results to mean that the RNA preparation contained the mRNA for bindin, and so proceeded with the cDNA cloning (Gao *et al.*, 1986). A random primed cDNA library was constructed in the bacteriophage vector λgt10. The random primer was sheared calf thymus DNA that was 8–12 nt



long. The cDNA was synthesized by the RNase H procedure of Watson and Jackson (1984). The library was screened for bindin cDNAs using a 17-nt oligonucleotide mix. This oligonucleotide mix was synthesized to contain all possible sequences that could code for the amino acids indicated in Fig. 4, overline 2. The oligonucleotide mix was labeled with [γ-^{32}P]ATP and hybridized to DNAs from clones in the cDNA library. Several clones were isolated from the random primed library, and the sequence of one of them is shown in Fig. 4.

III. INFORMATION FROM THE cDNA CLONE

The clone contains an open reading frame that codes for 437 amino acids, which would predict a protein with a molecular weight of 51,000. The C-terminal half of the reading frame contains 236 amino acids that can be aligned with the available protein sequence data with 97% fidelity. Since the tyrosine at amino acid position 246 (Fig. 4) is the N-terminus of mature bindin as isolated from acrosomal granules, bindin is evidently synthesized as a much longer precursor (which we call "prebindin"). The 236 amino acid mature bindin has a predicted molecular weight of 24,000, which is close to the value of 25,000 obtained from HPLC measurements of bindin. The 30,400 apparent molecular weight for mature bindin is probably due to anomalous migration in SDS–polyacrylamide gels. This may also explain the difference between the 51,000 predicted molecular weight for prebindin and the ~60,000 apparent molecular weight for the product of *in vitro* translation.

The cDNA clone was used as a probe in hybridizations with genomic DNA from sperm and poly(A)$^+$ RNA from testis. The pattern of hybridization of this probe with sperm genomic DNA indicates that this gene is single copy in the genome. This assured that the cDNA clone derives from the bindin gene and not from some related genomic sequence. The 3% disagreement between the DNA and protein sequence data could be due to cloning artifacts, sequencing errors, or more likely to single copy DNA sequence polymorphism between members of the species *S. purpuratus* (Britten *et al.*, 1978). Hybridization of the probe to testis poly(A)$^+$ RNA indicates that the bindin mRNA is about 2500 nt long.

A primer extension experiment using the primer in Fig. 4, overline 1 indicates that the cDNA clone is missing only 50 nt of 5′-untranslated sequence from the mRNA. It follows that the cloned bindin mRNA also lacks about 570 nt of untranslated 3′ sequence.

An analysis of the open reading frame of prebindin is shown in Fig. 5. The first 20 amino acids are highly hydrophobic (Fig. 5A), and have the charac-

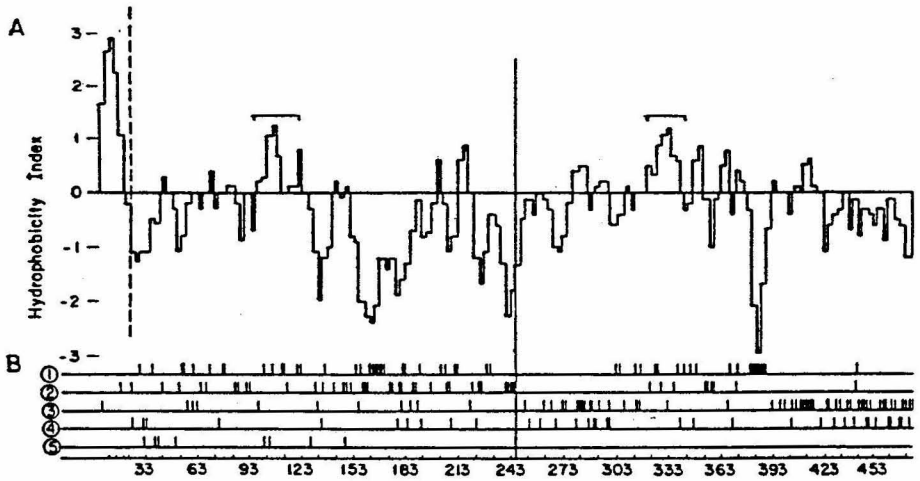


Fig. 5. (A) Hydrophobicity plot for the bindin protein sequence, obtained by the program of Kyte and Doolittle (1982), using a search length of nine amino acids. Positive values indicate hydrophobicity and negative values indicate hydrophilicty. The dotted vertical line denotes the putative leader sequence cleavage site. The N-terminus of the mature bindin is indicated by the solid vertical line. The brackets demarcate two regions that are relatively hydrophobic. (B) Distribution of selected amino acids in the bindin protein. Each vertical line indicates one amino acid. Line 1, Acidic amino acids including aspartic acid and glutamic acid. Line 2. Basic amino acids, including histidine, arginine, and lysine. Line 3, Glycine. Line 4, Proline. Line 5, Cysteine. The amino acids are numbered from the N-terminus of the probindin. [From Gao, B., Klein, L. E., Britten, R. J., and Davidson, E. H. (1986). *Proc. Natl. Acad. Sci. U.S.A.* 83, 8634–8638.]

teristics of a leader sequence (Watson, 1984). This putative leader sequence is probably responsible for the export of bindin to the acrosome vesicle, and is similar in sequence characteristics to the leader sequence of another secreted sea urchin protein, SM50 (a 50-kDa spicule matrix protein) (Sucov *et al.*, 1987). The four basic residues (Arg-Lys-Lys-Arg) preceding the N-terminal tyrosine of the mature bindin constitute a typical cleavage site for trypsin-like proteases (Bergmann and Fruton, 1941). Thus it is likely that the processing cleavage releasing mature bindin from prebindin occurs at this site. The distribution of amino acids along the prebindin chain is indicated in Fig. 5B. Although 34% of the amino acids of the N-terminal half of prebindin ("probindin") are hydrophilic, only 14% of amino acids of mature bindin are hydrophilic. This accounts for the insolubility of bindin in aqueous solutions that do not contain detergent (Glabe and Lennarz, 1979). There are eight cysteine residues in probindin, indicating the possibility of a compacted structure.

The abundance of proline and glycine suggests that mature bindin is an extended, rigid structure. This might account for its anomalous migration in polyacrylamide gels.

The derived protein sequence of prebindin was checked for homology against the National Biomedical Research Foundation Protein Identification Resource database. There were no highly significant matches, and the highest matches [basic proline-rich peptide (Kauffman *et al.*, 1986), procollagen α-1 (III) chain precursor (Loidl *et al.*, 1984), proline-rich phosphoproteins C and A (Wong and Bennick, 1980), and keratin (Hoffmann *et al.*, 1985)] were probably merely due to areas of high glycine and proline content. The chain of eight contiguous glutamic acids (Fig. 4, amino acids 380–387) was found in only three other proteins in the database [cytochrome c_1 (Wakabayashi *et al.*, 1982), sodium channel protein (Noda *et al.*, 1984), and mouse m6 "homeotic" protein (Colberg-Poley *et al.*, 1985)]. It is unlikely any of these matches is due to evolutionary conservation, or that there is any functional correspondence.

IV. BINDIN AS A TESTIS-SPECIFIC GENE

Both total and poly(A)$^+$ testis RNAs react strongly with the bindin cDNA probe. In contrast, total RNA from 40-hr gastrula stage embryos and adult tube feet, and poly(A)$^+$ RNA from eggs, ovaries, coelomocytes, lantern tissue, and intestine had no detectable reaction with the bindin cDNA probe. This indicates that bindin is expressed only in testes. Sea urchins also have testis-specific *H1* and *H2B* histone genes (Strickland *et al.*, 1980; Busslinger and Barberis, 1985). In contrast, sea urchin major yolk protein is expressed in both ovaries and testis. For these genes the pattern is that genes coding for sperm proteins are expressed only in the testis whereas the gene coding for egg yolk protein is expressed both in the ovaries and in the testis. The restricted expression of sperm protein genes is not because these genes are on a male restricted ("Y" type) chromosome, since the bindin cDNA probe reacts with both sperm DNA and DNA from the coelomocytes of female sea urchins (Gao, 1986). The testis-restricted expression of bindin is therefore due to a difference in the regulation of its expression in these tissues.

V. SPECIES SPECIFICITY OF THE BINDIN–RECEPTOR INTERACTION

Two of the species in which bindin has been extensively studied are *S. purpuratus* and *S. franciscanus*. These two species have widely overlapping

ranges on the Pacific Coast, and are frequently found in the same tidepools. In addition, the breeding seasons overlap (Boolootian, 1966). Since this means that gametes from the two species are in the same place at the same time, there is the possibility of cross-fertilization. Despite extensive intermingling of large populations, reports of naturally occurring species hybrids are rare (Swan, 1953), and even these animals have not been demonstrated to be *bona fide* hybrids. Therefore, there is a barrier to interspecies hybridization that is significant if not absolute. Although the deficiency in sperm binding can be attributed to the species specificity of the interaction of purified bindin with homologous eggs, the other steps in the fertilization pathway can be shown not to be barriers to cross-fertilization. Thus the steps (which include the egg-induced activation of sperm and the acrosome reaction) leading to the exposure of bindin proceed normally in cross-fertilization. Likewise, the processes occurring after sperm binding are also not barriers to cross-fertilization. The sperm-binding step may be bypassed by treating the eggs with trypsin (Moore, 1943). The cross of *S. purpuratus* sperm and *S. franciscanus* eggs arrests at the gastrula stage (Loeb *et al.*, 1910), but the reciprocal cross produces feeding sea urchins (J. Minor, A. Cameron, and P. Leahy, unpublished observation). Therefore, incompatibility of developmental programs is only a barrier in one of the two interspecies crosses. The bindin–receptor interaction therefore appears to be the only major barrier to cross-fertilization between these two species.

The species specificity of the bindin–receptor interaction of these two species means that (1) evolutionary changes have occurred in the bindin–receptor system responsible for the specificity, (2) these changes are the only identified barrier responsible for the current lack of cross-hybridization between the species, and (3) the changes in the bindin–receptor system may be responsible for establishing the species isolation barrier between these species. Species specificity has also been described for a number of other cellular processes at fertilization in sea urchins and other organisms. The demonstration of any species specificity implies that evolutionary changes have occurred between the species studied. However, only in the bindin–receptor system does the species specificity imply the second and third points. The biology or ecology of the other studied systems prevents their species specificity from being interpreted this way. For example, the resact peptide (Ward *et al.*, 1985) from *Arbacia* eggs acts as a chemoattractant for *Arbacia* sperm, but not for *Strongylocentrotus* sperm. The species specificity cannot be demonstrated reciprocally since the analogous compound (speract) from *Strongylocentrotus* eggs does not attract either *Strongylocentrotus* or *Arbacia* sperm. In addition, these two sea urchins are from different families and different oceans. This interaction is then a nonreciprocal one between taxonomically and ecologically separated species. In another example, the acrosome reaction generally does

not display species specificity (Summers and Hylander, 1975). In the case of *S. purpuratus* and *Strongylocentrotus droebachiensis*, the failure of *S. droebachinsis* sperm to undergo the acrosome reaction in the presence of *S. purpuratus* eggs blocks cross-fertilization in this direction. However, the reciprocal fertilization is nearly 100% complete (SeGall and Lennarz, 1979; P. Leahy, personal communication). Here, the species studied are closely related and their ranges overlap, but the barrier is only 50% effective since it is nonreciprocal. As these examples demonstrate, species specificity can be demonstrated in a number of cases with obscure ecological and evolutionary relevance. In contrast, the bindin–receptor interaction inhibits fertilization reciprocally (Sp ♂ × Sf ♀; Sf ♂ × Sp ♀) between two closely related species with overlapping ranges and breeding seasons. For these reasons the study of the bindin–receptor system may provide insights into how species isolation barriers are established at the molecular level.

VI. FUTURE PROSPECTS

Research into bindin has generated antibodies to the bindin protein and nucleic acid probes to bindin mRNA. The antibodies to bindin have already been shown to be useful for quantifying the number of sperm undergoing the acrosome reaction (Vacquier, 1984). Both the protein and nucleic acid probes should be useful for studying the development of the testis. By performing *in situ* hybridizations to the developing gonads of juvenile sea urchins, it should be possible to detect when bindin is first synthesized in the developing testis. These experiments can thus reveal the timing of gene expression during spermatogenesis, the onset of spermatogenesis, and concomitantly the onset of male sexual maturity.

The nature of the probindin sequence cleaved from prebindin remains a mystery. This N-terminal region accounts for nearly half of the prebindin molecule. It could have a separate function, or it may simply be responsible for delivering the mature bindin to the acrosomal vesicle. This function could be required by the insolubility of the mature bindin stored in the vesicle. The probindin sequence, which is more hydrophilic, might then serve as a carrier that is cleaved from the mature bindin on delivery to the acrosomal vesicle. The fate of the probindin molecule could be studied by generating antibodies to its predicted amino acid sequence and using these antibodies on preparations of testis cells and developing sperm.

Comparisons of bindin cDNAs from different species of sea urchins should provide information on what changes in the bindin molecule are important for changes in bindin activity between species. Genomic clones isolated from

several species could indicate how these changes took place. These ideas can be tested in an *in vitro* assay system for the fucose sulfate-binding activities of bindin (De Angelis and Glabe, 1987) using bindins that have been altered by genetic engineering. Finally, it should be possible to change the species specificity of fertilization by introducing the bindin gene from one species of sea urchin into another using established gene transfer techniques (Davidson *et al.*, 1985).

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Chapter 3

Comparison of the Bindin Proteins of *Strongylocentrotus franciscanus*, *S.purpuratus*, and *Lytechinus variegatus*: Sequences involved in the Species-Specificity of Fertilization[1]

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ABSTRACT

Bindin is the sea urchin sperm acrosomal protein that is responsible for the species-specific adhesion of the sperm to the egg. Two new bindin cDNA sequences that contain the entire open reading frame for the bindin precursor are reported: One for *Strongylocentrotus franciscanus* and one for *Lytechinus variegatus*. Both contain inverted repetitive sequences in their 3' untranslated regions and the *S.franciscanus* cDNA contains an inverted repetitive sequence match between the 5' untranslated region and the coding region. The middle third of the mature bindin sequence is highly conserved in all three species, and the flanking sequences share short repeated sequences that vary in number between the species. Cross fertilization data are reported for the species *S.purpuratus, S.franciscanus, L.variegatus,* and *L.pictus*: A barrier to cross fertilization exists between the sympatric *Strongylocentrotus* species but there is no barrier between the allopatric *Lytechinus* species.

Introduction

Sea urchin fertilization involves a complex series of cell recognition events that insure fertilization specificity (Rossignol et al., 1984a; Minor et al., 1989). This specificity is necessary since like many marine invertebrates, sea urchins are broadcast spawners: Gametes are released into open water where fertilization takes place. Sea urchins in general do not appear to go through premating isolating behaviors that would insure that the first egg a sperm would encounter would be one of the same species (Pennington, 1985; Pearse et al., 1988). Therefore fertilization specificity is largely, if not entirely, dependent upon the species-specifity of gamete recognition. In the case of the species *Strongylocentrotus purpuratus* and *S.franciscanus*, the species-specificity of fertilization has been shown to be due to the interaction of the sperm protein bindin with a glycoprotein receptor of the egg (Glabe and Vacquier, 1977, 1978). Bindin is packaged as a large insoluble granule in the sperm acrosome. Upon contact with the egg, factors from the egg induce the sperm to undergo the acrosome reaction that exposes bindin. Bindin is not an integral membrane protein, but remains associated with the sperm membrane and forms a contact with the receptor on the surface of the egg. In most cases, this contact must be species-specific for efficient fertilization.

The bindin protein from *S.purpuratus* has been isolated and sequenced at the protein level (Vacquier and Moy, 1977, 1978; T. Sasagawa and K. Walsh, personal communication). A bindin cDNA for *S.purpuratus* was cloned using this sequence and was used to show that the *S.purpuratus* bindin message is 2.5 kb long, and is a testis specific transcript. The sequence of the clone revealed that bindin is initially synthesized as a 51 kDa precursor polypeptide which is cleaved to yield the 24 kDa acrosomal bindin from its C-terminus, plus a 27 kDa N-terminal protein of unknown function (Gao et al., 1986). The bindin gene is transcribed relatively late in spermatogenesis (Cameron et al., 1990; Nishioka et al., 1990) and is quickly translated and packaged into the acrosomal vesicle (Nishioka et al., 1990).

The four species of sea urchin used in this study are *Strongylocentrotus franciscanus*, *S.purpuratus*, *Lytechinus variegatus*, and *L.pictus*. The relationships between the species are summarized in Table 1. The ranges of *Strongylocentrotus franciscanus* and *S.purpuratus* overlap from British Columbia to Baja California on the Pacific coast of North America and their breeding seasons overlap as well (Boolootian, 1966). *Lytechinus variegatus* was chosen as an outgroup for comparison since it is one of the most closely related species that does not have an overlapping range with the *Strongylocentrotus* species. *L.pictus* is from the Pacific coast and its range overlaps with the

Strongylocentrotus species. The two *Strongylocentrotus* species diverged 3.5 to 20 million years ago (Mya), and the total single copy divergence is 19%. The genus *Lytechinus* diverged from the genus *Strongylocentrotus* 30-40 Mya and the total single copy divergence is 31%. The sequences of the bindin cDNAs of *S.franciscanus* and *L.variegatus* are reported in this paper. Also reported are cross fertilization data between *S.purpuratus*, *S.franciscanus*, *L.variegatus*, and *L.pictus*. The sequence data describe the changes that have taken place in bindin evolution and the cross fertilization data allow inferences to be made about how changes in the bindin protein have affected bindin function.

Materials and Methods

Cross fertilizations

Gametes were collected by either electric shock or by intracoelomic injection of 0.5 M KCl. The eggs were dejellied by washing in Millipore filtered sea water (MPFSW) titrated to pH 5 by the addition of citric acid, returned to MPFSW, then 500 eggs in 1 ml were placed in each of the wells of a Falcon 3047 cell culture plate. All work was done in an 18°C room. Sperm were diluted in MPFSW containing 0.05% bovine serum albumin (BSA, Sigma Fraction V). A series of 1:1 dilutions was made and aliquots of these dilutions were added to the eggs producing the final

dilution levels indicated (Fig. 1). After 5 minutes, the eggs were fixed by the addition of an equal volume of 2% glutaraldehyde (Sigma) in MPFSW. The position of each species' eggs in the wells was randomized, and the plates coded before scoring. Fertilization was scored by viewing the elevation of the fertilization envelope at 100X in an inverted microscope. Dry sperm concentrations were determined by counting in a Hausser chamber and by counting sperm fixed in the bottoms of the fertilization wells. The two values obtained were similar, and were close to the value obtained from the yield of DNA extracted from dry sperm. The dry sperm concentrations were ($x10^{10}$ sperm/ml): *S.purpuratus* - 3.9, *S.franciscanus* - 9.5, *L.variegatus* - 4.6, and *L.pictus* - 4.0.

DNA and RNA Isolation, cDNA Synthesis and Cloning

Sperm DNAs were isolated from *S.purpuratus* and *L.variegatus* by the method of Lee et al. (1984). *S.franciscanus* sperm DNA was a gift from J.J. Lee and F. Calzone. Testis RNAs for *S.franciscanus* and *L.variegatus* were isolated as described by Gao et al. (1986). *S.purpuratus* testis RNA was a gift from B. Gao. PolyA^+ RNAs were purified by oligo-(dT) cellulose chromatography. *S.franciscanus* cDNA was synthesized by the S1 hairpin method, while *L.variegatus* cDNA was synthesized using the

RNAse H procedure (Amersham). *Eco*RI linkers were blunt end ligated on the cDNA, cDNAs were then size selected on a BioRad Biogel A-50m column, and then cloned into λgt10 (Stratagene). The libraries were screened with either the total *S.purpuratus* cDNA (*S.franciscanus* library) or with the Sp5' and Sp3' fragments (defined below) of it (*L.variegatus* library). The hybridization was performed at 65°C in 5X SET (SET=0.15 M NaCl, 30 mM Tris-HCl pH8, 2 mM EDTA), 5X Denhardt's solution, and 25 μg/ml sheared, denatured calf thymus DNA. The final wash was in 1X SET at 65°C. Multiple clones were isolated and subcloned into Bluescript vectors (Stratagene). Deletion subclones were made by the *Exo*III-Mung Bean nuclease deletion method, and one clone per species was sequenced completely on both strands. The sequenced clones were pBSf35 for *S.franciscanus* and pBLv22 for *L.variegatus*. The sequencing error is estimated to be 0.04% (sequences from each strand were obtained independently and differ by less than 2%).

DNA and RNA Gel Blot Analysis

Genomic DNAs were cleaved with a restriction enzyme (usually *Bgl*II), and separated by electrophoresis on agarose gels, then transferred to nitrocellulose. RNAs (both total and polyA$^+$) were separated by electrophoresis on formaldehyde gels and transferred to nitrocellulose. Hybridizations were conducted as above at either 65°C (final wash 1X SET, 65°C) or 55°C (final wash 1X SET, 55°C). The

resulting filters were hybridized with several different DNA fragments taken from the *S.purpuratus* and *S.franciscanus* cDNAs. The *S.purpuratus* cDNA (see Gao et al., 1986; Fig. 1) was divided into three fragments: Sp5' (bases 1-637), SpMID (638-1075), and Sp3' (1076-1873) by digestion with *Sal*I and *Eco*RI. The 3' repetitive DNA fragment of the *S.franciscanus* cDNA (Sf3') was isolated by digestion with *Bgl*II and *Eco*RI, yielding a 657 bp fragment (bases 1651-2307 in Fig. 2).

Results

Cross Fertilizations

The degree of cross fertilization between the four species (*S.purpuratus*, *S.franciscanus*, *L.variegatus*, and *L.pictus*) was evaluated in the experiment described in Fig. 1. The general result is that barriers to cross fertilization exist in most cases but can be overcome at very high sperm concentrations. The congeneric Pacific *Strongylocentrotus* species have overlapping geographical ranges, habitat preferences, and breeding seasons. Figure 1A shows that even at the lowest sperm concentrations used, *S.franciscanus* sperm are effective at fertilizing their own eggs. Significant cross fertilization with *S.purpuratus* eggs occurs only at 64-fold higher sperm concentrations. In Fig. 1B, *S.purpuratus* sperm are less effective at cross fertilization, even at the highest concentrations of sperm used. In Fig. 1C, *L.variegatus* sperm fertilize both species of *Lytechinus* well, and also fertilize *S.franciscanus* equally well at higher sperm concentrations (the fertilization of *L.variegatus* eggs was inhibited at 18°C because their usual temperature is 25-30°C). Figure 1D shows that *L.pictus* sperm also fertilize both species of *Lytechinus* well, but do not cross fertilize *S.franciscanus* eggs as efficiently. Unlike the barrier between the Pacific *Strongylocentrotus* species, the *Lytechinus* species

(*L.variegatus* Atlantic, *L.pictus* Pacific) show no barrier to cross fertilization.

There are three steps in the sea urchin fertilization pathway the failure of which could potentially result in the species-specificity of fertilization. These steps are, in order of occurrence, movement of sperm to the egg, induction of the acrosome reaction, and adhesion of the sperm to the egg vitelline layer. It is this last step that is mediated by bindin. In a study of cross fertilization between four sympatric species of sea urchins from Bermuda, Summers and Hylander (1975) concluded that in the majority of cases (9/11) the failure of cross fertilization was due to a failure of sperm to adhere to the egg vitelline layer. However, there were two of eleven cases where the failure of cross fertilization was due to a failure of the acrosome reaction. It is also possible that species differences could exist in egg chemotactic molecules which would result in species-specificities in fertilization. An example of a sea urchin egg-derived chemotactic molecule is the *Arbacia* peptide resact (Ward et al., 1985). Species-specific egg chemotactic molecules are unlikely to be a concern for the species *S.purpuratus* and *S.franciscanus*, since the eggs of both species activate the motility of the sperm of the other species (Loeb, 1915). The following considerations and observations show that neither chemotaxis nor acrosome reactions could have been responsible for the species-

specific differences in efficiency of cross fertilization seen in Fig. 1.

The possibility that the differences in fertilization could be due to a failure of chemotaxis resulting in differing numbers of sperm reaching the eggs was addressed by counting the number of sperm reaching the eggs. In the experiment described in Fig. 1, the eggs were fertilized, fixed, and scored in the same well to avoid the preferential loss of unfertilized eggs that occurs when eggs are transferred. After scoring fertilization, the number of sperm visible around the diameter of the unfertilized eggs was scored at 320X (the perimeter counting method of Kinsey et al., 1980). The sperm were counted around each of the four species of eggs at the sperm dilution that resulted in ~50% fertilization in the homotypic case. Since the eggs were not washed after fertilization, the sperm visible could be either sperm bound to the vitelline layer or sperm loosely "attached" in a nonspecific manner (cf. Summers and Hylander, 1975; Wasserman, 1990). There were no differences in the number of sperm per egg diameter, indicating no cross-species incompatibilities in the overall ability of sperm to locate the eggs.

The possibility that the species-specificities of fertilization observed in Fig. 1 were due to failures of the acrosome reaction can be ruled out in 11 of the 12 interspecies crosses performed. There is no species-

specificity in the induction of the acrosome reaction between the species *S.purpuratus* and *S.franciscanus* (A. Lopez and C. Glabe, personal communication), or between these species and *L.pictus* (B. Brandriff and V. Vacquier, personal communication). This eliminates the acrosome reaction as the cause of the species-specificity of fertilization for the crosses Sp♂ X Sf♀, Sp♂ X Lp♀, Sf♂ X Sp♀, Sf♂ X Lp♀, Lp♂ X Sp♀, and Lp♂ X Sf♀ (Fig. 1A,B,D). The acrosome reaction of *L.variegatus* is nonspecific (Summers and Hylander, 1975) and occurs spontaneously to a considerable degree (SeGall and Lennarz, 1979). This indicates there are no acrosome reaction compatibility problems for the crosses Lv♂ X Sp♀, Lv♂ X Sf♀, and Lv♂ X Lp♀ (Fig. 1C). There is no acrosome reaction incompatibility in the Lp♂ X Lv♀ cross since this cross fertilization proceeds efficiently (Fig. 1D). *L.variegatus* egg jelly efficiently induces the acrosome reaction of *S.purpuratus* sperm (SeGall and Lennarz, 1979), negating the possibility of a failure of the acrosome reaction in the Sp♂ X Lv♀ cross. Finally, the possibility of a failure of the acrosome reaction in the Sf♂ X Lv♀ cross cannot be excluded, but seems unlikely in light of the general nonspecificity of the acrosome reaction in cross fertilizations involving *S.franciscanus* sperm and *L.variegatus* eggs. Therefore, the only remaining cause for the species-specific inefficiencies in the cross fertilizations shown in Fig. 1 is the failure of the sperm

to bind productively with the egg, at least for 11 of the 12 cases tested.

In another experiment (data not shown), the barrier to cross fertilization between *S.purpuratus* and *S.franciscanus* was evaluated in detail by using eggs and sperm from different individuals at limiting sperm concentration. Gametes were collected from four *S.franciscanus* females, five *S.franciscanus* males, five *S.purpuratus* females, and four *S.purpuratus* males. All possible interindividual fertilizations were performed at a sperm dilution that resulted in an average of 72% fertilization in the homologous cases (Sp♂ X Sp♀ or Sf♂ X Sf♀). Cross fertilization (Sp♂ X Sf♀ or Sf♂ X Sp♀) rarely occurred, and averaged less than 0.2%. These results show that under limiting sperm conditions, such as might be expected in the ocean (Pennington, 1985), there is a very effective barrier to cross fertilization between *S.purpuratus* and *S.franciscanus*.

Bindin Protein Sequences

Figure 2 displays the sequence of a 2.3 kb *S.franciscanus* bindin cDNA clone and Fig. 3, of a 2.6 kb *L.variegatus* cDNA clone. RNA gel blots (data not shown) show that the bindin message size is 3.3 kb for *S.franciscanus* and 3.0 kb for *L.variegatus*. Both clones contain the entire open reading

frame for the bindin precursor, which in all three species is made as a large molecule approximately twice the size of the mature bindin. The predicted molecular weights for the bindin precursors are 52 kDa for *S.franciscanus* and 50 kDa for *L.variegatus*. The *S.franciscanus* precursor is known to be cleaved, since the acrosomal bindin has as its N-terminal residue the amino acid 248 of the precursor sequence (Vacquier and Moy, 1978; and Fig. 2). The *L.variegatus* precursor is assumed to be similarly cleaved, based on its sequence homology to the other two species in the region surrounding the cleavage site (Figs. 3-4). The predicted molecular weights for the cleaved C-terminal acrosomal bindins are 24 kDa for *S.franciscanus* and 22 kDa for *L.variegatus*. The sequence preceding the cleavage site (RKKR, marked A in Figs. 2-3) is conserved in all three species, and constitutes a cleavage site for a protease with trypsin-like specificity that is located in the Golgi apparatus (Strauss and Strauss, 1985; Whealy et al., 1990). All three species of bindin contain repeats of two related protein sequences: GMGG(A/P)VGGG and QGMGG(P/Q)(P/H). These sequences are called the "long" (L) and "short" (S) repeats and all the indicated sequences (Figs. 2,3, and 5) have at most one difference from the consensus sequence. The middle third of the bindin molecule is highly conserved in all three species (sequences in italics, Figs. 2-3).

Inverted and Repetitive Sequences on Bindin mRNAs

DNA gel blots using the SpMID fragment indicate that bindin is a single copy gene in all three species (data not shown). Figure 4 shows that the *S.franciscanus* cDNA (panel B) contains a repetitive sequence that is not present on the *S.purpuratus* cDNA. This repetitive sequence is present in the genomes of *S.franciscanus* (Fig. 4B1) and *S.purpuratus* (data not shown), but is absent from the genome of *L.variegatus* (Fig. 4B2,3). This repetitive sequence was shown to be limited to the 3' untranslated region of the *S.franciscanus* mRNA by using a probe (Sf3') that contains only 3' untranslated sequences. Using the Sf3' probe on gel blots of testis RNA, it was shown that this repetitive sequence is present only on the bindin mRNA of *S.franciscanus*, and not on any other testis RNA of *S.franciscanus* or any testis RNA (including bindin) of the other two species. The sequence of the *S.franciscanus* bindin cDNA reveals that this repetitive element (C,C' in Fig. 2) consists of a large inverted repeat. The size of this repeat accounts for most of the size difference between the bindin mRNAs of the two *Strongylocentrotus* species. Sequence analysis of the *L.variegatus* cDNA reveals a different 3' untranslated repetitive sequence (B in Fig. 3). The simplest explanation of these data is that a novel repetitive element present in the *Strongylocentrotus* lineage spread through the genomes after the divergence of the two species. During this process this repetitive element landed in the *S.franciscanus* bindin transcription unit, but is not

found in the *S.purpuratus* bindin though as noted above it is present elsewhere in the *S.purpuratus* genome. The *L.variegatus* bindin transcription unit would then have independently acquired a different 3' repetitive element.

The *S.franciscanus* cDNA contains a second inverted DNA sequence (B,B' in Fig. 2) that consists of a 29 out of 30 nt match between a sequence in the 5' untranslated region and its complement in the coding region. This "antisense pair" as well as the other repetitive elements could affect both transcript stability and translation control of bindin. The functional significance of all of these repeats, as well as the possibility of additional repetitive elements on the uncloned portions of all three bindin mRNAs, remains uninvestigated.

Phylogenetic Distribution of Bindin

A search was made for the presence of bindin DNA sequences in species other than sea urchins by probing "zoo blots" [blots containing the *Bgl*II digested genomic DNAs of three sea urchin species (*S.purpuratus*, *S.franciscanus*, and *L.variegatus*), a starfish (*Pisaster ochraceus*), a sea cucumber (*Thyone briareus*), *Xenopus laevis*, *Mus musculus domesticus*, *Homo sapiens*, *Aplysia californica*, *Mytilus edulis*, *Loligo pealii*, *Drosophila simulans*, *Caenorhabditis elegans*, *Cerebratulus lacteus*, *Tetrahymena thermophila*,

Macrocystis integrifolia, and *Saccharomyces cerevisiae*] with fragments of the *S.purpuratus* cDNA (Sp5', SpMID, and Sp3') at the 55°C criterion. No reliable signal was found outside the sea urchin species, a result consistent with earlier antibody studies (Moy and Vacquier, 1979). Bindin (and bindin precursor) sequences seem to be limited to the echinoids.

Discussion

The derived sequences from the entire open reading frames of three species of bindin are aligned in Fig. 5. There is a dramatic difference in the pattern of sequence evolution when comparing the N-terminal protein sequences (amino acids 1-245 of *S.purpuratus*) to the mature bindin sequences (amino acids 246-481 of *S.purpuratus*). In the N-terminal protein sequences, there is little sequence rearrangement (insertions, deletions, or repeated sequences) and many point changes while in the mature bindin there is extensive sequence rearrangement and comparatively fewer point changes. This is a variant of the "mosaic evolution" pattern seen in the *Drosophila* sgs genes (Martin and Meyerowitz, 1988), where there is a five- to ten-fold change in the frequency of nucleotide substitutions on either side of a boundary that is less than 50 nt long. In the case of bindin and N-terminal sequences, the differences in the

pattern of sequence evolution probably reflect the effects of selection for different functions on different parts of the protein. While the function of the N-terminal protein sequences remains unknown, it has been speculated based on sequence content that N-terminal protein might play a role in packaging the insoluble mature bindin for transport to the acrosome (Gao et al., 1986). The fact that the three species of bindin precursor all conserve their overall size and structure, including the exact relative positioning of eight cysteines despite multiple point changes (Fig. 5, arrows), tends to support this notion.

The extensive rearrangement of the mature bindin sequences directly contrasts the above pattern. The middle third of the molecule is highly conserved (there is a stretch of 68 amino acids that is perfectly conserved within *Strongylocentrotus*). This conserved region (amino acids 314-390 of *S.purpuratus*, Fig. 5; diagrammed as a black box in Fig. 6) corresponds to the region that in *S.purpuratus* is thought to bind sulfated fucans (DeAngelis and Glabe, 1988, 1990) and to possibly interact with the sperm acrosomal membrane (Kennedy et al., 1989). There are three areas of sequence rearrangement between the three species. The first involves a polyglycine stretch (G in Figs. 2,3, and 6) that is longer in *S.franciscanus* and *L.variegatus* than in *S.purpuratus*. The second and third areas involve differences in the number and position of two related

repeated protein sequences: GMGG(A/P)VGGG (L in Fig. 6) and QGMGG(P/Q)(P/H) (S in Fig. 6). The difference in the number of L repeats between *S.purpuratus* and *S.franciscanus* was first noticed in N-terminal protein sequences of bindin determined by Vacquier and Moy (1978). Around the conserved repetitive regions are short areas of species-unique sequence (e.g. amino acids 288-314 of Sp or 312-333 of Sf, Fig. 4). Presumably some of these sequence differences are responsible for the differences in fertilization specificity seen between the species.

The cDNA sequence has recently been reported for the bindin of a fourth species of sea urchin, *Arbacia punctulata* (Glabe and Clark, 1990). *Arbacia* is a distant relative of the sea urchins in this study, having diverged from them ~200 million years ago (Smith, 1984). Despite this long divergence time, the *Arbacia* bindin shares several features with the other bindins. The *Arbacia* bindin is also synthesized as a larger precursor, and the N-terminal sequences conserve the eight cysteines found in the *Strongylocentrotus* and *Lytechinus* bindins. It also conserves a large section of the core sequences conserved among the species in this study. The *Arbacia* bindin differs from the the bindins reported here in that it contains an extra hydrophobic region, and it does not conserve the "L" and "S" sequences.

The uniqueness of bindin does not allow for predictive structural comparisons to be made with other recognition proteins. Although it mediates cell-cell recognition and possibly membrane fusion (Glabe, 1985a,b), bindin is not an integral membrane protein. Bindin is insoluble in seawater, and in electron micrographs is visible as a "blob" of protein both in the sperm acrosomal vesicle (Nishioka et al., 1990) and between the sperm and egg at fertilization (Moy and Vacquier, 1979). In addition, it binds in a strong field of contact (capable of holding a moving sperm) that involves many bindin monomers. The *S.purpuratus* bindin receptor is a high molecular weight ($>10^7$ Da) complex that requires both protein and carbohydrate for its specificity (Rossignol et al., 1984b; Foltz and Lennarz, 1990). The bindin-receptor interaction takes place in seawater, which has a higher ionic strength than that in which recognition proteins with solved structures operate. It is not surprising then that bindin is unrelated in sequence to other proteins: Bindin and bindin precursor sequences were searched against the National Biomedical Research Foundation's Protein Identification Resource Database (releases 22.0 and 40.0, Sept. 1989) and Genbank (release 63, March 1990) and no significant matches were found.

The differences in the bindin proteins could result in changes in the species-specificity of fertilization if some of the altered sequences encoded species-specific domains of

interaction between bindin and its receptor. This idea has been tested in our laboratory by assaying the ability of bindin derived peptides to inhibit fertilization. We have found that a peptide derived from a unique region of the *S.franciscanus* bindin inhibits fertilization species-specifically (unpublished observation), supporting the view that the species-specificity of the bindin-receptor interaction is due to altered binding domains. In addition to continuing these structural studies, it will be interesting to study the population distribution of bindin alleles in groups of species and subspecies that may be currently undergoing speciation (e.g. the *Echinometra* species complex, Lessios and Cunningham, 1990; Palumbi and Metz, 1991).

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TABLE 1

Relationships Among the Sea Urchin Species Used in This Study

Species	Range[a]	Divergence time (Mya)[b]	Divergence % scDNA[c]
S.purpuratus	Pacific	-	4
S.franciscanus	Pacific	3.5-20	19
L.pictus	Pacific	30-40	31
L.variegatus	Atlantic	30-40	ND

[a]Geographic ranges from Boolootian (1966).

[b]Divergence times from *S.purpuratus* are based on a cladistic analysis of morphological data and on the fossil record from Smith (1988).

[c]Total single copy DNA diverges are median divergence values from Hall et al. (1980) and Britten et al. (1991); the 4% value is interindividual polymorphism within the species *S.purpuratus*.

Figure Legends

FIG. 1 - Cross fertilization between *S.franciscanus* (Sf), *S.purpuratus* (Sp), *L.variegatus* (Lv), and *L.pictus* (Lp). Each panel of the figure shows the result of fertilizing eggs of all four species (Sf, ♦———♦; Sp, +·····+; Lv, o——o; Lp, ❐-----❐) with decreasing amounts of sperm from the species indicated in the upper right corner. Eggs were dejellied and placed 500 per 1 ml well and sperm were added at the final dilution indicated. X-axis is logarithmic; each point has half the sperm concentration of the preceding point. The curves shown result from fitting the experimental data to the equation $P = ae^{-bD}$, where P is the percent fertilization that occurs at the sperm dilution D. The value e is the base for natural logarithms, and the value a was the maximum percent fertilization that occurs in the particular cross (for all the homologous crosses, a = 100). The data were fit to find the value for b that would minimize the difference between the experimental data and the curves.

FIG. 2 - Sequence and translation of a *S.franciscanus* bindin cDNA. The sequence is 2307 bp in length (including the *Eco*RI cloning linkers). The sequences are numbered to the left; the numbers for the protein sequences are underlined. The open reading frame extends from base 194 to base 1648. A hydrophobic leader sequence (underlined and marked "H")

extends from amino acid 1 to 20, where it ends with a putative leader cleavage site (von Heijne, 1983,1984). The double underlined sequences marked "A" denote a signal sequence for protease cleavage that precedes the cleavage between amino acids 247 and 248 that separates bindin from the N-terminal protein. L1,L2,L3 (underlined) mark repeats of the long consensus sequence GMGG(A/P)VGGG. S1,S2 (underlined) mark repeats of the short consensus sequence QGMGG(P/Q)(P/H). A highly conserved block of sequence (italics) extends for 76 amino acids from 334 to 409. "G" marks a stretch of glycine residues. The double underlined DNA sequences (B,B') denote an inverted repeat (29 nt/30 nt match) that covers part of the coding region. C and C' (underlined) denote a 149 nt inverted repeat (with stretches of perfect match up to 35 nt) that is in the 3' untranslated region. This sequence has been submitted to Genbank, accession number M59490.

FIG. 3 - Sequence and translation of a *L.variegatus* bindin cDNA. The sequence is 2584 bp in length (including the EcoRI cloning linkers). The open reading frame extends from base 163 to base 1548. A hydrophobic leader sequence (underlined and marked "H") extends from amino acid 1 to 19, where it ends with a putative leader cleavage site. The double underlined sequences marked "A" denote a signal sequence for protease cleavage that precedes the putative cleavage between amino acids 242 and 243 that separates

bindin from the N-terminal protein. L1-L4 (underlined) mark repeats of the long consensus sequence; S1 (underlined) marks a short consensus sequence site. A highly conserved block of sequence (italics) extends for 76 amino acids from 335 to 410. "G" marks a stretch of glycine residues. The underlined sequence "B" from base 2196 to 2404 indicates an area in the 3' untranslated region consisting of multiple direct and inverted repeats of the sequence TTCG. This sequence has been submitted to Genbank, accession number M59489.

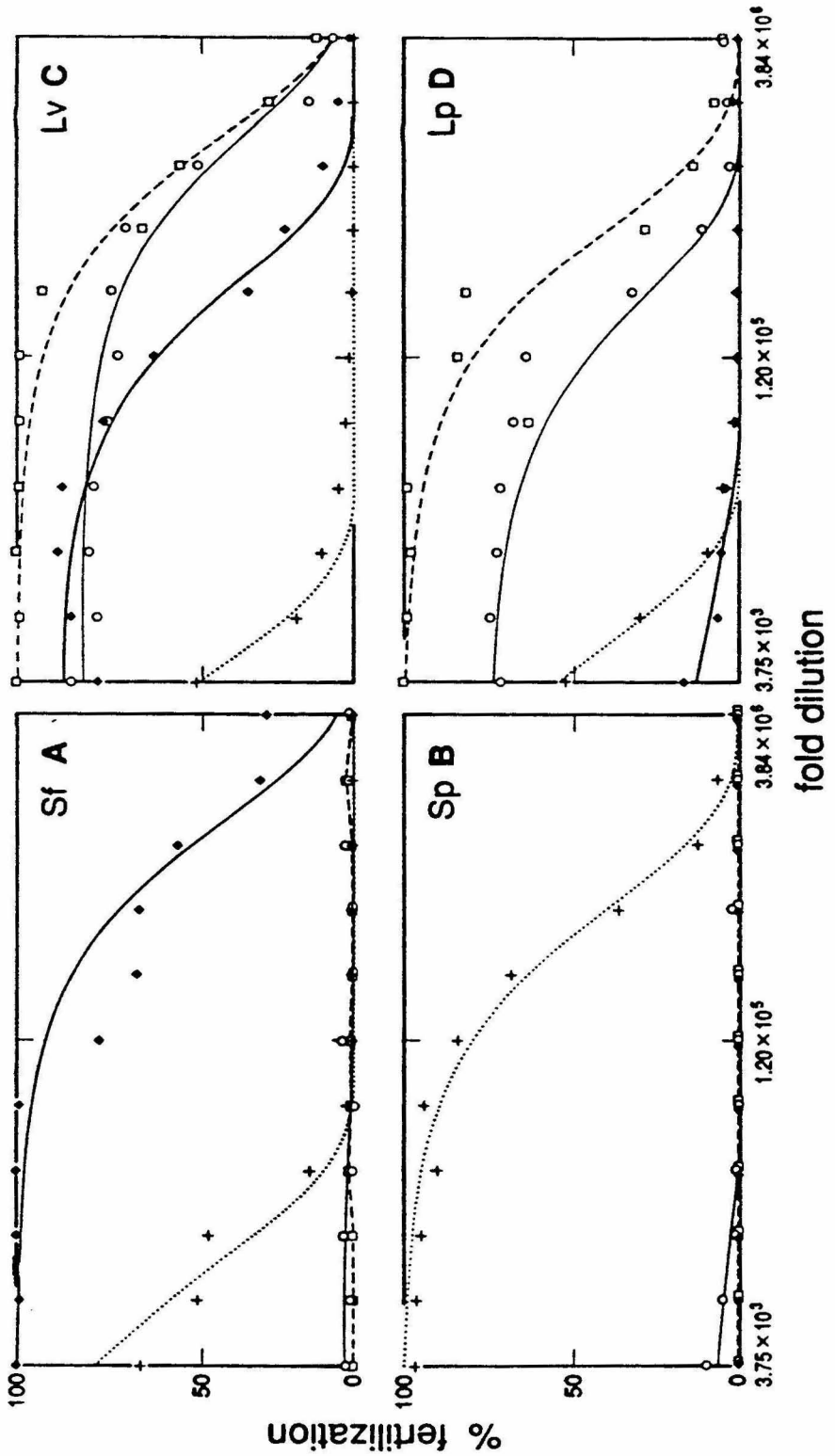
FIG. 4 - Genomic DNA blots probed with bindin cDNAs. Sperm genomic DNAs from a *S.franciscanus* individual (lane 1) and two *L.variegatus* individuals (lanes 2 and 3) were cut with *Bgl*II, separated on an agarose gel, and transferred to nitrocellulose. The filter was then hybridized to labeled cDNA from the entire *S.purpuratus* bindin cDNA (panel A) or the entire *S.franciscanus* bindin cDNA (panel B). The bindin cDNAs are single copy sequences in *Lytechinus*, and the *S.franciscanus* cDNA contains a sequence that is repetitive in the genome of *S.franciscanus*.

FIG. 5 - Comparison of the entire bindin precursor sequences of *S.purpuratus* (Sp), *S.franciscanus* (Sf), and *L.variegatus* (Lv). The sequences are aligned relative to *S.purpuratus*, amino acid identities are shown as overlined capital letters, mismatches in lower case letters. Deletions in sequence relative to *S.purpuratus* are marked with dashes

(-), while insertions are shown right justified above the amino acid that they follow. Large repeats in sequence are shown underneath each other, with their order in the sequence shown connected by lines. The vertical line that follows amino acid 245 in Sp marks the cleavage site that frees the mature acrosomal bindin from the precursor. Numbers 1 to 7 appearing beneath the *S.purpuratus* sequence after amino acid 420 mark repeats of the short consensus sequence.

FIG. 6 - Diagram of the protein sequence relationships between the bindins of *S.purpuratus* (Sp), *S.franciscanus* (Sf), and *L.variegatus* (Lv). The major block of conserved sequence is shown as a dark box at the core of the three bindins. Tracts of polyglycine are marked G. L marks repeats of the long consensus sequence GMGG(A/P)VGGG, while S marks repeats of the short sequence QGMGG(P/Q)(P/H).

Figure 1


Sf A
Lv C
Sp B
Lp D
% fertilization
100
50
0
3.75×10³
1.20×10⁵
3.84×10⁶
fold dilution

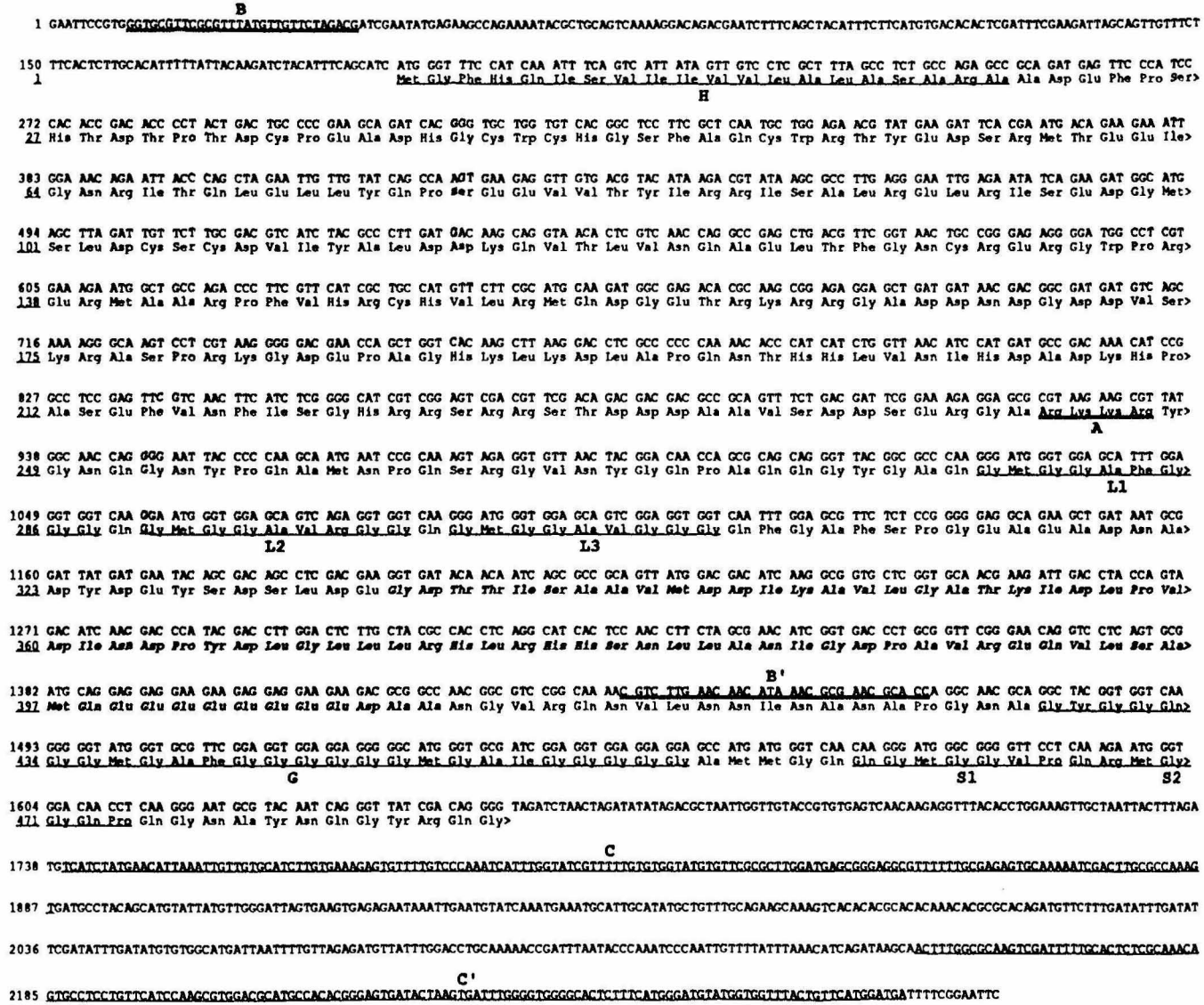


Figure 2

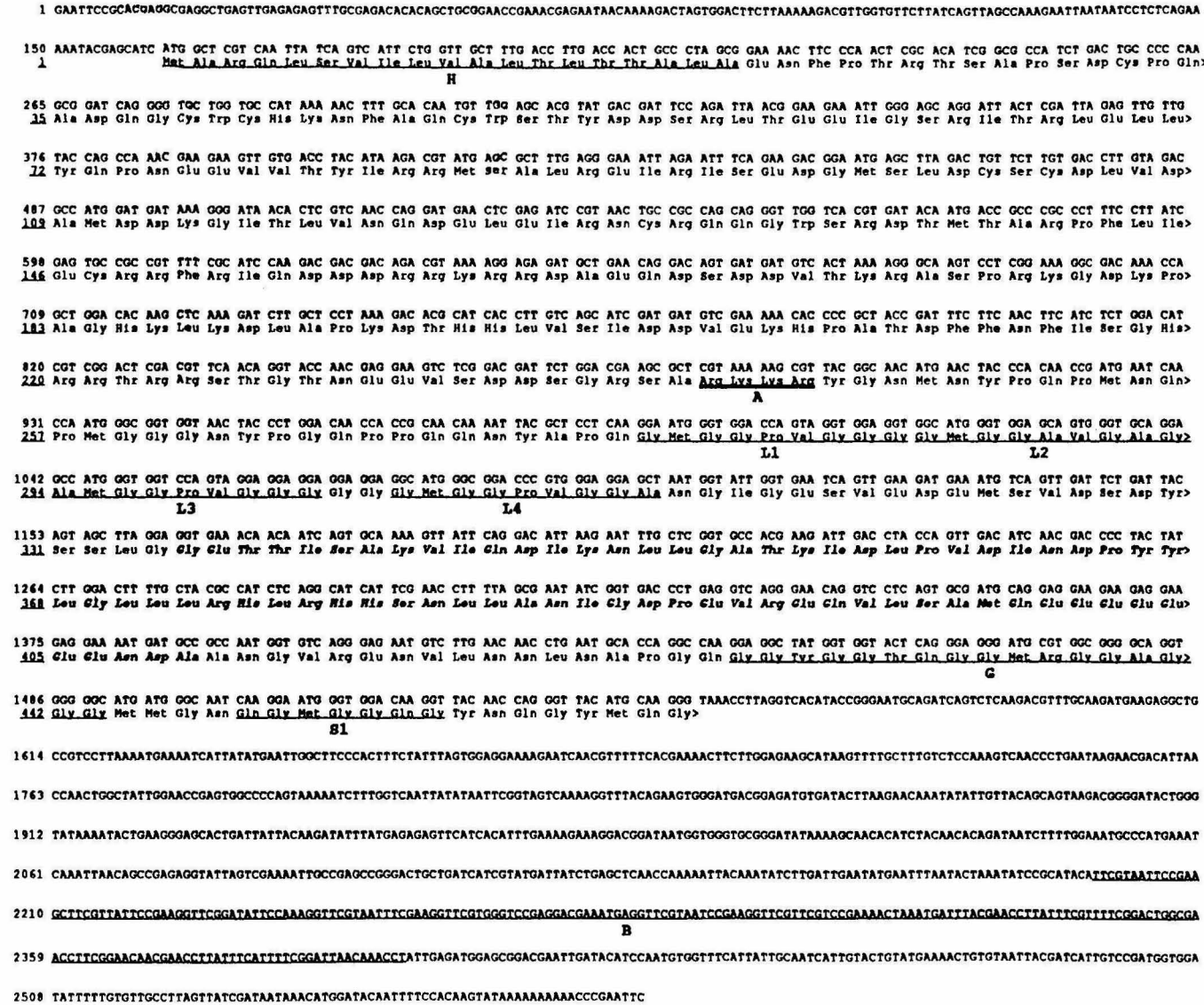
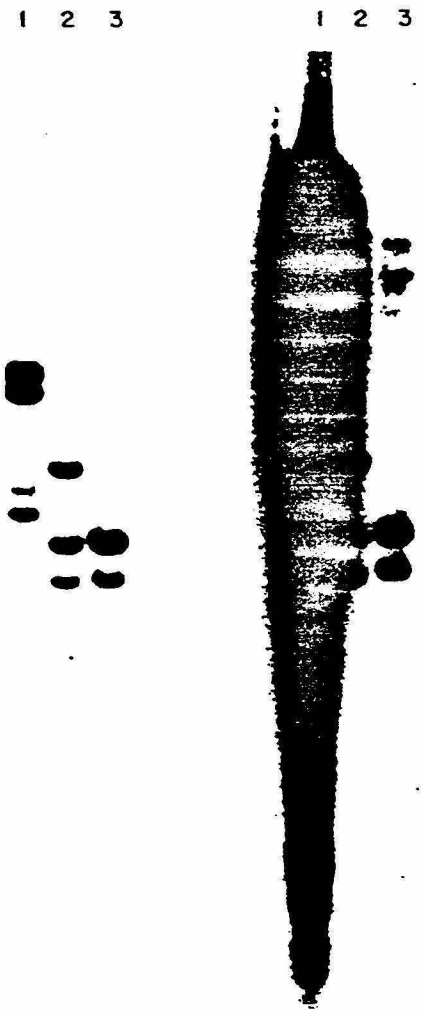


Figure 3

C-35

Figure 4



Probe-Sp bindin cDNA (A)

Probe-Sf bindin cDNA (B)

Figure 5

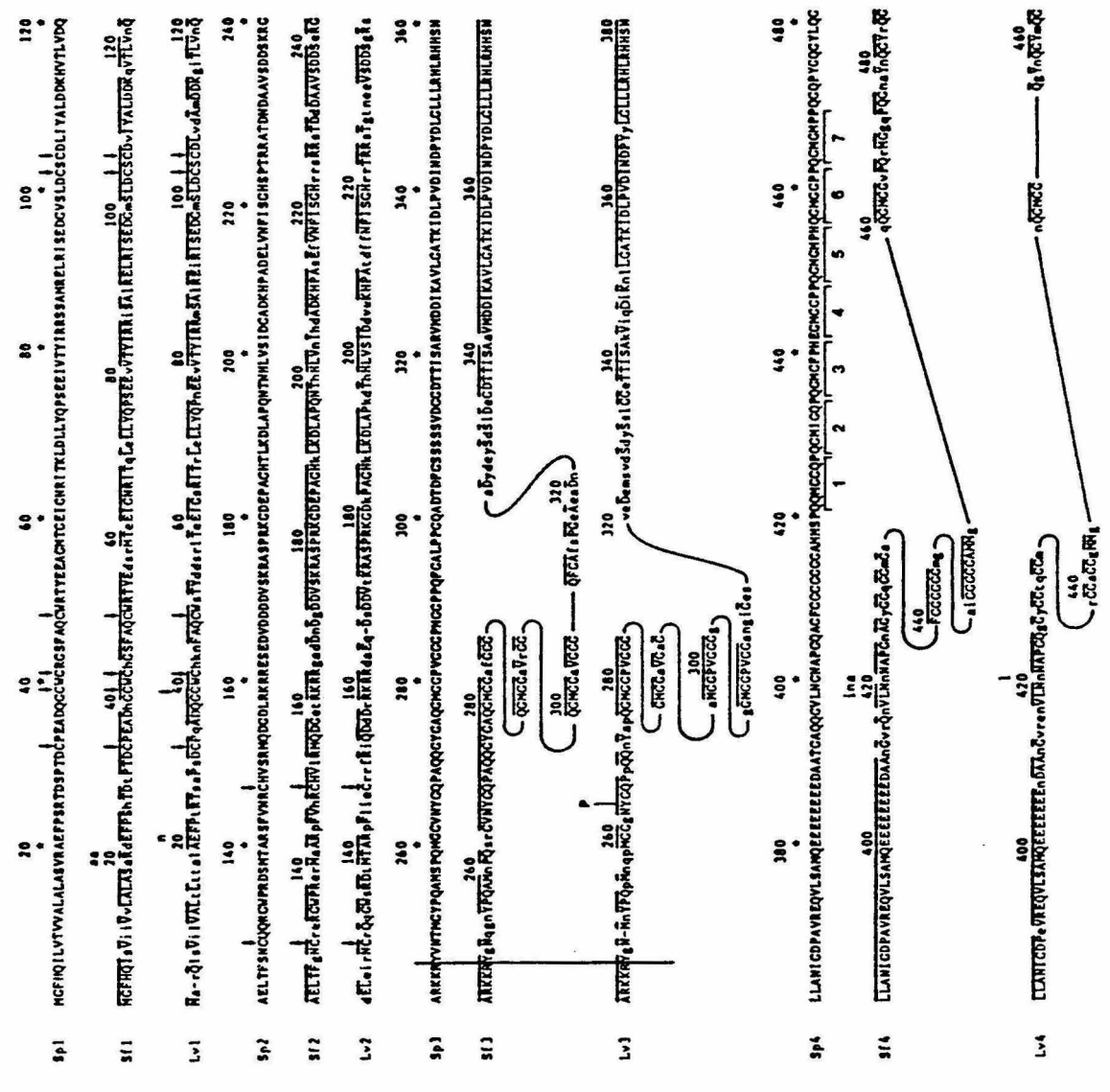
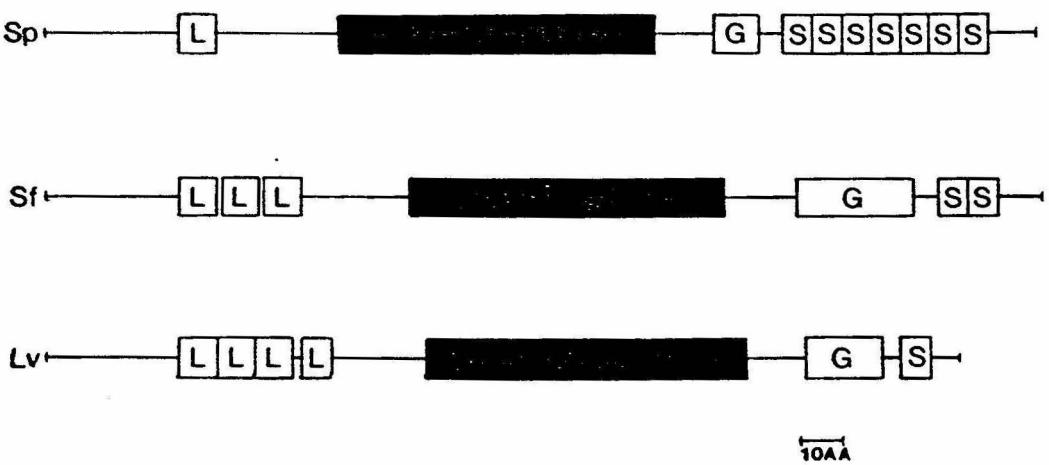


Figure 6



Chapter 4

Species-Specific Inhibition of Fertilization by a Peptide Derived from the Sperm Protein Bindin

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Running title: Bindin peptides inhibit fertilization

ABSTRACT

The sperm protein bindin is responsible for the species-specific adhesion of the sperm to the egg. The regions of the bindin molecule responsible for forming the contact between the sperm and the egg were investigated by assaying the ability of bindin-derived peptides to inhibit fertilization. Twenty-four peptides were studied: seven based on the *Strongylocentrotus purpuratus* bindin sequence, eleven based on the *S.franciscanus* bindin sequence, and six control peptides. Values for IC_{50}, the concentration of peptide required to inhibit 50% of the productive sperm contacts, were extracted from experimental measurements of the extent of fertilization in the presence of various concentrations of these peptides. The IC_{50} value averaged 220 μM for the control peptides. Five subregions of bindin are represented by peptides that had IC_{50} values less than 20 μM; the most potent peptide (SfO) had an IC_{50} value of 2.2 μM. Peptide SfR, derived from a region of the *S.franciscanus* bindin that differs from the *S.purpuratus* bindin, inhibited fertilization species-specifically. The peptides inhibit fertilization with a steep dose-response relationship, which probably reflects a requirement for the engagement of multiple bindin monomers in the initiation of the sperm-egg bond. These results demonstrate that a few specific regions of the bindin molecule are involved in the sperm-egg contact, and that these regions mediate the species-specificity of the interaction.

INTRODUCTION

Fertilization in sea urchins includes a series of interactions between the sperm and the egg (for reviews see Vacquier and Moy, 1978; Glabe *et al.*, 1982; Rossignol *et al.*, 1984a; Minor *et al.*, 1989). The specificity of fertilization can be due to an interspecies incompatibility at any of three steps in the fertilization pathway. These steps are chemotaxis of the sperm to the egg (Ward *et al.*, 1985), induction of the acrosome reaction (Summers and Hylander, 1975; SeGall and Lennarz, 1979), and binding of the sperm to the egg (Summers and Hylander, 1975; Glabe and Vacquier, 1977). Summers and Hylander (1975) determined that in most (9 of 11) cases the species-specificity of fertilization is due to the failure of sperm to bind productively to the eggs of other species. This is the case for the species *Strongylocentrotus purpuratus* and *S.franciscanus*, where the specificity of fertilization has been shown to be due to the specific interaction of the sperm adhesive protein bindin with a glycoprotein receptor of the egg (Glabe and Vacquier, 1977, 1978; see also Minor *et al.*, 1991). Bindin is packaged as an insoluble granule in the sperm acrosome. Upon contact with the egg, the sperm is induced to undergo the acrosome reaction that exposes bindin. Bindin remains associated with the sperm membrane and forms a contact with the receptor on the surface of the egg. For the species *S.purpuratus* and *S.franciscanus*, the

lowered efficiency of cross fertilization is due to a mismatch between bindin and its receptor. Bindin and its receptor mediate the specificity of this contact, and bindin may be involved in the subsequent membrane fusion as well (Glabe, 1985a, 1985b; Kennedy *et al.*, 1989).

The bindin protein from *S.purpuratus* was purified and partially sequenced (Vacquier and Moy, 1977, 1978; T. Sasagawa and K. Walsh, personal communication). This sequence was used to clone an *S.purpuratus* bindin cDNA, and the clone was used to show that the bindin gene is transcribed only in the testes (Gao *et al.*, 1986). The *S.purpuratus* bindin is synthesized initially as a 51 kDa precursor polypeptide that is cleaved to yield the 24 kDa acrosomal bindin from its C-terminus, plus a 27 kDa N-terminal protein of unknown function (Gao *et al.*, 1986). The bindin gene is transcribed relatively late in spermatogenesis (Cameron *et al.*, 1990; Nishioka *et al.*, 1990): the mRNA is immediately translated and bindin is then packaged into the acrosomal vesicle (Nishioka *et al.*, 1990). Bindin cDNAs representing the complete open reading frame have also been cloned and sequenced from three other species of sea urchin: *Arbacia punctulata* (Glabe and Clark, 1990), *Strongylocentrotus franciscanus*, and *Lytechinus variegatus* (Minor *et al.*, 1991). Like the *S.purpuratus* bindin, all of these bindins are cleaved from the C-terminus of much larger precursors. The bindins differ in several regions, and some or all of these differences must be responsible for the

species-specific activity of bindin.

In this study we attempted to determine whether there are particular elements of the bindins of *S.purpuratus* and *S.franciscanus* that are responsible for species-specific sperm adhesion. Peptides were synthesized to match various regions of the bindins of both species. If such peptides occupy the regions in the receptor that would normally be occupied by the corresponding areas of bindin, fertilization would be inhibited, were the bindin-peptide/receptor complex sufficiently stable, and were the complex capable of sterically preventing bindin from occupying the receptor. Some of the peptides we tested indeed block fertilization at very low concentrations. Peptide inhibition experiments used in other systems have been useful in mapping intermolecular contacts. For example, the contacts have been mapped between antibody and antigen (Haam *et al.*, 1988), α–bungarotoxin and the acetylcholine receptor (Lentz *et al.*, 1988), and transducin and rhodopsin (Koenig *et al.*, 1989). The results we report demonstrate that the species-specificity of bindin function is due to a sequence dependent, direct recognition process. It follows that the species-specificity of bindin function is due to the formation of species-specific molecular contacts between bindin and its receptor.

MATERIALS AND METHODS

Peptides

Peptides were synthesized and characterized by Suzanna Horvath and coworkers in the Microchemical Facility at Caltech. All peptides were greater than 80% pure (as judged by HPLC profiles). All peptides were checked for amino acid content, and those that differed from the expected molar ratios were sequenced (the errors in synthesis of peptides SpA and SfN were detected in this way). Peptide SfQ was further purified by preparative FPLC.

Peptides derived from the *Strongylocentrotus purpuratus* sequence are shown in Fig. 1; those derived from the *S.franciscanus* sequence are shown in Fig. 2. Six peptides were selected for control experiments on the basis that they showed no significant sequence similarity to bindin. The sequences of these peptides are (from N-terminus to C-terminus): CP1 (ASRDCQNGAV), CP2 (QNGGICIDGINGYT), CP3 (LNISEVKSQFYRYPFICVVKN), CP4 (IWDNWGVDKFDVYLSRRKC), CP5 (KPPSPISEAPRTLASC), and CP6 (MAKTAMAYKEKMKELSMLSLIC).

Inhibition of Fertilization Assay

All work was done in a 16°C room. Gametes were collected by either electric shock or by intracoelomic injection of 0.5 *M* KCl. The eggs were dejellied by washing in Millipore-filtered sea water (MPFSW) titrated to pH 5 by

the addition of citric acid, washed in MPFSW, then returned to MPFSW in an 800 egg/ml suspension. Six hundred µl of this suspension were placed in each well of a Falcon 3047 cell culture plate. Peptide solutions were prepared at a concentration 2.5-fold higher than the test concentration, and NaOH was added to bring the solutions to pH 8 (when necessary). The amount of peptide to be used was determined gravimetrically, assuming that the peptide samples were 100% pure. Four hundred µl of these solutions were added to the eggs. After a 1 hr incubation with peptide, 40 µl of a freshly prepared sperm dilution were added to the eggs. The sperm dilution was prepared by diluting dry sperm in MPFSW containing 0.005% bovine serum albumin (BSA, Sigma Fraction V). The dry sperm was titrated to determine the dilution necessary to achieve 75% fertilization in the controls. The eggs were fixed 5 min after the addition of sperm by the addition of an equal volume of 2% glutaraldehyde (Sigma) in MPFSW. The positions of the treatments and controls in the wells were randomized, and the plates coded before scoring. Over 100 eggs were counted for each experimental data point. Eggs were scored as fertilized on the basis of the elevation of the fertilization envelope when viewed at 100X in an inverted microscope. Eggs were treated with peptide, fertilized, fixed, and scored all in the same well to avoid the preferential loss of unfertilized eggs that occurs when eggs are transferred.

Definition of Terms

Let C be the fraction of eggs fertilized in the control (no peptide added)

$$C = \frac{\textit{number eggs fertilized (no peptide)}}{\textit{total number of eggs counted}} \qquad (1)$$

Let E be the fraction of eggs fertilized when peptide is added

$$E = \frac{\textit{number eggs fertilized (peptide present)}}{\textit{total number of eggs counted}} \qquad (2)$$

Then the fraction of eggs whose fertilization was blocked by the peptide, B, is given by

$$B = C - E \qquad (3)$$

Thus, the fraction of *fertilizations* inhibited by the peptide, *FFI*, is

$$FFI = \frac{B}{C} = 1 - \frac{E}{C} \qquad (4)$$

An expression was derived to quantify the fraction of productive sperm contacts inhibited, *F*. This was necessary for comparison of experiments that had differing degrees of fertilization in the control. The expression for *F* is derived here based on the assumption that the distribution of the sperm among the eggs is approximated by the Poisson distribution. In this case, the percentage of eggs in the sample that receive *R* active sperm is given by

$$\textit{fraction of eggs with R active sperm} = \frac{\mu^R}{R!e^{\mu}} \qquad (5)$$

where μ is the mean number of active sperm per egg

calculated from the Poisson zero class,

$$\mu = ln\left(\frac{1}{1 - C}\right) \qquad (6)$$

The fraction of the eggs with R active sperm whose fertilization is inhibited by peptide is given by the inhibitory factor, F, to the power R. For example, if F = 0.5, then of the eggs with only one sperm, one-half (F^1) of the eggs will not fertilize. Of the eggs with two sperm, only one-quarter (F^2) will not fertilize since both sperm must be inhibited to keep the egg from being fertilized. One-eighth (F^3) of the eggs with three sperm will not fertilize, one-sixteenth (F^4) of the eggs with four sperm will not fertilize, etc. The fraction B of eggs in the entire sample whose fertilization is inhibited is given by

$$B = \sum_{R=1}^{\infty} \left(\frac{\mu^R}{R!e^{\mu}} \left(F^R\right)\right) \qquad (7)$$

This can be rearranged to

$$B = \left(e^{(\mu F-\mu)}\right)\left(\sum_{R=1}^{\infty}\left(\frac{(\mu F)^R}{R!e^{\mu F}}\right)\right) \quad (8)$$

The right hand term is the Poisson series summation (minus the zero class), so equation 8 can be expressed as

$$B = \left(e^{(\mu F-\mu)}\right)\left(1-e^{-\mu F}\right) \quad (9)$$

Substituting equation 3 for B and equation 6 for μ, equation 9 can now be expressed as

$$F = 1 - \frac{ln(1 - E)}{ln(1 - C)} \quad (10)$$

This expression allows the fraction of sperm contacts inhibited to be determined from the experimental data. Note that equation 10 states that F is independent of sperm concentration.

Hill Analysis of μM versus F Data

A single determination of peptide inhibition consists of a set of data points of the inhibitory effect F of a peptide at a given μM concentration (e.g., see Fig. 3). In order to fit these data and extract the value for the amount of peptide necessary to achieve 50% inhibition of sperm contacts, a Hill analysis of the data was used (see, e.g., Marshall, 1978). This analysis assumes peptides (I) are filling sperm binding sites (S) in an equilibrium manner. If n peptides are required to inactivate a sperm binding site, then

$$S + nI \leftrightarrow SI_n \tag{11}$$

where SI_n signifies sites inactivated by being filled with n inhibitory peptides. An equilibrium constant K_I can then be defined as

$$K_I = \frac{[S][I]^n}{[SI_n]} \tag{12}$$

Since the filled binding sites are inaccessible for

fertilization, then

$$F = \frac{[SI_n]}{[S] + [SI_n]} \qquad (13)$$

Substituting equation 13 into equation 12 and rearranging yields

$$\log\left(\frac{F}{1 - F}\right) = n\log(I) - \log(K_I) \qquad (14)$$

In a plot of $\log(I)$ versus $\log[F/(1-F)]$ (Hill plot), n is the slope and $-\log(K_I)$ is the Y-intercept. The concentration of peptide, I (in μM), necessary for 50% inhibition of sperm contacts (IC_{50}), i.e. when $F = 0.5$, is given by

$$IC_{50} = (K_I)^{(1/n)} \qquad (15)$$

In this work a single determination of the value of n for a given peptide consists of at least four data points, of which the least inhibited has $0 < F < 0.75$ and the most inhibited has $0.75 < F < 1$. The value $F = 0.75$ is an important value for these titrations to span. When F = 0.75, FFI ≈ 0.5 (equation 4) which is where the highest transition from fertilized to unfertilized eggs is taking place. Of these determinations, only those that had $n \geq 1$ were used for the determination of IC_{50}. The majority of determinations for all of the peptides had $n > 1$ and the occasional titration curves yielding values of $n < 1$ show little dose response relationship due to high data scatter. These titrations are therefore likely to be misleading for determining IC_{50}, and have been omitted for the determinations of IC_{50} only.

The data for each determination were individually fit by least squares linear regression to the Hill equation, and values for n and IC_{50} were extracted. For all determinations, the fraction of eggs fertilized in the control was $0.50 < C \leq 0.90$, and averaged 0.75. Since the control fertilizations were performed within a narrow interval, the relative difference between fitting the peptide inhibition data to F or FFI was minimal. When the inhibition of fertilization data are fit to FFI rather than F, the values for n average 2% higher and the values for

IC_{50} average 40% higher. The relative values for IC_{50} are largely unaffected when fit to *FFI* rather then *F*, since all of the values increase to the same degree when fit to *FFI*.

Statistical Test of Species Differences in IC_{50}

Differences in IC_{50} for a given peptide between species were tested for statistical significance using the approximate *t*-test for the equality of two means (Sokal and Rohlf, 1981). Peptide SfR is the only peptide that has a significant difference in IC_{50} between the two species ($\alpha < 0.05$) [the species difference in IC_{50} for this peptide is also significant ($\alpha < 0.05$) if the determinations where n < 1 are also included].

Control Experiment Protocols

Observation of eggs through cleavage. The experiment is performed as in the inhibition assay, and all of the treatments are performed in duplicate. One of the treatments is fixed five minutes post sperm addition as in the inhibition assay, but the other treatment is fixed two hours after the addition of sperm. All of the treatments are scored for unfertilized eggs, fertilized eggs, and fertilized embryos that have cleaved.

Treatment of peptide inhibited eggs with A23187. The experiment is performed as in the inhibition assay, and all of the treatments are performed in duplicate. One of the treatments is fixed five minutes post sperm addition as in

the inhibition assay, but the other treatment has no sperm added and is fixed five minutes after the addition of 2.5 µM A23187 (Sigma). All of the treatments are scored for eggs with and without an elevated fertilization membrane.

Toxicity of peptide on sperm. In this assay, the peptide is added to the sperm dilution prior to its addition to the eggs. The sperm and peptide are coincubated for 5 min, then added to the eggs (which have no peptide added). Forty µl of the sperm-peptide mix were added to the eggs as in the inhibition of fertilization assay, and the assay is completed as described above for the inhibition assay.

Toxicity of peptide on eggs. After the 1 hr coincubation as in the inhibition assay, the MPFSW containing peptide is removed by aspiration and replaced with fresh MPFSW. After times varying from 1 min to 1 hr, sperm were added, and the assay proceeds as described above in the inhibition assay.

Evaluation of the acrosome reaction. The extent of the acrosome reaction in sperm treated with peptide was monitored by a modification of the phenyl bead method of Yamada and Aketa (1988). This method takes advantage of the fact that only acrosome reacted sperm stick to phenyl Sepharose beads. Phenyl Sepharose CL-4B beads were washed and equilibrated in MPFSW. Egg jelly was prepared as in Vacquier (1986). Experiments were performed in 13X100mm glass test tubes. Five hundred µl of MPFSW with or without

peptide and with or without egg jelly (1/8 dilution) were added to the tubes. Fifty µl of a 7% suspension (v/v) of phenyl beads were added to the tubes. Dry sperm was diluted 10 µl to 3 ml in MPFSW with 10 mM $NaHCO_3$ (Vacquier, 1986) and 0.005% BSA (Sigma). One hundred µl of this dilution were added to the tubes. One minute later, the sperm/beads were fixed with 8 ml of 2% glutaraldehyde in MPFSW. The beads were spun down (clinical centrifuge on low), and the supernatent was removed by aspiration. The beads were resuspended in 9 ml of fixative, spun down, and the supernatent was again removed by aspiration. This wash was repeated once more. The pelleted beads were resuspended and the number of sperm per bead diameter was counted at 320X under phase contrast.

Time course of peptide inhibition. Peptide is added to the eggs as in the inhibition of fertilization assay, but the peptide and eggs are coincubated for times ranging from 1 min to 1 hr. The eggs are then fertilized and fixed as described above in the inhibition assay.

RESULTS

Peptide Inhibition of Fertilization

The peptides used in this study are shown in Fig. 1 (*S.purpuratus* derived peptides), Fig. 2 (*S.franciscanus* derived peptides), and in Materials and Methods (control peptides). The inhibitory effects of peptides SfO and SpE on both *S.purpuratus* and *S.franciscanus* fertilizations are shown in Fig. 3. Peptide SpE does not inhibit the fertilization of either species very well (mean 50% inhibition concentration is 300 μM), and peptide SfO inhibits fertilization of both species at low concentrations (3.6 μM). Each experiment was separately fit to the Hill equation (equation 14, Materials and Methods) and an example is shown in Fig. 4. The regression is used to obtain the slope of the line, n, and the concentration at which 50% inhibition of sperm contacts occurs, IC_{50}. A summary of the data from all the inhibition experiments performed is shown in Tables 1 (*S.purpuratus* fertilizations) and 2 (*S.franciscanus* fertilizations).

The IC_{50} value ranges from 2.2 μM up to 740 μM for different peptides. The magnitude of this range means that the inhibition cannot be due to any contaminant common to the peptide synthesis or purification. The consistency between different synthesis preparations can be seen in the results for peptides SfM and SfN. These peptides were made

on different synthesis runs and differ only at the N-terminal amino acid. The values for *n* and IC_{50} (Tables 1 and 2) are similar for both of these peptides, so the results obtained do not depend on a particular peptide synthesis preparation. Peptide SfQ was further purified by FPLC, and the results obtained after FPLC were the same as before the FPLC purification (data not shown).

Control Experiments

Several experiments were performed to help determine when the peptides acted to inhibit fertilization. All of the peptides were tested for their ability to activate eggs and none of the peptides caused an elevation of the fertilization membrane when incubated with eggs.

The effect of the peptides on the development of fertilized eggs was monitored by observing the eggs through cleavage. Peptides SpC, SfO, and SfR were tested on both *S.purpuratus* and *S.franciscanus* fertilizations. Duplicate treatments were fixed at either 5 minutes or 2 hours after the addition of sperm. At peptide concentrations ranging from the IC_{50} value up to 4 times the IC_{50} value, the number of fertilized (fertilization envelope elevated) eggs that had cleaved at 2 hours was the same as the number of eggs that had an elevated fertilization membrane at 5 minutes. This indicates that the presence of these peptides did not inhibit or delay the cleavage of the fertilized eggs. There were no observed cases of eggs that had cleaved without an

elevated fertilization membrane, indicating that these peptides were not causing an apparent inhibition of fertilization by preventing the elevation of the fertilization envelope.

The possibility that the peptides were preventing the elevation of the fertilization envelope was addressed directly by adding the calcium ionophore A23187 to the peptide treated eggs. Peptides SpC, SfO, and SfR were tested on both *S.purpuratus* and *S.franciscanus* eggs. At peptide concentrations that were 8 times the IC_{50} values, when over 90% of the sperm contacts were inhibited in the duplicate treatments with added sperm, the addition of A23187 caused the elevation of all of the fertilization envelopes. This is a direct demonstration that these peptides do not inhibit the elevation of the fertilization envelope.

In the inhibition assay, eggs are treated with peptide, fertilized, fixed, and scored all in the same well without washing. After scoring fertilization, the number of sperm visible around the diameter of the unfertilized eggs was scored at 320X in an inverted microscope (the perimeter counting method of Kinsey *et al.*, 1980). The sperm were counted around the diameters of both peptide-treated eggs and untreated (control) eggs. Since the eggs were not washed after fertilization, the sperm visible are both sperm bound to the vitelline layer and sperm loosely "attached" in

a nonspecific manner (cf. Summers and Hylander, 1975; Wasserman *et al.*, 1986; Wasserman, 1990). The peptides tested were SpA, SpC, SpF, SpG, SfJ, SfK, SfL, SfM, SfO, SfP, and SfQ on *S.purpuratus* eggs. For all these peptides, the number of sperm around unfertilized eggs was the same in both the peptide-treated and control cases. This indicates that these peptides do not prevent the sperm from reaching the eggs.

The possibility of toxic effects of the peptides on both eggs and sperm was evaluated by washout experiments. The egg washout experiment tested peptides SpF, SfH, SfN, and SfP on *S.purpuratus* eggs. In this experiment, after the 1 hr coincubation of eggs with peptide, the MPFSW containing the peptide was removed by aspiration and replaced with fresh MPFSW. After 1 min, the eggs were fertilized as usual. The eggs in this experiment (peptide treated then washed) fertilized at the same levels as the untreated eggs, which indicates both that the eggs had not been poisoned by the peptides and that the inhibitory effects of the peptides are removed quickly by washing. The sperm washout experiment tested peptides SpB, SpC, SpD, SpE, SfI, and SfP on both *S.purpuratus* and *S.franciscanus* eggs. In this experiment the sperm are diluted into MPFSW containing the peptide. After a 5 min coincubation of sperm and peptide, 40 µl of the sperm-peptide mixture were diluted into 1 ml of MPFSW containing the eggs and the assay proceeds as in the inhibition assay. In all cases, there was no inhibition of

fertilization observed when IC_{50} concentrations of peptide were added to the sperm. These two washout experiments indicate that at the IC_{50} concentrations these peptides do not inhibit fertilization by poisoning either the sperm or the egg.

The effects of the peptides on the acrosome reaction was monitored by the phenyl bead method of Yamada and Aketa (1988). This method takes advantage of the fact that only acrosome reacted sperm stick to phenyl Sepharose beads. Sperm and phenyl beads are mixed either in the presence or absence of acrosome reaction inducers. When *S.purpuratus* sperm are induced to undergo the acrosome reaction by egg jelly, an average of ~20 sperm adhere per bead diameter (compared with ~1 in the absence of egg jelly). In the case of *S.franciscanus* sperm, ~30 sperm adhere per bead diameter. Higher numbers of sperm per bead diameter are obtained when the acrosome reaction is induced (in the absence of egg jelly) with either A23187 (Vacquier, 1986) or MPFSW containing 30 mM Tris 9.8 (Kinsey *et al.*, 1980). The effects of peptides SfM, SfO, SfQ, and SfR were tested on both *S.purpuratus* and *S.franciscanus* sperm. In the absence of egg jelly, IC_{50} concentrations of the peptides did not increase the number of sperm per bead diameter (0.64-2.2 for *S.purpuratus*, 0.5-1.5 for *S.franciscanus*). At 8 times the IC_{50} concentrations (in the presence of egg jelly), the peptides did not decrease the number of sperm per bead diameter (15-24 for *S.purpuratus*, 26-36 for *S.franciscanus*).

These results indicate that these peptides do not either induce or inhibit the acrosome reaction.

In the inhibition assay, peptide is coincubated with the eggs for 1 hr. To determine the minimum coincubation time required for inhibition of fertilization, shorter periods of coincubation ranging from 1 min to 1 hr were performed. Peptides SpF, SfH, SfN, and SfP were tested on *S.purpuratus* eggs. For these peptides, the fertilizations in the 1 min coincubation were as inhibited as those in the 1 hr coincubation. Taken together, the results of this experiment and the washout experiment indicate that the peptides inhibit fertilization quickly and reversibly.

Bindin Surface Contacts

Peptides were selected that collectively cover all areas of the *S.purpuratus* bindin where there were two or more consecutive amino acid sequence differences with the *S.franciscanus* bindin sequence (Fig. 1) and all areas of the *S.franciscanus* bindin sequence where there were three or more differences with the *S.purpuratus* sequence (Fig. 2). Of the 24 peptides tested for their ability to inhibit fertilization, 10 were effective at inhibiting fertilization (defined as those peptides with IC_{50} values less than 20 μM). None of the control peptides inhibited fertilization effectively. Only about one-third of the bindin derived

peptides are effective inhibitors of fertilization, if the fact that peptides SfJ-SfQ cover the same binding site is taken into account.

The size and sequence requirements of the contact defined by the peptide SfJ were investigated by using peptides related in sequence to peptide SfJ (see Fig. 2). Peptides SfK, SfL, SfM, and SfN were synthesized to cover the ends and middle of peptide SfJ. Peptides SfK and SfL were not potent inhibitors of fertilization, but peptides SfM and SfN retained the inhibitory activity of peptide SfJ. These two peptides correspond to the C-terminus of peptide SfJ, and contain only four amino acids that are not on the inactive peptide SfL. These amino acids are SLDE; if the serine is altered to threonine (TLDE), the peptides carrying the alteration (SfO, SfP, and SfQ) have increased inhibitory activity (Fig. 2). While this might seem a large effect for a conservative amino acid change, the reciprocal conservative change (threonine to serine) made in potassium channels results in changes in both the ionic conductivity (Yool and Schwarz, 1991) and TEA sensitivity (Yellen *et al.*, 1991) of the channels. Peptides carrying this inhibitory site are effective if they are as small as 10 amino acids (SfM, SfN, and SfO) or as large as 42 amino acids (SfQ).

The peptides selected for study contain several features of the *Strongylocentrotus* bindin sequences that have been previously defined by sequence comparisons (Minor

et al., 1991). Peptide SpD contains the polyglycine stretch (Fig. 1) and is a poor inhibitor of fertilization. Peptide SfI contains copies of the L repeat (Fig. 2) and is ineffective. Peptides SpE, SpF, and SpG all contain copies of the S repeat (Fig. 1) and have varying effects on fertilization: SpE is ineffective, SpF is effective, and SpG is borderline. Thus although some of the amino acids carried on peptide SpF that vary between the S repeats might confer inhibitory activity, these results suggest that the S repeats themselves do not define effective inhibitory regions.

Peptide SpC was synthesized to cover part of the conserved core sequences (Fig. 1) and is centered on the sequence LRHLRHHSN. This region was previously identified by DeAngelis and Glabe (1990a) as a sulfated fucan binding site in bindin. DeAngelis and Glabe showed that the peptide LRHLRHHSN binds to sulfated fucans, causes egg adhesion, and inhibits fertilization. Both the adhesive and inhibitory properties of LRHLRHHSN require added zinc. The concentration of LRHLRHHSN necessary for half maximal inhibition of fertilization was 400 μM. Peptide SpC shares both the egg adhesive (data not shown) and fertilization inhibitory properties of LRHLRHHSN (Fig. 1, Tables 1 and 2). Unlike LRHLRHHSN, peptide SpC does not require added zinc for either its adhesive or inhibitory activities. In addition SpC is a much more potent inhibitor of fertilization, with an IC_{50} value of 15 μM. Since the half

maximal inhibitory concentration (400 μM) of LRHLRHHSN places it among the least potent of the inhibitory peptides in this study (cf. control peptides CP1, CP2, CP4, and CP5; Table 1), it is possible that the higher fertilization inhibitory activity of peptide SpC is due to sequences on SpC that are not contained on LRHLRHHSN. The bindin sequence corresponding to peptide SpC is identical in both species of *Strongylocentrotus*, and SpC inhibits the fertilization of both *Strongylocentrotus* species equally well (Fig. 1, Tables 1 and 2).

Species-Specificity of Inhibition of Fertilization by Peptide SfR

The only peptide used in this study that showed a statistically significant difference in IC_{50} values between the two species was SfR, a peptide from a species-unique region of the *S.franciscanus* bindin (see Fig. 2). The inhibitory effects of peptide SfR on both *S.franciscanus* and *S.purpuratus* fertilizations are shown in Fig. 5. Figure 5A shows the inhibitory effect of peptide SfR on the fertilization of *S.franciscanus*. Four separate experiments were performed, each using a different male and female. The mean IC_{50} value for these experiments is 8.6 μM (Table 2, last line). Figure 5B shows the inhibitory effects of peptide SfR on three different *S.purpuratus* fertilizations. The mean inhibitory value for these experiments is 120 μM (Table 1, last line). Representative Hill plots for one

experiment from each species are shown in Fig. 6. As can be seen in both figures, there is a greater than tenfold difference in the IC_{50} concentration for this peptide between the two species. This difference between the species indicates a high degree of species-specificity in the inhibitory activity of peptide SfR.

DISCUSSION

Comparison to Other Peptide Inhibition Studies

The results reported here indicate that small, soluble peptides derived from the sperm protein bindin are capable of inhibiting fertilization. Eleven of the 24 peptides tested had IC_{50} values below 20 μM, the most inhibitory being SfP (IC_{50} = 2.2 μM). The IC_{50} concentrations of these peptides are in the range of the most effective peptides found to inhibit association in other studies. These include peptide inhibition of the interaction of: HEL(46-61)-peptide with the MHC protein I-A^k (IC_{50} = 10 μM, Buus *et al.*, 1987); transducin with anti-tranducin monoclonal antibody 4A (IC_{50} = 10 μM, Haam *et al.*, 1988); α-bungarotoxin with the acetylcholine receptor (IC_{50} = 17 μM, Lentz *et al.*, 1988); and transducin with rhodopsin (IC_{50} = 50 μM, Koenig *et al.*, 1989). The most active peptides from each study are compared here; in all these studies peptides with much higher IC_{50} values were also tested.

With the exception of the bindin derived peptide

LRHLRHHSN used by DeAngelis and Glabe (1990a), we are unaware of other studies using unmodified peptides to inhibit fertilization. The peptide LRHLRHHSN 50% inhibits fertilization at 400 µM, and only in the presence of 0.4 µM zinc (DeAngelis and Glabe, 1990a). The modified peptide chymostatin inhibits fertilization at 150-200 µM (Hoshi *et al.*, 1979; Glabe *et al.*, 1981). Roe *et al.* (1988) reported two modified peptides, succinyl-alanyl-alanyl-phenylalanyl-4-aminomethylcoumarin and carbobenzoxy-glycyl-phenylananyl-NH_2, 50% inhibited fertilization at 450 µM and 1,370 µM respectively. Matsumura and Aketa (1991) reported that the modified peptide succinyl-lysyl-lysyl-valinyl-tyrosinyl-metylcoumaryl-7-amide inhibited the acrosome reaction at 800 µM. Hoshi *et al.* (1979) reported that soybean trypsin inhibitor, leupeptin, and antipapain did not inhibit fertilization but did inhibit the elevation of the fertilization envelope. Peptides SpC, SfO, and SfR were shown to not inhibit the elevation of the fertilization envelope of either species of sea urchin (both by monitoring eggs through cleavage and by using the calcium ionophore A23187). The concentrations of these modified peptides required to inhibit fertilization are in the range of the control peptides we used (see Tables 1 and 2), and all are much higher than the concentration limit we set for active inhibitory peptides (20 µM).

Cooperative Peptide Inhibition of Fertilization

A general property of the inhibitory effects of these peptides is a steep dose response, i.e., the inhibition changes from no inhibition to complete inhibition over a narrow interval of concentration (Figs. 3 and 5). The steepness of this dose response is reflected in the Hill coefficient, *n*, which is the slope of the line fit to the Hill equation (equation 14, Figs. 4 and 6). The average of the Hill coefficients for all the peptides used in this study is 2.1 (± 0.8); the average of the effective peptides on *S.franciscanus* eggs is 2.7 (± 0.4). This Hill coefficient indicates that the concerted action of a minimum of 2 to 3 peptides is necessary to inhibit fertilization. An analogous situation occurs in the peptide inhibition of rhodopsin-transducin association (Koenig *et al.*, 1989). The rhodopsin-derived inhibitory peptides also demonstrate a steep dose response, with Hill coefficients of 2-3, because they interfere with a cooperative binding process.

Bindin is packaged as an insoluble granule of protein inside the sperm acrosome. When the sperm contacts the egg, the exocytosis of the acrosome exposes bindin on its surface. The bindin granule remains associated with the sperm acrosomal membrane and forms a large contact with the surface of the egg. The size of this contact varies, but can be estimated to be about 0.25 μm^2 from electron micrographs of sperm-egg contacts in which bindin has been

stained with immunoperoxidase (Moy and Vacquier, 1979). Given this area and the molecular weight of bindin, there could be as many as 10^4 bindin monomers in the contact region between the sperm and the egg, though only a small fraction of these might be productively engaged with the bindin receptor on the egg surface. The intact bindin receptor is a very large glycoprotein complex (Glabe and Vacquier, 1978; Rossignol *et al.*, 1984b). Digestion of the intact receptor with lysylendoproteinase C releases a 70 kDa fragment that retains the ability to bind species-specifically to bindin (Foltz and Lennarz, 1990). The specificity of the peptide inhibitions reported here implies that the peptides are occupying portions of the recognition sites on the receptor. Thus the average Hill coefficient of 2.7 can be interpreted as indicating that at each sperm binding site at least 2-3 receptor target sites must be occupied by peptides to prevent the sperm from binding. The data cannot be used to determine how many bindin-receptor interactions are involved in the formation of the sperm-egg contact; the data only indicate that within the sperm-egg contact region a minimum of 2-3 of these interactions must be inhibited to prevent fertilization.

Species-Specificity of the Bindin-Receptor Interaction

Earlier sequence comparisons between the mature bindins of *S.franciscanus* and *S.purpuratus* revealed that the middle third of the protein is conserved between the two species,

while the N- and C-terminal thirds display both point changes and variations in the numbers of direct repeats of 7-10 amino acid long sequence elements (Minor *et al.*, 1991). These changes can be seen by comparing the bindin sequences in Figs. 1 and 2. One explanation of how these changes in bindin affect species-specificity is to assume that the variable sequences encode species-specific binding domains on the surface of the bindin molecule, and that these sequences are matched by complementary species-specific target sites on the receptor. An alternative explanation of the cause of species-specificity, the "supramolecular theory" (Glabe, 1978, 1979), postulates that specificity could be due to the same binding site being presented in different, species-specific, ordered arrays on the surfaces of the sperm of the two species. In the supramolecular model, a randomly packed field of bindin-receptor contacts would have insufficient strength to maintain sperm binding. A "threshold" value for binding could only occur between species-compatible arrays of ordered monomers in the bindin granule and ordered receptors in the vitelline envelope.

Peptide SfR is derived from a unique region of the *S.franciscanus* bindin, and this peptide species-specifically inhibits *S.franciscanus* fertilization. This fact directly supports the notion that the species-specificity of the bindin-receptor interaction is due to a sequence specific recognition process. This result would not be expected if species-specificity were due to different ordering of arrays

presenting the same binding site, as predicted by the "supramolecular theory."

Five different areas of the bindins were identified that could be involved in direct surface contacts between bindin and its receptor. These regions are represented by the peptides SfR, SpC, SpA, SpF, and SfM. Peptide SfR inhibits fertilization species-specifically, while the other four peptides inhibit the fertilization of both species equally well. The lack of a species difference in the activity of the peptide SpC is the easiest to understand, since the amino acid sequence of peptide SpC is identical in both species of bindin. Therefore, peptide SpC represents an area of bindin involved in a contact (probably to sulfated fucans; DeAngelis and Glabe, 1988, 1990ab) that is conserved between the two species. Peptides SpA, SpF, and SfM might also be involved in conserved contacts, despite the differences between the two bindins in these areas. Thus some amino acids are conserved between the bindins of these two species in these areas, and the ability of the SpA, SpF, and SfM to inhibit the fertilization in both species could be explained if it is the conserved amino acids that form the important contacts with a conserved receptor structure. It is also possible that the bindin regions represented by these peptides have indeed changed functionally between the two species, but the corresponding target site on the receptor has not changed. Such "vestigial" characters, if present in the receptor, could

make it possible for a peptide derived from a unique area of the bindin of a given species to inhibit the fertilization of both species. In any case, the data indicate that there are five subregions of the bindins that are involved in the sperm-egg contact, but only one of these regions (that included on the 30 amino acid long peptide SfR) seems to constitute a species-specific binding domain.

There are many sequence differences in the region homologous to SfR in the sea urchin bindins that have been sequenced (Fig. 7). The most notable change between the *Strongylocentrotus* species is a three amino acid insertion in the *S.franciscanus* bindin sequence, relative to the homologous area of the *S.purpuratus* bindin. Curiously, the sequence encoding these 3 amino acids is included in a 30 nt long stretch of DNA also present as an inverted repeat (29/30 nt match) in the 5' untranslated region of the *S.franciscanus* bindin mRNA (Minor *et al.*, 1991).

Evolutionary Implications

Many marine invertebrates utilize external fertilization and planktonic larvae. The species-specificity of gamete interaction is likely to play a major role in establishing the reproductive isolation necessary for speciation in these lineages. The results reported here imply that changes in the species-specificity of gamete interaction could result from a small change in the primary sequence of the sperm recognition protein. Peptide SfR is

derived from a unique area of the *S.franciscanus* bindin and specifically inhibits *S.franciscanus* fertilization. The peptide covering the homologous region of the *S.purpuratus* bindin (SpD) is an ineffective inhibitor of fertilization of both species. These results can be interpreted to indicate a gain of a unique interaction region on one molecule (e.g., the bindin of *S.franciscanus*) accompanied by a compensatory change in its partner (i.e., the *S.franciscanus* receptor) that creates a novel and specific sperm binding site. Further understanding of how changes in sperm-egg recognition evolved will require studies of the structures of both bindin and the receptor responsible for specific recognition. The recent demonstration that a soluble receptor fragment retains the ability to species-specifically recognize bindin (Foltz and Lennarz, 1990) greatly improves prospects for such structural studies. Studies of variant functional domains and their distribution in populations will elucidate the structures responsible for the specificity of sperm adhesion and will provide information on how these changes are involved with speciation.

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TABLE 1

HILL COEFFICIENTS AND 50% INHIBITORY CONCENTRATIONS FOR PEPTIDES USED TO INHIBIT *S.PURPURATUS* FERTILIZATION

Peptide	n^a	SDn[b]	Det.[c]	IC50[d]	SDIC[e]	Det.[f]
CP2	3.0	-	1	670	-	1
SpE	1.4	0.5	3	510	170	2
SfH	1.8	0.8	4	420	100	3
SpB	2.1	2.1	4	210	130	2
CP1	3.2	0.5	2	200	80	2
SpD	1.8	0.8	3	190	70	2
CP4	2.6	1.7	2	170	160	2
CP5	1.5	0.1	4	110	60	4
SfL	1.5	0.4	4	92	77	3
CP6	1.8	0.4	2	58	13	2
CP3	1.3	0.1	4	40	31	4
SfK	2.6	0.4	3	37	12	3

SpG	1.5	0.4	3	26	6	3
SfI	1.5	0.6	2	22	2	2
SfJ	2.2	0.4	3	17	6	3
SpA	1.6	0.6	4	16	8	3
SpF	0.8	0.2	2	16	-	1
SpC	1.6	0.3	4	15	6	4
SfQ	2.1	0.4	4	12	4	4
SfN	2.5	0.4	5	12	9	5
SfM	2.2	0.8	4	10	7	4
SfO	2.3	0.7	5	3.6	1.8	5
SfP	1.7	0.5	5	2.2	1.6	5
SfR	1.2	0.6	6	120	40	3

[a] Average Hill coefficient. [b] Standard deviation of the Hill coefficient measurements. [c] Number of determinations. [d] Concentration (in μM) of peptide necessary for 50% inhibition of sperm contacts; average of experiments where $n \geq 1$. [e] Standard deviation of the 50% inhibitory values.

[f] Number of determinations with $n \geq 1$. Peptides are arranged from the least inhibitory to the most inhibitory. The first group of peptides has inhibitory values greater than 20 μM. The second group has inhibitory values less than 20 μM. Twenty μM was chosen as the arbitrary dividing line between "inactive" and "active" peptides since the lowest inhibitory value seen in the control peptides was 40 μM (CP3). The last peptide (SfR) was the only peptide that had a statistically significant species difference in its inhibitory concentrations (see also Table 2).

TABLE 2

HILL COEFFICIENTS AND 50% INHIBITORY CONCENTRATIONS FOR PEPTIDES USED TO INHIBIT *S.FRANCISCANUS* FERTILIZATION

Peptide	*n*	SDn	Det.	IC50	SDIC	Det.
CP2	5.0	-	1	740	-	1
CP1	2.3	-	1	260	-	1
SpE	1.3	0.7	7	220	170	5
SfH	1.8	1.2	4	100	90	3
SpB	1.5	0.6	3	92	40	2
SfL	2.0	1.3	3	64	16	3
SpD	2.3	0.8	4	38	25	4
SfK	1.9	0.1	2	36	11	2
SfI	0.7	0.3	3	-	-	-
SfN	2.5	0.9	4	18	7	4
SfJ	2.8	0.8	4	16	5	4

SpC	1.9	0.5	3	13	5	3
SfM	3.2	0.8	5	13	3	5
SpG	3.0	1.1	5	12	6	5
SpF	2.8	1.1	2	12	17	2
SfQ	2.6	0.9	5	8.2	5.1	5
SfP	2.6	2.3	3	6.5	4.1	2
SpA	3.3	0.9	3	5.5	4.8	3
SfO	2.5	1.0	4	3.8	2.0	4
SfR	2.4	0.9	4	8.6	5.2	4

Note. Column headings and arrangement are the same as for Table 1. The last peptide (SfR) was the only peptide that had a statistically significant species difference in its inhibitory concentrations (see also Table 1).

FIG. 1. *Strongylocentrotus purpuratus* bindin sequence and *S.purpuratus* derived peptides. The *S.purpuratus* bindin protein sequence (Gao *et al.*, 1986) is shown numbered every 10 amino acids. Features of bindin are shown above the sequence and are defined in Minor *et al.* (1991). "L1" marks a copy of the long consensus sequence; "glyc." indicates a polyglycine stretch; and "S1-S7" mark copies of the short consensus sequence. Underlined amino acids are those that differ between the two species. Peptides (SpA to SpG) synthesized to match portions of the bindin protein are shown beneath the regions that they match, and are labeled in bold. Lowercase letters in the peptide sequences indicate amino acids that were added during synthesis that do not match the bindin sequence. The two dashes in the SpA sequence indicate that this peptide was synthesized without the amino acids AQ. The values in italics indicate the concentration (in μM) of peptide necessary to inhibit sperm contacts 50%. The value "p" indicates the effect of the peptides on *S.purpuratus* fertilizations, the value "f" indicates the effect on *S.franciscanus* fertilizations. **a** This value is from one experiment only.

FIG. 2. *Strongylocentrotus franciscanus* bindin sequence (Minor *et al.*, 1991) and *S.franciscanus* derived peptides. The nomenclature is as in Fig. 1. "L1-L3" mark copies of the long consensus sequence, "inv rep" marks 10 amino acids translated from a 30 nt stretch that is present as an

inverted repeat in the 5' untranslated region of the *S.franciscanus* bindin mRNA, and "S1-S2" mark copies of the short consensus sequence. Underlined amino acids are those that differ between the two species. SfH-SfR indicate the synthesized peptides. The double underlined "t" in peptides SfO, SfP, and SfQ indicates that these peptides were synthesized with a serine altered to threonine. [a] This value is from data where $n < 1$ (see Materials and Methods).

FIG. 3. Inhibitory effect of bindin derived peptides on fertilization. The data are graphed as μM peptide concentration (log scale, X-axis) versus fraction of sperm contacts inhibited (F, Y-axis). Different data symbols represent independent experiments done on different days with different batches of eggs and sperm. A-D show measurements done with peptides SpE and SfO on *S.franciscanus* eggs (Fran) or *S.purpuratus* eggs (Purp). Mean inhibitory levels are: SfO/Fran - 3.8 μM, SfO/Purp - 3.6 μM, SpE/Fran - 220 μM, and SpE/Purp - 510 μM.

FIG. 4. Hill plot of peptide inhibition of fertilization. The data plotted are for a single experiment using peptide SfP to inhibit *S.purpuratus* fertilization. The line is fitted by least squares linear regression using the Hill equation (equation 14, Materials and Methods) and its slope is the Hill coefficient n (in this experiment $n = 2.5$). The point where fertilization is half inhibited ($F = 0.5$) occurs when $\log[F/(1-F)] = 0$. The dotted line indicates the

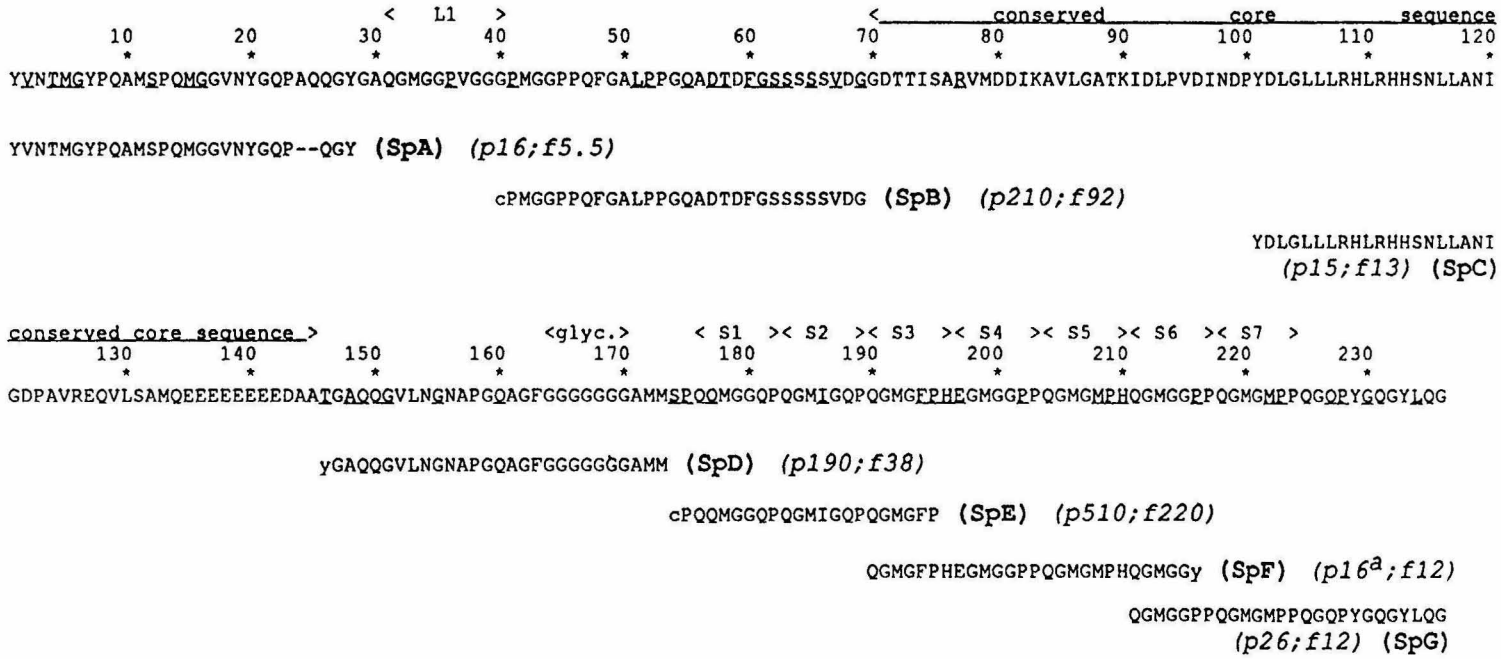
concentration of peptide required for 50% inhibition. In this case, at F = 0.5, log(μM) = 0.7, and IC_{50} = 5 μM.

FIG. 5. Species-specific inhibition of fertilization by peptide SfR. The format is the same as for Fig. 3. Different data symbols represent independent experiments done on different days with different batches of eggs and sperm. (A) Inhibition of *S.franciscanus* fertilization by peptide SfR; mean IC_{50} value is 8.6 μM. (B) Inhibition of *S.purpuratus* fertilization by peptide SfR; mean IC_{50} value is 120 μM.

FIG. 6. Hill plot of peptide SfR inhibition of fertilization of both *S.purpuratus* and *S.franciscanus*. One individual experiment is shown for each species. The data symbols (*X* for the *S.franciscanus* experiment; $\oplus$ for the *S.purpuratus* experiment) correspond to the experiments with the same symbols in Fig. 5. The fit for the *S.franciscanus* experiment (solid line) yields a Hill coefficient *n* of 2.2 and a 50% inhibitory value (IC_{50}) of 2.6 μM. For the *S.purpuratus* fit (dotted line) *n* = 2.3 and IC_{50} = 160 μM.

FIG. 7. Sequence of the species-specific inhibiting peptide, SfR. Shown beneath the SfR sequence are the homologous regions of the bindins from *Strongylocentrotus franciscanus* (Minor *et al.*, 1991), *S.purpuratus* (Gao *et al.*, 1986), *Lytechinus variegatus* (Minor *et al.*, 1991), and *Arbacia punctulata* (Glabe and Clark, 1991). Amino acids deleted relative to the *S.franciscanus* bindin are shown as

dashes, mismatched amino acids are shown in lowercase. A threonine insertion in the *Lytechinus* sequence is shown as a "t" over the glycine that it follows. The double underlined sequence in the *S.franciscanus* bindin sequence indicates 10 amino acids encoded by a DNA sequence in the coding region of the *Strongylocentrotus franciscanus* bindin mRNA that is also contained in the 5' untranslated region as an inverted repeat (Minor *et al.*, 1991).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YVNTMGYPQAMSPQMGGVNYGQP--QGY (SpA) (p16;f5.5)
cPMGGPPQFGALPPGQADTDFGSSSSSVDG (SpB) (p210;f92)
YDLGLLLRHLRHHSNLLANI
(p15;f13) (SpC)
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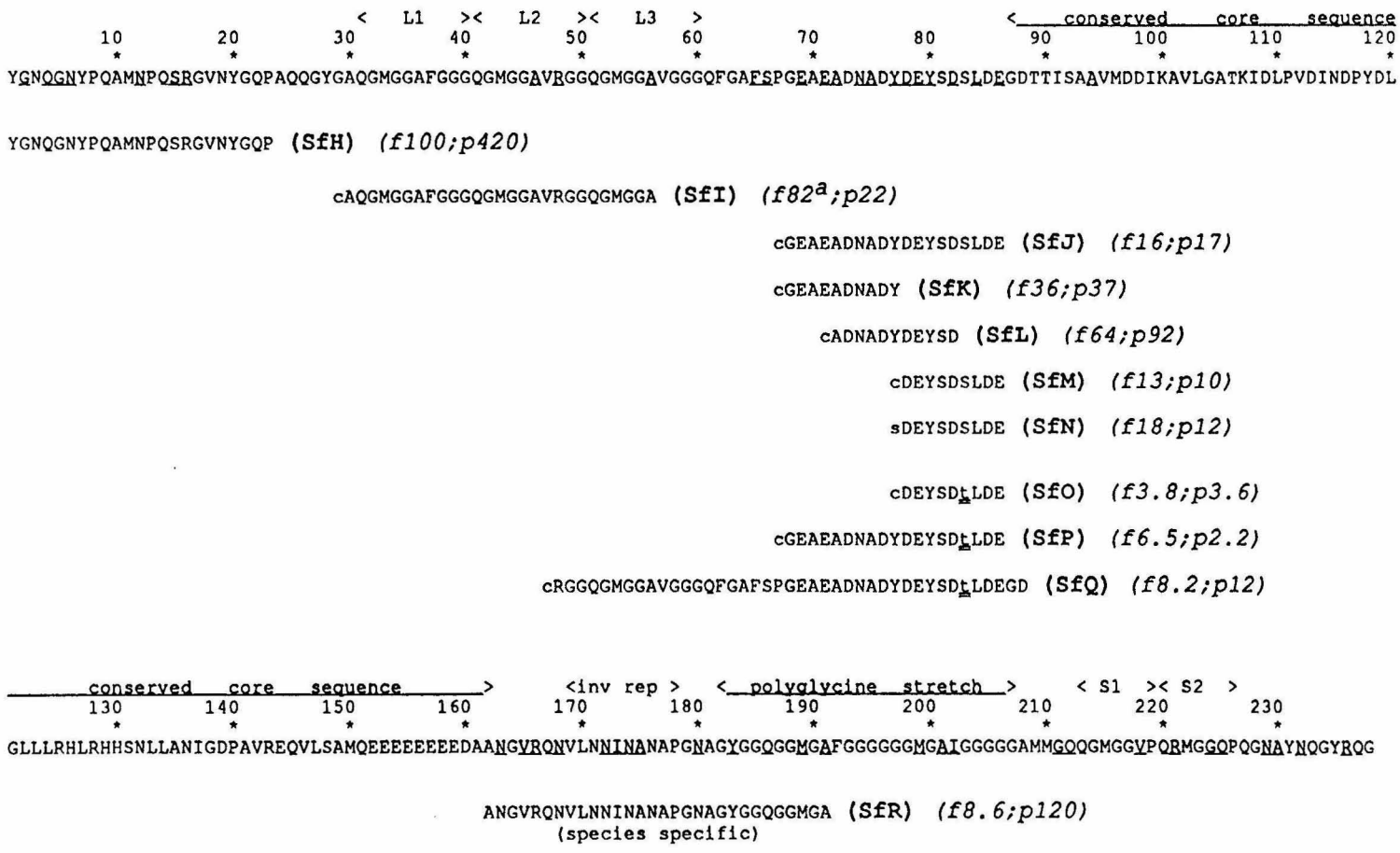
yGAQQGVLNGNAPGQAGFGGGGGGGAMM (SpD) (p190;f38)
cPQQMGGQPQGMIGQPQGMGFP (SpE) (p510;f220)
QGMGFPHEGMGGPPQGMGMPHQGMGGy (SpF) (p16a;f12)
QGMGGPPQGMGMPPQGQPYGQGYLQG
(p26;f12) (SpG)

Figure 1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YGNQGNYPQAMNPQSRGVNYGQP (SfH) (f100;p420)
cAQGMGGAFGGGQGMGGAVRGGQGMGGA (SfI) (f82a;p22)
cGEAEADNADYDEYSDSLDE (SfJ) (f16;p17)
cGEAEADNADY (SfK) (f36;p37)
cADNADYDEYSD (SfL) (f64;p92)
cDEYSDSLDE (SfM) (f13;p10)
sDEYSDSLDE (SfN) (f18;p12)
cDEYSDtLDE (SfO) (f3.8;p3.6)
cGEAEADNADYDEYSDtLDE (SfP) (f6.5;p2.2)
cRGGQGMGGAVGGGQFGAFSPGEAEADNADYDEYSDtLDEGD (SfQ) (f8.2;p12)
conserved core sequence > <inv rep > < polyglycine stretch > < S1 >< S2 >
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ANGVRQNVLNNINANAPGNAGYGGQGGMGA (SfR) (f8.6;p120)
(species specific)

Figure 2

Figure 3

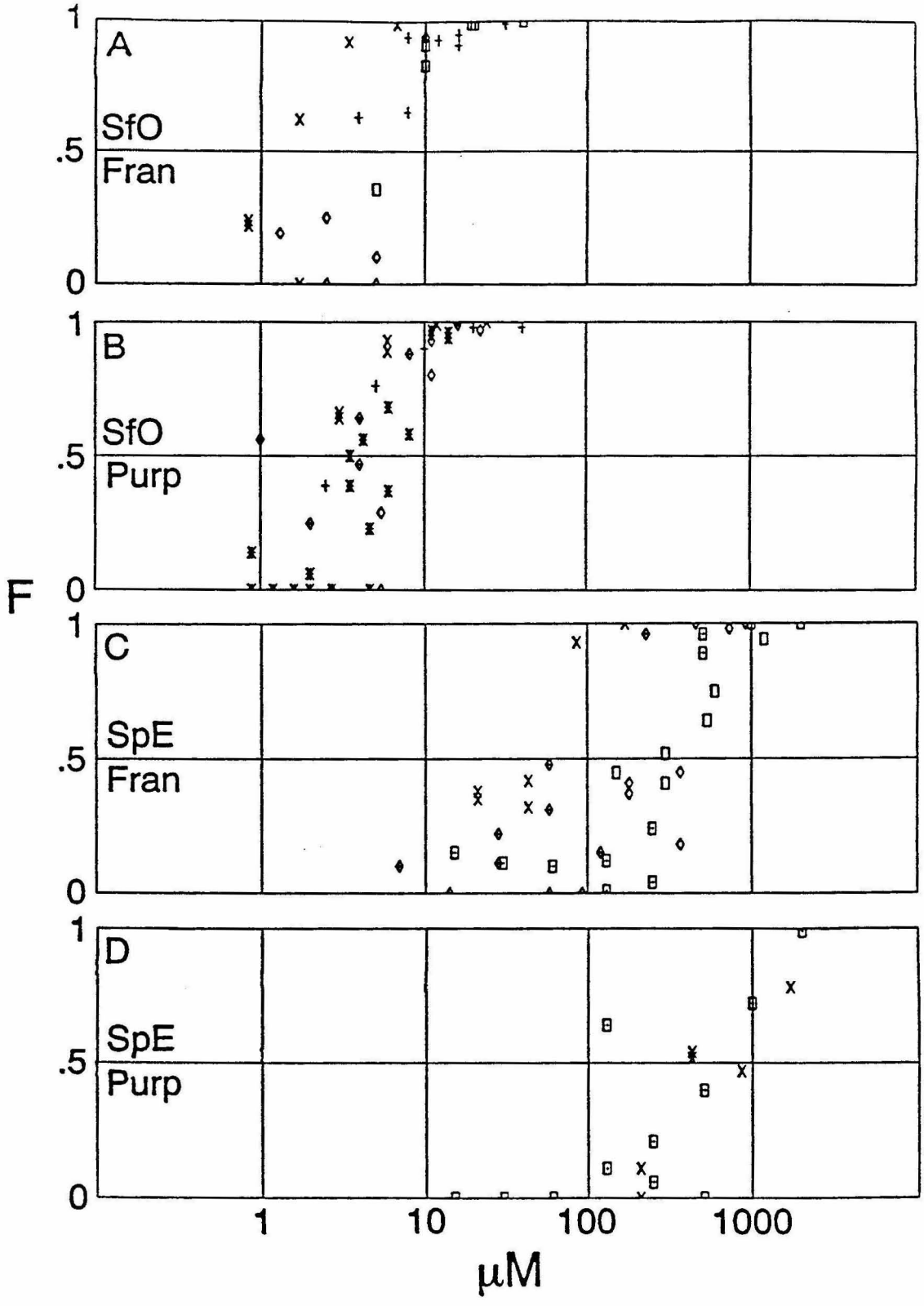


Figure 4

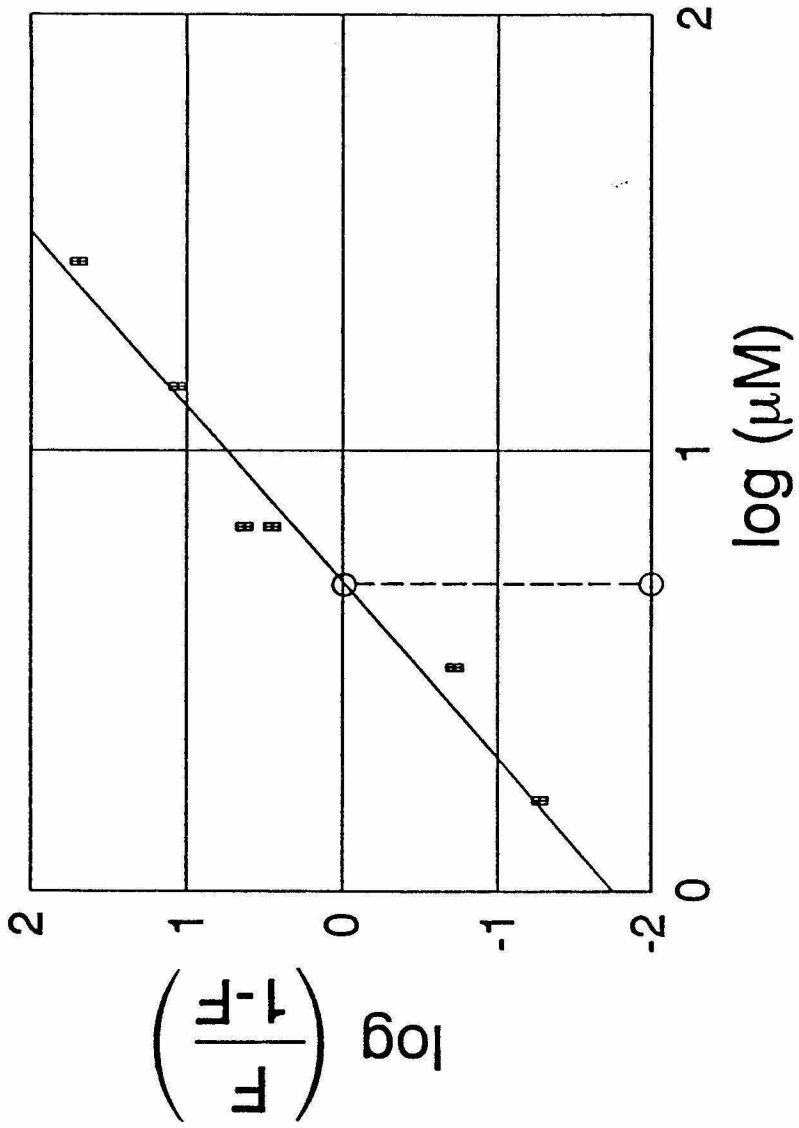


Figure 5

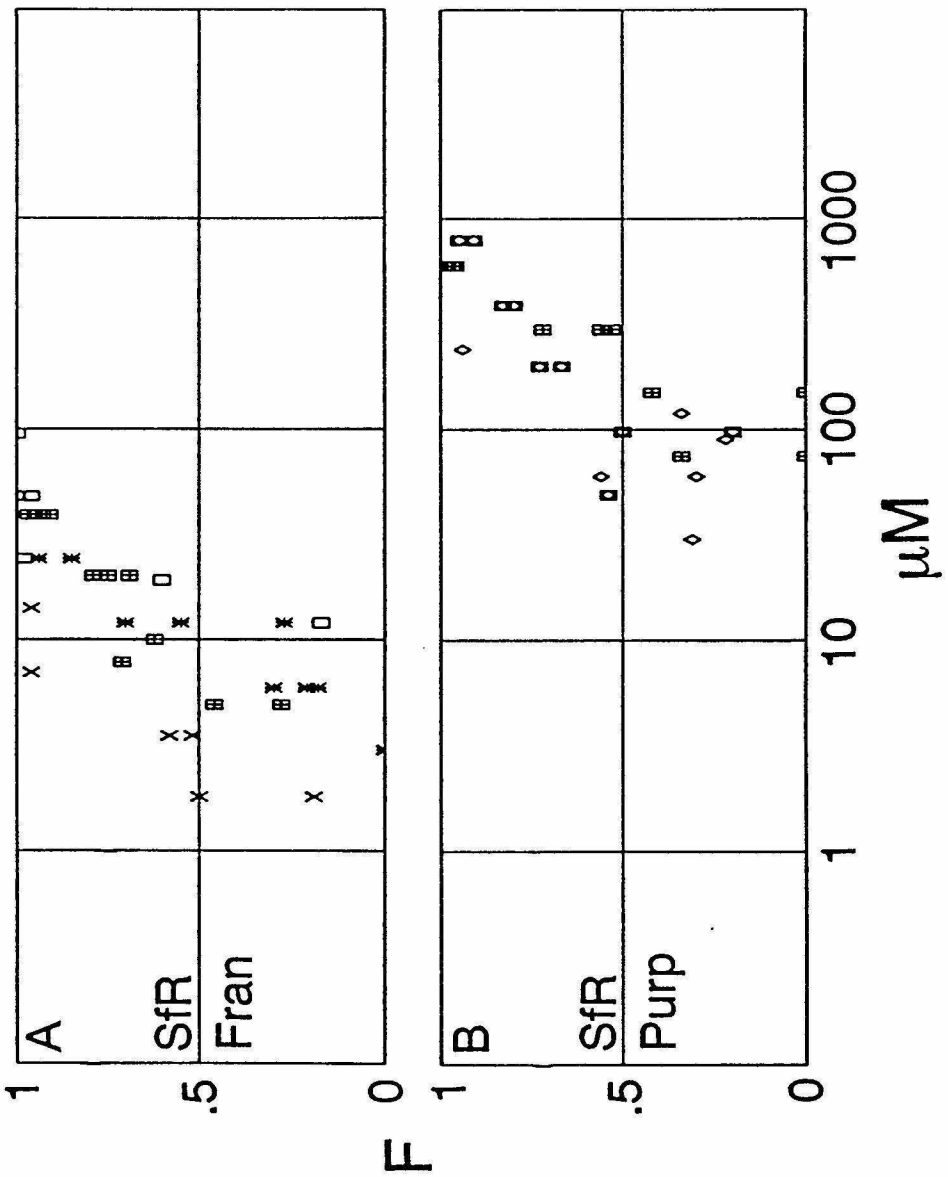


Figure 6

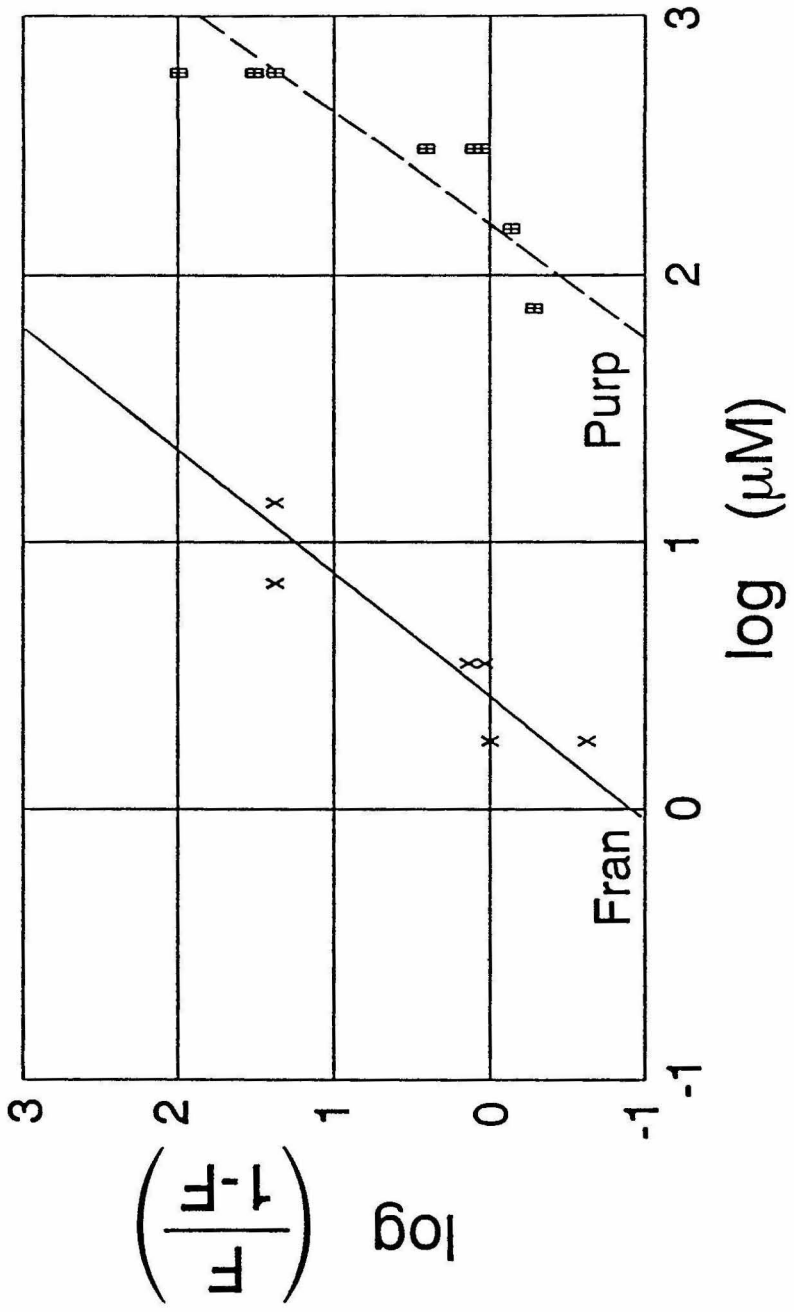
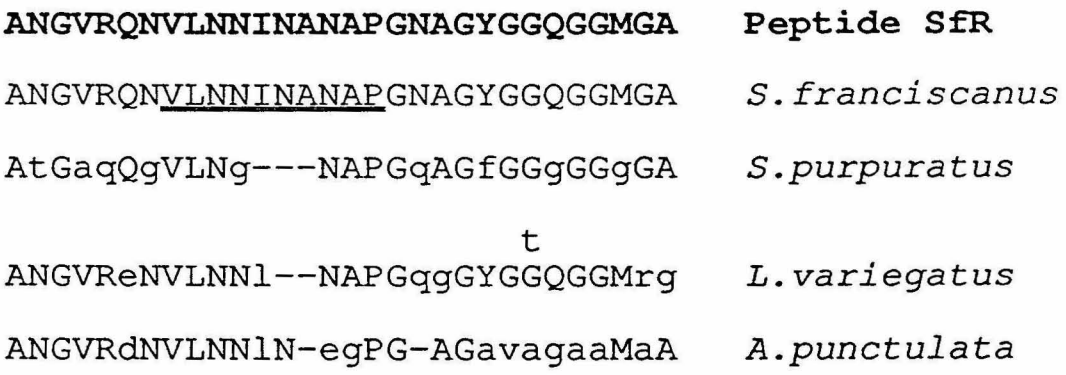


Figure 7


ANGVRQNVLNNINANAPGNAGYGGQGGMGA Peptide SfR
ANGVRQNVLNNINANAPGNAGYGGQGGMGA S.franciscanus
AtGaqQgVLNg---NAPGqAGfGGgGGgGA S.purpuratus
t
ANGVReNVLNNl--NAPGqgGYGGQGGMrg L.variegatus
ANGVRdNVLNNlN-egPG-AGavagaaMaA A.punctulata

Chapter 5

Summary and Conclusions

In this final chapter, I briefly review both our work on bindin and the work done in other labs during the period of my stay that pertains to bindin/receptor structure and function. The chapter considers some ideas that might be considered too speculative for inclusion in the published papers. The chapter concludes with some prospects for future research, and the relevance of the current work to our understanding of speciation in sea urchins.

Evolutionary relationships of sea urchin species revealed by comparison of bindin sequences

In Chapter 3 we reported the sequences of the cloned preprobindins of *S.franciscanus* and *L.variegatus* (Minor et al., 1991a). These sequences were compared to the bindin sequence for *S.purpuratus* that had been previously determined in our lab (Gao et al., 1986). Another bindin sequence that has also been determined is that of the preprobindin of *Arbacia punctulata* (Glabe and Clark, 1991).

Two things should be kept in mind when considering all of these sequences. The first is the relative divergence of the species involved (see Chapter 1, Fig. 1): *Arbacia* is

only distantly related to the other three species. Second, there is an unusual aspect to comparison and analysis of sequences that encode a *species-specific* function. In most sequence comparison studies (e.g., actin, histone) where the function of the proteins compared is conserved, the conserved amino acid sequences observed are presumed to be responsible for the function of the protein. While this may be true for some of the conserved bindin sequences, the species-specificity of bindin implies that at least some of the sequences that are not conserved are also important for the function of the protein.

The mature *Arbacia* bindin sequence has three noteworthy characteristics: 1) a conserved core, 2) an absence of "L" and "S" repeats (cf. Chapter 3, Fig. 5), and 3) an extra hydrophobic segment. The middle third of the *Arbacia* bindin sequence is highly conserved, just as it is in the other three species (Chapter 3, Fig. 5; Glabe and Clark, 1991, Fig. 2). This conservation is even more striking in the *Arbacia* case, since *Arbacia* is ~200 million years diverged from the other species. The *Arbacia* bindin lacks the "L" and "S" repeats that are present in the other three species (Chapter 3, Fig. 6). This result is not that surprising considering the long divergence time and since these repeats do not seem to be directly involved in the sperm-egg contact (Chapter 4). Finally, the *Arbacia* bindin contains an extra hydrophobic region in the C-terminal third of the bindin

sequence. This region is more hydrophobic than any other area of all four of the bindins, and may account for the affinity of *Arbacia* bindin for phospholipid and detergent (Glabe and Lennarz, 1979).

Outside of the conserved core sequences, it is difficult to align the *Arbacia* bindin sequence with the bindin sequences of the other three species. Short regions of apparent homology exist, but alignment over any large distance requires numerous insertions/deletions/ rearrangements. Note that this contrasts with the bindin precursor sequences (the N-terminal half of preprobindin that is cleaved to release mature bindin), which can be aligned with few insertions/deletions. Since bindin is a single copy gene in sea urchins (Chapter 3), the aligned precursor sequence can be used to confirm the phylogenetic relationship of *Arbacia* to the other species. Confirmation of the relationship of *Arbacia* to the other species by the criterion of sequence conservation was not possible using the traditional method for sea urchins, single copy DNA hybridization (e.g., Hall et al., 1980), because the single copy DNA divergence of even *Lytechinus* to *Strongylocentrotus* is such that 60-80% of the sequences fail to form duplexes under the normal conditions.

The bindin precursor sequences of all four species were aligned [using the default settings of the Pearson-Lipman

algorithm (1988) in the IBI Pustell sequence analysis package], relative to the *S.franciscanus* sequence. The alignment scores were (as percent of *S.franciscanus* score): *S.franciscanus*, 100%; *S.purpuratus*, 84%; *L.variegatus*, 77%; and *A.punctulata*, 41%. This comparison provides a crude estimate of relative amino acid similarity. The relative amino acid identities are: *S.franciscanus*, 100%; *S.purpuratus*, 78%; *L.variegatus*, 68%; and *A.punctulata*, 36%. The greater divergence of the *A.punctulata* bindin precursor sequences relative to the other species confirms the phylogenetic assignment made by Smith (1981, 1984) based on morphological criteria.

Despite numerous amino acid substitutions, the *A.punctulata* bindin precursor sequence retains the same basic structure as the other bindin precursors. The *A.punctulata* bindin precursor sequence is exactly the same length as the *S.franciscanus* bindin precursor sequence. All four bindin precursors contain eight cysteines, and the spacing of the cysteines is identical in all four species (cf. Chapter 3, Fig. 5). The conservation of these features of the bindin precursor, over 200 million years, is interesting since the function of the bindin precursor remains unknown. It has been proposed that the bindin precursor may be responsible for assisting the delivery of the otherwise insoluble bindin to the acrosomal vesicle (see Gao et al., 1986; Chapter 2).

Lysin, bindin, and positive selection for sequence change

Lysin is an abalone sperm acrosomal protein that is at least partially responsible for the species-specificity of fertilization in abalones (Vacquier et al., 1990). Lee and Vacquier (1991) have recently concluded, based on an analysis of sequences of lysin, that lysin is under selective pressure for changes in its protein sequence. To understand the basis for this statement, it is necessary to consider the general pattern of protein evolution.

Li et al. (1985) compared the changes in the DNA sequences that encoded 36 mammalian proteins (see Table 1). They found that, on average, DNA sequence changes that result in amino acid changes (nonsilent) are outnumbered by changes that do not change the protein sequence (silent) by a factor of 5 (on a per site basis). The ratio of silent (S) to nonsilent (NS) changes varied from a low of 2.1 (insulin) to a high of 350 (histone H4). The majority of the difference in the values of S/NS was due to the differences in the rate of nonsilent (amino acid replacement) substitutions. The slower rate of nonsilent changes is due to natural selection against changes in the sequence of the proteins, and the large difference in the nonsilent rates therefore implies varying amounts of selection pressure on the different proteins (for earlier

reviews reaching the same conclusion see Zuckerkandl and Pauling, 1965; Wilson et al., 1977). Thus a protein without any constraints on the evolution of its sequence should have S/NS ≈ 1. Again, it should be noted that S and NS are frequencies of change per available site (in the case of the Li et al., 1985 data, "the number of nucleotide substitutions per site per 10^9 years"). This differs from the ratio of the number (as a raw count) of silent changes (SC) to the number of nonsilent changes (NSC). A protein without any constraints on the evolution of its sequence should have S/NS ≈ 1, but SC/NSC ≈ 0.31. The reason for this is evident from an analysis of a table of the genetic code: the number of base changes that result in a change in an amino acid (an NSC) exceeds the number resulting in no change (an SC) by a factor of 3.2.

Hughes and Nei (1988) studied silent and nonsilent changes in the antigen recognition site (ARS) of the class I major histocompatibility locus gene (MHCI). The limits of this site had been determined in the X-ray crystallographic studies of Bjorkman et al. (1987ab). As can be seen in Table 1, the S/NS value for the ARS region is less than one, while the value for the rest of the extracellular residues is close to the average value for other proteins. The low value of S/NS within the ARS must be due to either: (1) a mutational apparatus that is specifically skewed towards changes in codon positions responsible for changes in the

amino acids of the ARS, or (2) selection *for* changes in the amino acid sequence of the ARS. While (1) seems unlikely, the basis for (2) is understandable since allelic diversity in MHCI should increase the ability of the organism carrying the changes to present novel antigens. Additional sequence evidence supporting the idea that *positive selection* acts to promote the diversity of the MHCI has also been presented (Hughes et al., 1990; Watkins et al., 1990). S/NS ratios less than one have also been reported for the putative antigen recognition region of the MHC class II protein (Hughes and Nei, 1989), the complementarity-determining regions of immunoglobins (Tanaka and Nei, 1989), and the epitopes of the *Plasmodium* circumsporozite cell surface protein recognized by T-cells (Hughes, 1991). Since all of these molecules involve the immune response, it has been proposed that positive selection is acting to promote changes in sequence that improve the immune response (or, in the *Plasmodium* case, the chance to escape it).

In the lysin study of Lee and Vacquier (1991), the S/NS ratio for the entire lysin coding sequence was found to be less than one (Table 1). This result is not an artifact of alignment, since the lysin sequences of all four species can be aligned with only a single amino acid insertion in the sequence of one species (see Vacquier et al., 1990). The low S/NS ratio is striking since it does not involve the selection of any specialized area, although regions with

even lower S/NS ratios are easily seen in the sequences. The function of lysin is to open a hole in the egg vitelline envelope (which in abalones is elevated before fertilization) to allow passage of the sperm. The ability of lysin to dissolve egg vitelline layers is species-specific. It is interesting to speculate that the low S/NS ratio of lysin may be due to positive selection for changes in its amino acid sequence (Lee and Vacquier, 1991).

The S/NS ratio for bindin is much harder to determine because of the extensive sequence rearrangement and differences in the numbers of L and S repeats bindin (Chapter 3, Fig. 5). The ends and core of the bindin molecule are the only areas that are suitable for this analysis, and the values for these areas are reported in Table 1. Also, the values for the bindin precursor sequences, which are easily aligned, are shown as prebindin in Table 1. The changes were counted manually: SC and NSC are the raw count of the number of silent changes and nonsilent changes that occur between the sequences of *S.purpuratus* and *S.franciscanus*. The value of S/NS for bindin and the bindin precursor reported in Table 1 are SC/NSC times 3.2, which should be comparable to the other values of S/NS in the table.

The values for mature bindin are based on sequences that are too short for statistical analysis, but the low S/NS

ratio in the ends of the protein is interesting. A low S/NS ratio is also seen for the bindin precursor sequences. While the S/NS ratio is not less than 1, as it is for the immune proteins and lysin, it is surprisingly low considering the entire sequence is being used. Since some of the bindin precursor residues are clearly conserved (even in *Arbacia*), there may be regions of the bindin precursor (and bindin) that exhibit the results of positive selection for change in protein sequence. A better analysis of the question of positive selection in bindins will require an analysis of the bindin sequences from closely related sea urchins whose bindins have not undergone extensive rearrangements. This may be possible in the *Echinometra mathaei* species complex (Palumbi and Metz, 1991), and the preliminary result from the partial sequences of bindin that have been obtained is that the S/NS ratio is very low (E. Metz and S. Palumbi, personal communication).

The idea that positive selection is acting to promote sequence changes in proteins involved in the species-specificity of fertilization is an interesting one. The prospects for ascertaining the validity of the positive selection hypothesis will be greatly improved when the basis of their species-specific action is better understood. It was the determination of the MHCI contact sites (Bjorkman, 1987ab) that allowed for the meaningful interpretation of

the sequence comparisons of the ARS region (Hughes and Nei, 1988).

In the following two sections, I report recent work on bindin and the receptor from other labs. I give my view of the the meaning of the results, particularly with reference to some of my data.

New information on the bindin receptor

An important new discovery concerning the bindin receptor is the isolation of a soluble fragment of the *S.purpuratus* receptor that retains the ability to species-specifically inhibit fertilization (Foltz and Lennarz, 1990). As reviewed in Chapter 2, previous studies on the bindin receptor were only able to show species-specific activity of the receptor in crude preparations of the egg surface. Treatment of these preparations with either glycosylases or proteases destroyed the species-specificity of the preparation. These preparations could not be purified further, apparently because the receptor was present in large, and possibly heterogeneous, complexes. Efforts were made to release a discrete, soluble fragment of the receptor by treating eggs with specific proteases. The first effort, using trypsin, was largely unsuccessful because the released fragments were heterogeneous in size (Ruiz-Bravo et al., 1986). Foltz and Lennarz (1990) then tried lysylendoproteinase C, which released a soluble 70 kDa

glycoprotein fragment. This fragment was purified to homogeneity and was shown to species-specifically inhibit fertilization and species-specifically interact with bindin. The species-specific inhibitory activity of the fragment was destroyed by Pronase digestion, confirming the role of the protein backbone for receptor function.

New information on bindin

Biochemical studies on *S.purpuratus* bindin continue in C. Glabe's laboratory (U.C. Irvine). The work relies on assays of two properties of bindin: affinity for sulfated fucans and interaction with phospholipid bilayers.

As reviewed in Chapter 2, Glabe (1985ab) had previously shown that bindin is incorporated into gel-phase phospholipid vesicles and that bindin induces the fusion of mixed-phase vesicles. Kennedy et al. (1989) mapped the domains of bindin that interact with gel-phase vesicles using two different methods. First, tryptic fragments of bindin were assayed for their ability to interact with vesicles. The peptides that did interact with the vesicles were derived from amino acids 77 to 126 of bindin. In a second assay, bindin was allowed to interact with vesicles and then the bindin/vesicles were digested with proteases. The N-terminal two-thirds of bindin were protected (amino

acids 1-126). In my opinion, the relevance of the interaction of bindin with lipid bilayers remains uncertain. The purified bindins of *S.purpuratus*, *S.franciscanus* (Glabe and Vacquier, 1977), *Arbacia punctulata* (Glabe and Lennarz, 1979), and *Lytechinus pictus* (Foltz and Lennarz, 1990) have all been shown to agglutinate the eggs of the homologous species, but in no case has bindin been observed to cause the fusion of egg membranes. Kennedy et al. (1989) propose that the lipid interaction activity of bindin may serve to keep bindin associated with the inner acrosomal membrane of the sperm after the acrosome reaction.

A second set of studies in the Glabe lab involves the affinity of bindin for sulfated fucans. Despite early reports of the lectin-like activities of bindin (Glabe et al., 1982), recent work by DeAngelis and Glabe (1988, 1990ab) suggests bindin is recognizing the sulfates and not the fucose residues of the fucans. Hence desulfated fucans do not bind to bindin, and other highly sulfated polymers (e.g., dextran sulfate and polyvinyl sulfate) bind avidly to bindin (DeAngelis and Glabe, 1990a). It is therefore not surprising that the basic amino acids of bindin seem to mediate the interaction with sulfated fucans. DeAngelis and Glabe (1988) chemically modified the basic amino acids of bindin and showed this modification decreased the affinity of bindin for sulfated fucans. DeAngelis and Glabe (1990b) then synthesized a peptide (LRHLRHHSN) corresponding to the

region of bindin with the highest density of basic residues and showed that this peptide bound to sulfated fucans.

In my opinion, the relationship of the affinity of bindin for sulfated fucans to the bindin-receptor interaction remains uncertain. Crude preparations of the bindin receptor (Glabe and Vacquier, 1978) and the purified receptor fragment (Foltz and Lennarz, 1990) do contain fucose and sulfate (and also significant amounts of protein, mannose, galactose, and galactosamine). It is clear that the activity of the receptor requires both protein and carbohydrate (Rossignol et al., 1984b; Ruiz-Bravo et al., 1986; Foltz and Lennarz, 1990). What is puzzling is that the major source of sulfated fucans at fertilization is not the bindin receptor but is the egg jelly layer (SeGall and Lennarz, 1979; Mikami-Takei, 1991). For example, the egg jelly layer of *Hemicentrotus* contains ~2mM fucose (Yamaguchi et al., 1987). If the primary ligand of bindin is sulfated fucan, then the egg jelly should be a potent inhibitor of fertilization but it is not. DeAngelis and Glabe (1990b) report that the peptide LRHLRHHSN inhibits fertilization only at the relatively high concentration of 300 μM (cf. values in Tables 1 and 2, Chapter 4), and only in the presence of zinc, which is itself a potent inhibitor of fertilization.

It is also difficult for me to understand what role the fucose-sulfate binding site could play in the species-specificity of the bindin-receptor interaction. The sequence LRHLRHHSN is in the middle of a sequence that is highly conserved between all of the sea urchin species whose bindins have been sequenced (Chapter 3, Fig. 5; Glabe and Clark, 1991). In the case of *S.purpuratus* and *S.franciscanus,* the fucose-sulfate binding site is centered in a stretch of 68 amino acids that are identical between the two species. In contrast, the results we report in Chapter 4 indicate that peptides derived from the variable regions of bindin are capable of inhibiting the sperm-egg contact. The inhibition caused by the *S.franciscanus* species-unique peptide SfR is specific for *S.franciscanus* fertilizations. We feel that this demonstration that species-specific inhibition of fertilization is due to a species-unique bindin sequence is a more compelling explanation of the cause of bindin-mediated species-specific fertilization.

Inhibition of fertilization by bindin-derived peptides

The bindin sequences reported in Chapter 3 were used to select the regions of the bindins of *S.franciscanus* and *S.purpuratus* that we felt were most likely to be involved in the species-specificity of the bindin-receptor interaction. The regions that were selected (Chapter 4, Figures 1 and 2)

were areas of the bindins that contained clusters of amino acids that differed in sequence between the two species. Peptides that corresponded to these regions were synthesized by Suzanna Horvath and coworkers in the Microchemical Facility. An experimental procedure was developed to quantitatively measure the degree of inhibition of fertilization. Using this assay system, the inhibitory activity of the bindin derived peptides was determined.

One result of these experiments was the confirmation of the role of bindin in sperm-egg adhesion and fertilization. While purified bindin of four species of sea urchins does species-specifically aggregate the eggs of those species (Glabe and Vacquier, 1977; Glabe and Lennarz, 1979; Foltz and Lennarz, 1990), bindin at concentrations of even hundreds of μg/ml fails to inhibit fertilization. The probable reason for this is the insolubility of bindin. In the egg agglutination assay (Glabe and Vacquier, 1977; Glabe and Lennarz, 1979), the bindin that is added is present as particles that adhere to the surfaces of the eggs. As a result of this insolubility, the agglutination assay is purely qualitative in nature. Measures of the degree of agglutination caused by a given quantity of bindin cannot be quantitatively assessed since the "activity" of the added bindin varies with the size of the particles. The solubility of bindin derived peptides allows them quantitatively controlled access to sites on the egg

surface. Our measurements that show that certain of these peptides inhibit fertilization at μM concentrations provide further experimental confirmation of the role of bindin and its receptor in fertilization.

Prospects for future research

There is still much to be learned about bindin, its receptor, and the other steps in sea urchin fertilization. Some of the questions that are raised from the current results and that can be addressed directly are mentioned here. First, the functions of the bindin precursor sequences, which comprise half of preprobindin, remain unknown. Antibodies raised to these precursor sequences could be used to investigate where the precursor is routed during spermatogenesis (as was done in the analysis of the pathway of bindin processing; see Appendix C).

The stoichiometry of the interaction of bindin and the receptor in fertilization can be addressed further. The inhibition of fertilization data reported in Chapter 4 do not provide data on the fraction of receptors occupied by peptide as a function of peptide concentration. This issue can be approached by studying the interaction of labeled peptides with eggs or with the 70 kDa receptor fragment. The relationship of the 70 kDa receptor fragment to the intact receptor needs to be addressed: will each fragment interact with one or more bindin molecules? This question

can be approached by a Hill analysis of data from experiments of peptide inhibition of the interaction of bindin particles with the 70 kDa receptor fragments.

A major unresolved question regarding sea urchin fertilization is how the sperm activates the egg. The causal relationships among the events that occur between sperm binding and the induction of the calcium wave (see Chapter 1) remain obscure. One hypothesis is that when the sperm and egg membranes fuse the sperm introduce a substance that activates the egg (Iwasa et al., 1990; Swann and Whitaker, 1990). The conventional view is that the sperm bind to a transmembrane receptor of the egg, that may be coupled to a G-protein which in turn produces an activation signal (Turner et al., 1986; Shilling et al., 1990; Jaffe, 1990). Since the bindin-receptor interaction is multivalent, it is possible that that a concentration range exists at which fertilization is inhibited but sperm adhesion is not. Studies of the morphology, electrophysiology, and biochemistry of peptide blocked fertilizations may provide clues about how the sperm activates the egg.

Another area that needs to be explored is the distribution of variant alleles of bindin and its receptor within species. For the species *S.purpuratus* and *S.franciscanus*, this search will be aided by the results of

this thesis that have mapped some of the surface contacts between the sperm and the egg. It will also be interesting to search for these variants in recently evolved species, such as the *Echinometra* species complex (Lessios and Cunningham, 1990; Palumbi and Metz, 1991).

Bindin and speciation

In order to understand the role bindin might play in speciation, it is necessary to consider the following question: How can an altered bindin/receptor pair become fixed in a population when the altered molecules are likely to be less effective in fertilizing the gametes of the parent species? This problem is similar to the problem of the acquisition of assortative mating in animals with internal fertilization (e.g. Muller, 1940; Crow and Kimura, 1965; Dobzhansky and Pavlovsky, 1971; Nei, 1975; Carson, 1975; and Kaneshiro, 1980). In the following discussion I consider a small part of the larger problem: the chances of bindin and its receptor changing from one form to another via "drift" in small populations.

In the model that follows, the bindin gene is symbolized by capital letters, the receptor by lowercase letters. The hypothetical starting "isolated" population has all but one of the animals homozygous for the dominant form of bindin "AA" and one male heterozygous with an allele for a new form of bindin "AB." The starting population also has all but

one of the animals homozygous for the matching form of the receptor "aa" and one female "ab," where "b" matches "B." (This model does not deal with the probably low chance that a matching bindin and receptor arise simultaneously. The model will show that the expectations for fixation are still low even when this starting situation is granted.)

The complete genotype for the "mutant" male is (ABaa) and for the "mutant" female is (AAab). From what we know about the timing of bindin expression in the testis (Appendices B and C), it is likely that a sperm contains bindin in its acrosome that corresponds only to the allele of the gene that it carries. The egg, however, has both alleles of the receptor expressed on its surface even though it carries only one of the two alleles of the gene itself (it is assumed here that the specificity of the receptor is encoded by a single gene). The gametes produced by these heterozygous individuals are diagrammed in Figure 1.

Assumptions for the following calculations include: 1) equal gamete production (all males produce the same number of sperm, all females the same number of eggs), 2) independence of generations (no back mating to parents), 3) complete mixing at each generation (no assortative mating), and 4) no penalty in fertilization efficiency for an egg carrying multiple receptor forms (a "B" sperm mates equally

well with eggs that have either all "b" receptors or a mix of "b" and "a" receptors).

The calculation for the direct path to producing a "BBbb" population is as follows. The steps are to cross the "mutant" animals to produce a double heterozygote "ABab," the mating of two "ABab"'s to produce a homozygote "BBbb," and then the mating of two of these homozygotes to produce a "BBbb" population. If there are a number "X" of females where one is "AAab" and the rest are "AAaa," then the fraction of eggs produced by the population that are genotypically "Ab" are:

$$E = \frac{1}{2X}$$

Likewise, if the number of males is given by Y, the fraction of genotypically "Ba" sperm is:

$$S = \frac{1}{2Y}$$

The fraction of offspring produced that are double heterozygotes "ABab" is then:

$$F_1 = (E)(S)$$

The fraction of "BBbb" offspring produced by two double heterozygotes mating is:

$$F_2 = \frac{(F_1)^2}{16}$$

where $(F_1)^2$ is the chance of two double heterozygotes mating and 1/16 is the fraction of "BBbb" individuals produced by this cross. For this BBbb individual to survive, the minimum requirement is that two individuals of this genotype must be produced and mate:

$$F_3 = (F_2)^2$$

The quantity F_3 is the fraction of the total population that after 3 generations should be "BBbb" individuals. F_3 can be expressed as:

$$F_3 = \frac{1}{(16XY)^4}$$

This analysis points to three conclusions. First, the probability of random co-fixation of a mutant bindin/receptor pair is unlikely, and rapidly becomes increasingly less likely as the size of the starting population increases. Second, the chances of co-fixation are enhanced when the starting population has a skewed sex ratio. Third, the chances for fixation would be increased if the starting population was small and expanded during the first few generations.

This analysis indicates that the chance that bindin and its receptor could change forms by drift is unlikely even if the co-occurrence of matching mutant forms of bindin is granted. Furthermore, the chances are even less likely than the analysis would suggest since the starting condition assumed a matching mutant bindin-receptor pair. On the other hand, speciation is a rare occurrence and the geologic time scale allows for many attempts to be made. The model would predict that the probable mode of speciation involving a change in bindin and its receptor would be "peripatric": it would occur in a small isolated population, preferably

with a skewed sex ratio. Similar conclusions have been reached in studies regarding speciation via sexual selection in Hawaiian *Drosophila* (Carson, 1975; Kaneshiro, 1980; Templeton, 1980; Giddings and Templeton, 1983). These studies all agree that even though sexual selection could speed the acquisition of reproductive isolation, it is unlikely to result in speciation except during "founder" events: times when new habitat is colonized by a small number of individuals (e.g. a single gravid female) and the population expands. For the sake of comparison, changes in bindin and its receptor can be thought of as sexual selection at the gametic level: i.e. the egg "chooses" the appropriate sperm.

This view of bindin and the receptor allows for speculation on how a compatible mutant bindin-receptor pair could arise. Imagine a small peripherally isolated population with a skewed sex ratio (in this case there is only one male, and it is heterozygous for bindin: "AB"). This means at the first breeding cycle one-half of the sperm available for fertilization will be presenting the altered "B" bindin. If, under normal circumstances, there was some plasticity in the fit between bindin and the receptor, the 4% sequence polymorphism of the sea urchin genome might extend into the receptor gene (particularly since there are two copies of the receptor per female, allowing for two receptor forms on the surface of the egg; see Figure 1). In

this case, a female carrying any altered form of the receptor that allowed for binding of the mutant bindin should be at an advantage since the range of sperm that could be received would be expanded (this assumes that the haploid presence of the "normal" receptor gene is sufficient for full efficiency in binding to the "normal" sperm). Under these circumstances, there would be a potential reward for polymorphism in the receptor which might in turn increase the odds of the production of a matching altered bindin-receptor pair. The chances of producing a match would be further improved if it were found to be the case that either bindin or the receptor was constrained in evolution: e.g., the nature of the receptor glycoprotein were to be such that only a few shapes are likely to be produced by mutation.

Another scenario under which the chances of the production of a novel bindin-receptor pair might be improved involves the possibility that intermediates in the change from one form to another retain some ability to productively interact with both forms. This would be in contrast to the foregoing analysis, where the ancestral form of bindin and its receptor ("A" matching "a") was considered to have no probability of interacting with the new forms ("B" matching "b"). The possibility of evolutionary intermediates of bindin that retain the ability to bind to both forms of the

receptor ("a" and "b") exists since several regions of bindin seem to be involved in the sperm-egg contact.

The data reported in Chapter 4 indicate that peptides representing five different regions of bindin are capable of inhibiting the sperm-egg contact. Four of these regions of bindin differ between the two species. If these areas are considered to be independent binding domains, then the possibility exists that not all of them are strictly required for sperm-egg adhesion. In the following analysis, the number of independently functioning contacts on each bindin monomer is considered to be four, and only half of these are considered necessary for bindin-receptor adhesion.

These four sites would be arrayed in some way on the surface of bindin and the receptor, represented here as a line (receptor in lowercase; bindin in uppercase). The starting condition would be:

```
a1-a2-a3-a4     RECEPTOR SUBREGIONS
|  |  |  |
A1-A2-A3-A4     BINDIN MONOMER SUBREGIONS
```

where all four sites match. Since only two of the contacts (1/2) are strictly required, then both bindin and its receptor have a certain "freedom" to mutate without affecting sperm binding. After a period of time, the dominant form of the receptor in some isolate of sea urchins could be:

a1-b2-a3-b4

A number of altered bindins could also be present in the population, all of which would match a1-b2-a3-b4:

BINDIN FORM	ABILITY TO MATCH a1-b2-a3-b4
A1-A2-A3-A4	yes
A1-B2-A3-B4	yes
A1-B2-A3-A4	yes
B1-B2-A3-A4	yes

Now, if due to a second "bottleneck" the dominant form of the receptor changed to:

a1-b2-b3-b4

then some of the bindins would match and some would not:

BINDIN FORM	ABILITY TO MATCH a1-b2-b3-b4
A1-A2-A3-A4	no
A1-B2-A3-B4	yes
A1-B2-A3-A4	yes
B1-B2-A3-A4	no

Under this scenario cryptic forms of bindin and its receptor could preexist in the general sea urchin population without a selective disadvantage. These cryptic forms would be able to match a novel partner if one appeared under the right "bottleneck," negating the problem of having to create both matching partners in one step. Now that discrete, contiguous regions of bindin have been shown to be involved in gamete recognition it should be possible to search for variants of these sites in sea urchins. Primers flanking

these regions could be made and variants could be searched for using the polymerase chain reaction. When variants were found, they could be assayed for their functional role in fertilization by either the peptide inhibition methods used here or by incorporating the changes into chimeric bindins and producing transgenic animals. The search for variant regions of the receptor will remain more difficult, mainly because the function of the receptor requires carbohydrate. The recent purification of a functional fragment of the receptor by Foltz and Lennarz (1990) raises hope that the contact sites on the receptor may also be mapped to an extent that will alow for the synthesis of functional analogs of the receptor structures. If this were to be the case, then it should be possible to investigate both the degree of bindin and receptor polymorphism in sea urchins and the functional importance of the variations.

Founder effects, skewed sex ratios in the starting population, population expansion in peripherally isolated populations, and redundancy in the bindin-receptor contact could all contribute to increasing the probability of an evolutionary shift in the specificity of the bindin receptor interaction. Bindin and its receptor may therefore be viewed as replacing the opportunities to take advantage of geographic isolation that are afforded by other means in species with internal fertilization. It will be interesting to see to what extent this view is correct; hopefully

further studies of the acquisition of reproductive isolation in marine invertebrates will provide further insight into the processes involved.

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Table 1

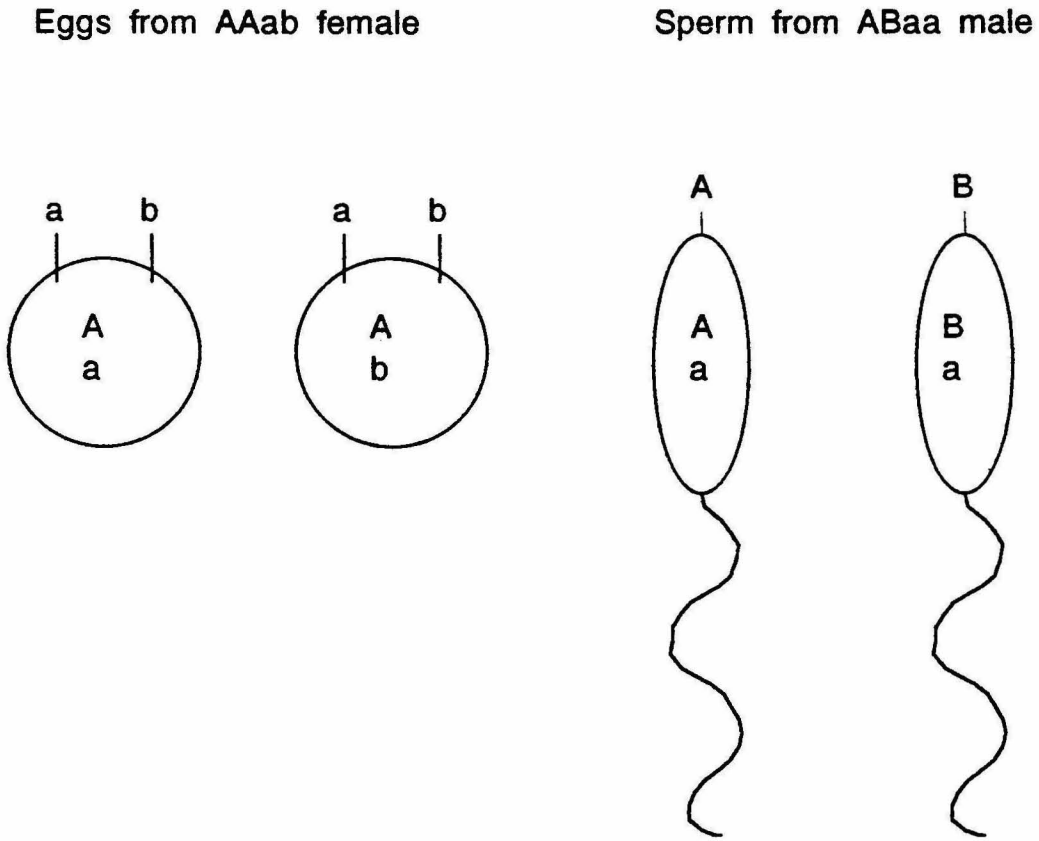
Silent and nonsilent substitutions in DNA sequences that encode proteins

Protein	L	S	NS	S/NS
Histone H4[a]	101	1.4	0.004	350
Insulin[a]	23	2.4	1.2	2.1
Avg. of 36[a]	-	4.6	0.88	5.3
MHCI (ARS)[b]	57	4.7	14	0.33
MHCI (rest)[b]	217	5.4	1.8	3
Lysin[a]	136	0.75	1.8	.42
		SC	NSC	S/NS[d]
BindinC[c]	12	0	6	0
BindinN[c]	15	3	10	0.96
Bindincore[c]	82	11	8	4.4
Prebindin[c]	245	20	55	1.2

This table is a compilation of sequence data from several sources; due to the different methods used to score S and NS (see below), the only comparable numbers are the S/NS ratios. L - number of codons in the sequence. S - silent sequence substitutions per available site. NS - nonsilent sequence substitutions per available site. S/NS - ratio of silent to nonsilent changes. [a]For these proteins, the S and NS are the number of nucleotide substitutions per site per 10^9 years (Li *et al.*, 1985; Lee and Vacquier, 1991). [b]For these regions of MHC-I, S and NS are the number of nucleotide

substitutions per 100 sites for alleles of MHC-I within *Homo sapiens* (Hughes and Nei, 1988). [c]For these regions of preprobindin, SC and NSC are the raw count of the number of silent and nonsilent changes between *S.purpuratus* and *S.franciscanus*. [d]In a random DNA sequence, the number of sites available for nonsilent change exceeds those for a silent change by a factor of 3.2 (see text). Thus S/NS for the bindin sequences (c) is given by 3.2(SC/NSC). Avg. of 36 (a) is the average value for 36 coding regions compared between orders of mammals; histone H4 and insulin had the highest and lowest values, respectively, of S/NS (Li *et al.*, 1985). MHC-I (ARS) is the antigen recognition sequence region of MHC-I (Bjorkman, 1987ab). MHC-I (rest) indicates the remaining extracellular amino acids. The value reported for lysin is the average value from the six pairwise comparisons made between four species of abalone that diverged 25 million years ago. BindinC, bindinN, and bindincore indicate the three alignable regions of bindin. BindinC is the C-terminal 12 amino acids of bindin (Chapter 3, Fig. 5). Bindincore is the region centered on the highly conserved middle third of bindin and extends from amino acid 316 to amino acid 398 of the *S.purpuratus* preprobindin (Chapter 3, Fig. 5). Prebindin represents the binding precursor sequences which align with only a single 6 bp insertion (which was ignored in this count). The ARS region of MHC-I and lysin both have S/NS ratios less than one, which probably indicates these proteins are under selection for change in their protein sequences.

FIGURE 1. Expected genotypes and phenotypes resulting from sea urchins heterozygous for bindin and the receptor (see text).



APPENDIX A

DEVELOPMENTAL BIOLOGY 122, 291-295 (1987)

Sea Urchin Actin Gene Linkages Determined by Genetic Segregation

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Genetic linkage between the actin genes of *Strongylocentrotus purpuratus* was investigated by observing the segregation of restriction fragment length polymorphisms (RFLPs). Specific RFLPs of actin gene pairs CyI/CyIIa and CyIIIa/CyIIIb always cosegregated, confirming the linkage groups CyI–CyIIa–CyIIb and CyIIIa–CyIIIb previously determined by molecular cloning. In contrast, RFLPs of actin genes CyI/CyIIa, CyIIIa/CyIIIb, and M all segregated at random with respect to one another. This demonstrates that the known actin gene clusters CyI–CyIIa–CyIIb, CyIIIa–CyIIIb, and the M actin gene are not closely linked.

INTRODUCTION

The genome of the sea urchin *Strongylocentrotus purpuratus* contains five cytoskeletal (Cy) actin genes, one muscle (M) actin gene, and two actin pseudogenes (Lee *et al.*, 1984; Akhurst *et al.*, 1986). It is known from analysis of overlapping genomic clones, and of genome blot patterns obtained with gene-specific probes, that genes CyI–CyIIa–CyIIb and genes CyIIIa–CyIIIb are located in two clusters, in the order indicated, while gene M is not known to be associated with any other actin gene. However, current clone maps extend only a few kilobases beyond the gene clusters themselves. The present study was undertaken to determine whether genetic linkage could be demonstrated between the known cytoskeletal actin gene clusters or between these and the M actin gene.

Genetic linkage can be demonstrated by showing that particular alleles of two genes always cosegregate in the progeny of a cross. The "alleles" used in this study were restriction endonuclease length polymorphisms (RFLPs). The RFLPs were detected as differently sized reactive restriction fragments when the genomic DNA of an individual sea urchin was cut with a restriction enzyme and, after agarose gel electrophoresis, blotted onto nitrocellulose and probed with a labeled cloned sequence. The probes used in this study were specific actin gene probes that have been described elsewhere (Lee *et al.*, 1984; Akhurst *et al.*, 1986). *S. purpuratus* displays a relatively high degree of intraspecific genomic variation, so that the average single copy DNA sequence divergence between individual genomes is about 4% (Britten *et al.*, 1979). It follows that when DNA of an *S. purpuratus* individual is digested with a restriction enzyme that recognizes a six nucleotide (nt) target site and that reacted with a single copy gene probe, two RFLPs are likely to be revealed, due to sequence differences in and around the allelic copies of the gene. This has in fact been observed in many recent studies (e.g., Posakony *et al.*, 1983; Lee *et al.*, 1984; Gao *et al.*, 1986; Sucov *et al.*, 1986). The multiplicity of RFLPs observed with specific actin gene probes was exploited by Lee *et al.* (1984) to deduce the number of actin genes in *S. purpuratus* and is again demonstrated here for the actin genes of several sea urchins all drawn from the same local population. In this study we tested for genetic linkage between the known actin gene clusters by observing the segregation of these RFLPs in the offspring of crosses between parental sea urchins that display characteristic RFLP patterns. This provides a method for mapping sea urchin genes at distances not easily accessible by molecular cloning.

MATERIALS AND METHODS

Sea urchin gametes were collected, the embryos and larvae were cultured to metamorphosis, and juveniles were raised in the laboratory as previously described (Leahy, 1986). DNA from male sea urchins was extracted from sperm following the procedure of Lee *et al.* (1984). DNA from individual juvenile sea urchins was extracted by the method of Flytzanis *et al.* (1985) from whole sea urchins approximately 4 mm in diameter (~5 months old). The amount extracted was quantitated by DAPI fluorescence (Brunk *et al.*, 1979), using a Turner Model 111 fluorometer. The yields obtained were 2-50 μg per juvenile sea urchin.

The genomic DNAs were cut with the restriction enzyme *Bgl*II, the fragments were separated by electro-

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phoresis on 0.7% agarose gels and transferred to nitrocellulose (Millipore) or GeneScreen Plus (NEN) filters. The genome blots were hybridized with gene specific probes prepared by nick-translation (CyI, CyIIa, CyIIIa-3', M), transcription from Sp6 vectors (CyIIa, M), or M13 primer extension (CyIIIa-5'). The probes for CyI, CyIIa, CyIIIa-3', and M are gene-specific probes that recognize 3' untranslated trailer regions of the respective mRNAs (Lee *et al.*, 1984). The CyIIIa-5' probe recognizes the promoter regions of both CyIIIa and CyIIIb (Shott-Akhurst *et al.*, 1984; Akhurst *et al.*, 1986). The hybridizations were carried out under the conditions described by Lee *et al.* (1984).

RESULTS

The RFLPs for the actin genes of 12 male sea urchins were determined from genome blots of sperm DNA probed with the five actin gene probes (CyI, CyIIa, CyIIIa-3', CyIIIa-5', and M). Three of the males were chosen for further study because of their high heterozygosity at the loci examined. The actin gene alleles for these three (M1, M2, M11) are listed in Fig. 1.

The RFLPs for the actin genes of five female sea urchins were inferred from blots of genomic DNA prepared from pools of gastrulae resulting from the cross of a female sea urchin to the male sea urchin M1. Three of the females (F1, F3, F5) were chosen for further study and their actin gene alleles are also listed in Fig. 1.

The six possible matings between the three females and males M2 and M11 were performed, and the resulting progeny were raised through metamorphosis. Genome blots showing the RFLPs of eight of the progeny

IND	CyI-3'	CyIIa-3'	CyIIIa-5'	CyIIIa-3' CyIIIb-5'	M-3'
M1	BD	IJ	NP	RR	UU
M2	BC	EJ	LM	RS	UU
M11	BB	EJ	KO	RR	TU
F1	BB	FG	MP	RR	UU
F3	BB	HJ	MM	OS	UU
F5	AB	JJ	MP	RR	UU

FIG. 1. RFLP chart for the parental sea urchins used in the linkage study. Each letter in the body of the table designates an allele with a distinct migration in the gel system used. The column M1–F5 lists the six parental sea urchins used in the study (*M*, male; *F*, female). The actin gene fragments whose RFLPs were determined are listed in the top row. The CyIIIa-3' probe reacts only with fragments containing the 3' region of the CyIIIa gene while the CyIIIa-5' probe hybridizes with the 5' ends of both the CyIIIa and the CyIIIb genes. Due to the close linkage of the CyIIIa and CyIIIb genes, RFLPs detected with the CyIIIa-3' probe are the same as the RFLPs that contain the 5' end of the CyIIIb gene.

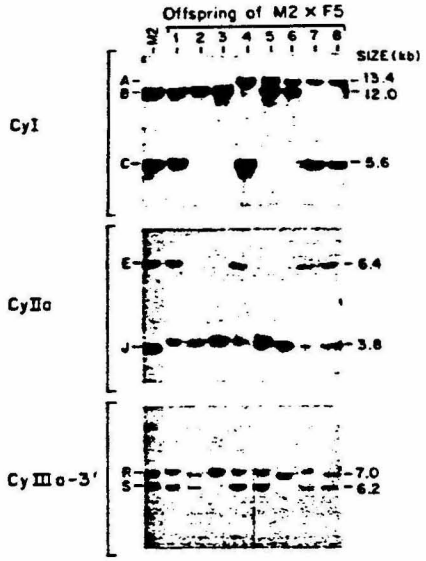

FIG. 2. Genome blots of the progeny of a cross between individuals M2 and F5. Genomic DNAs from sperm (M2) or whole juvenile sea urchins (1–8) were digested with *Bgl*II, run on a 0.7% agarose gel, and blotted onto nitrocellulose. The resulting blots were hybridized with one of three actin gene-specific probes (CyI, CyIIa, or CyIIIa-3'). See text for interpretation.

are shown in Fig. 2. The left lane (Fig. 1) is genomic DNA from the male parent M2. The top panel shows that for actin gene CyI this individual has the actin gene alleles designated *BC* (cf. Fig. 1). The female F5 has the CyI alleles *AB* (data not shown). As can be seen from the figure, the eight progeny have all the expected combinations of the parental RFLPs. Hence 1 inherited *B* from the female and *C* from the male; 2 and 3 both inherited *B*s from both the male and female; 4, 7, and 8 inherited *A* from the female and *C* from the male; and 5 and 6 inherited *A* from the female and *B* from the male.

Similar segregation can be observed for the other two genes shown in Fig. 2. For CyIIa F5 is homozygous *JJ* (Fig. 1, data not shown), while M2 is *EJ* (middle panel, left lane). The progeny segregate the male's RFLPs: half (1, 4, 7, 8) inherit the male's *E* while the other half (2, 3, 5, 6) inherit the male's *J* (all of the offspring inherit a *J* from the female). For CyIIIa, the female is homozygous *RR* while the male is *RS*. The offspring again segregate the male's RFLPs: 1, 2, 4, 5, 7, 8 inherit the male's *S* while 3 and 6 inherit the male's *R*.

The question of linkage between the genes shown is addressed by asking if a particular allele for one gene

always cosegregates with a particular allele for another gene. The top and middle panels of Fig. 2 contain information regarding the linkage of CyI and CyIIa. Since F5 is homozygous for its CyIIa alleles (i.e., *JJ*), informative segregation of RFLPs can only be observed with the male alleles. In Fig. 2 it can be seen that whenever an individual inherits the male's *C* allele for gene CyI it also inherits the male's *E* allele for gene CyIIa. This indicates that the two genes are linked (as is of course already known for this case). In order to use all the data in Fig. 2 it is also necessary to score the segregation of the male's other RFLPs (*B* of CyI and *J* of CyIIa). If the CyI allele *C* is linked to the CyIIa allele *E* on one chromosome (symbolized *C-E*), then the CyI *B* must be linked to the CyIIa *J* on the other chromosome (*B-J*). As can be seen in Fig. 2, it is also true that whenever an individual inherits a *B* allele it also inherits a *J* allele. Thus the data shown in Fig. 2 indicate that in eight out of eight cases the specific RFLPs of the CyIIa and the CyI genes cosegregate. Considering all the data obtained, there were 21 individuals that had the allelic combinations *C-E* or *B-J*, but none that had the opposite allelic combinations (*C-J* or *B-E*); i.e., in 100% (21/21) of the cases the specific RFLPs cosegregate. The probability of this cosegregation occurring by chance is less than 10^{-6}.

The data were scored for all the RFLPs as described above. Thus for any animal heterozygous at two separate loci (see Fig. 1) linkage could be tested. The general form of the analysis is as follows: the offspring of an animal having *WX* alleles for gene 1 and *YZ* alleles for gene 2 are scored for a particular combination of alleles, expressed as a percentage of the total number examined (e.g., the number of *W-Y* offspring plus the number of *X-Z* offspring divided by the total cases observed, which here could include (*W-Y*) + (*X-Z*) + (*W-Z*) + (*X-Y*). Where the result is 100% the genes are linked as *W-Y* on one chromosome and as *X-Z* on the other. A result of 0% would indicate that the genes are linked, but in the opposite orientation (i.e., *W-Z* on one chromosome and *X-Y* on the other). If the genes are unlinked so that their allelic segregation occurs at random, the value obtained would ideally be 50%.

The middle and bottom panels of Fig. 2 contain information regarding the linkage relationship of CyIIa to CyIIIa. Again F5 is homozygous for both CyIIa and CyIIIa, so that segregation can only be observed for the RFLPs of M2. In this case there are four *E-S* combinations (1, 4, 7, 8), two *J-R* combinations (3, 6), and two *J-S* combinations (2, 5). The fact that there are more than two combinations indicates that some assortment has taken place. Out of a total of 22 individuals examined, 13 had the RFLP combinations *E-R* or *J-S* (the other nine had the opposite combinations: *E-S* or *J-R*). The value of 59% (13/22) obtained is close to the 50% expected for random segregation, and thus the genes CyIIa and CyIIIa behave as if they are genetically unlinked.

The data reviewed above are summarized in Fig. 3, in combination with the sum of the data obtained using all five probes and a total of about 60 individual sea urchins. The linkage of CyI to CyIIa demonstrated in Fig. 2 is indicated by the line connecting CyI with CyIIa with the cosegregation value of 100% (21/21) indicated. Likewise the RFLPs of actin genes CyIIIa and CyIIIb also cosegregated (100%, 20/20), confirming that these genes are also linked. In contrast, all other combinations of the RFLPs of actin gene pairs indicated a lack of genetic linkage (cosegregation values of 54-65%). In two instances more than one data set comparing two genes has been obtained. In these cases two of the sea urchins used in the study were heterozygous for the two actin genes checked for linkage. Thus individuals M2 and F5 were both heterozygous for genes CyI and CyIIIb; and

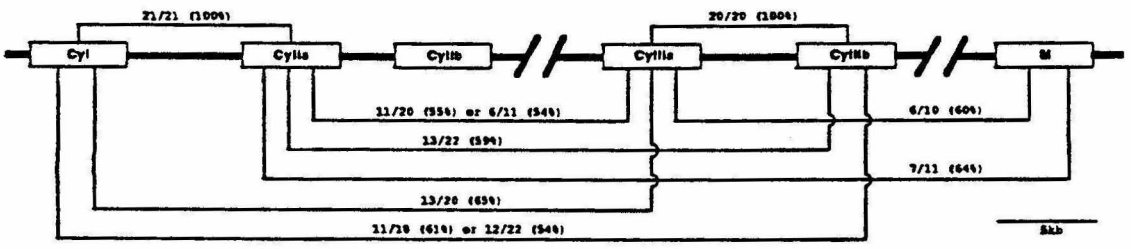

FIG. 3. Actin gene linkage map. The linkage relationships known from overlapping λ clones are indicated by thick lines (reviewed in Davidson *et al.*, 1985). The thin lines connecting the genes indicate genetic linkage relationships based on RFLP segregation described in this study. The fractions are the number of individuals who have inherited a particular combination of RFLPs divided by the total number of individuals examined (see text). The connections drawn above the gene map are RFLP segregations that are significantly different from random expectancy (χ^2 value greater than the $\alpha = 0.005$ value), while those drawn below are not significantly different from random expectancy (χ^2 value less than the $\alpha = 0.1$ value). The data confirm the linkages discovered by molecular cloning and indicate that the actin gene clusters are not closely linked in the genome.

both M2 and M11 were heterozygous for genes CyIIa and CyIIIa (see Fig. 1).

DISCUSSION

The genetic segregation of RFLPs described in this study confirms the linkage groups already known from molecular cloning and provides the new information that the two actin gene clusters, CyI–CyIIa–CyIIb and CyIIIa–CyIIIb, and the M actin gene are not closely linked.

Independent segregation and linkage of genetic markers have not previously been studied in sea urchins, to our knowledge, and thus data do not exist by which recombination intervals (kb per percent recombination, cM) can be estimated. The mouse is the closest evolutionary relative to sea urchins for which there is a good estimate of the recombination interval. For purposes of discussion we apply the recombination interval for rodents, which have a genome size about 4× that of the sea urchin and a very similar interspersed DNA sequence organization (Bonner *et al.*, 1974; Graham *et al.*, 1974). This value is estimated as 1900 kb/cM (Roderick *et al.*, 1981). The haploid genome size for *S. purpuratus* is about 8×10^5 kb (Hinegardner, 1974), and sea urchin diploid cells contain 42 chromosomes almost all of which are approximately the same size (Gerhart, 1983). Sea urchin chromosomes therefore average 40,000 kb and, assuming the mouse recombination interval, would be about 20 cM long. Linkage of any of the genes in our study that are at a distance of 20 cM or less apart would have been detected, since the segregation of their RFLPs would have been noticed as significantly different from random (at the $\alpha = 0.1$ level, see Fig. 3). Were the sea urchin recombination interval similar to (or longer than) the mouse value, the preceding calculations would imply that the two actin gene clusters and the M actin gene are probably each on different chromosomes.

The identity and location of the actin genes in other species of sea urchin is less well understood. Genomic cloning studies by Johnson *et al.* (1983) have shown that some actin genes are closely linked (i.e., recovered on the same genomic clone) in *S. franciscanus* while in *Lytechinus pictus* all cloned actin genes obtained so far occur singly. Lee *et al.* (1984) showed that in *S. franciscanus* the actin genes homologous to CyIIa and CyIIb are also located within a few kilobases of one another. Thus at least the CyIIa–CyIIb linkage is conserved between the two Strongylocentrotid species, and this is probably also true of the CyIIIa–CyIIIb linkage (Akhurst *et al.*, 1986).

Internal similarities in intron sequences of the actin genes within the CyI–CyIIa–CyIIb and CyIIIa–CyIIIb gene clusters of *S. purpuratus* show that the genes within each group are far more closely related than are the genes in different clusters (Schuler *et al.*, 1983; Akhurst *et al.*, 1986). Although the CyIIIa and CyIIIb genes are both expressed in aboral ectoderm cells, though on different temporal schedules (Cox *et al.*, 1986; Lee *et al.*, 1986), and the CyI and CyIIb genes are regulated similarly, the CyIIa gene is utilized differently from the CyI and CyIIb genes in respect to both time of expression in the embryo and the cell types in which the gene transcripts accumulate. Thus the linkage clusters are likely the consequence of recent evolutionary gene duplications and they may have little regulatory significance per se (Akhurst *et al.*, 1986). However, it is also true that with respect to one another, all the unlinked actin genes are diverse in their ontogenic pattern of expression. Though they no doubt originated as gene duplications, they are apparently associated with distinct regulatory sequences in the remote locations at which these genes and gene clusters now reside.

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APPENDIX B

Locale and Level of Bindin mRNA in Maturing Testis of the Sea Urchin, *Strongylocentrotus purpuratus*

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This first study of the onset of spermatogenesis in the sea urchin, *Strongylocentrotus purpuratus*, was undertaken using individuals reared in the laboratory. Spermatogenesis commences about 11-12 months after metamorphosis in these animals. Bindin message accumulates in late spermatocytes and early spermatids which lie in the luminal germinal layer. Bindin message accumulates later than does the testis-specific histone, H2b-1, suggesting that different classes of genes are sequentially activated during the differentiation of sperm. We correlate the number of bindin mRNA molecules with morphological structure and with quantitative aspects of gonad maturation including the number of nuclei and of sperm. The results suggest that the bindin mRNA concentration in total RNA from testis at different stages of maturation reflects the change in the proportion of expressing cells in the total cell population of the testis. © 1990 Academic Press, Inc.

INTRODUCTION

Soon after metamorphosis the gonad primordium of the sea urchin *Strongylocentrotus purpuratus* can be identified as an accumulation of large cells in the dorsal mesentery. Later this group of cells proliferates and spreads in a circular fashion around the periproct underneath the aboral ring of test plates. The individual gonads bud from this genital rachis extending down each interradius (reviewed in Delavault, 1966; Houk and Hinegardner, 1980). Only two cell types are identifiable in the immature gonads at this early stage, primordial germ cells and the forerunners of the accessory cells (Houk and Hinegardner, 1980). The primordial germ cells are characterized by unique cellular inclusions (Houk and Hinegardner, 1981). At maturity, the sea urchin testis consists of tubules containing accessory cells, spermatogonia, and differentiating spermatocytes. The relative proportions of these cell types vary during the annual reproductive cycle (Pearse, 1981). Proliferation of spermatocytes from spermatogonia at the periphery of the tubule occurs in clusters and differentiating spermatocytes are found nearer the center of the tubule. They are probably pushed inward by further proliferation of spermatocytes at the periphery. The reduction in cell size during spermiogenesis occurs in the most luminal region and consequently narrows the column of cells to virtually a point (Holland and Giese, 1965; Simpson and Poccia, 1987). Thus cellular dynamics result in loosely organized pyramidal groups of cells, triangular in cross section, in which the distance from the gonad wall corresponds to the stage of spermatogenesis. The lumen of the testis tubule in gravid males is packed with sperm.

The process of differentiation leading to the highly specialized sperm results from the expression of stage-specific gene products. Several sperm-specific gene products have been identified: bindin, a major protein constituent of the sperm acrosomal granule (Vacquier and Moy, 1977), and unique forms of H1 and H2b histones (Lai and Childs, 1986; Lieber *et al.*, 1986; Lai and Childs, 1988). The bindin protein is found in the acrosomal granule of the sperm and is implicated in species-specific sperm–egg interactions. Bindin was isolated and the sequence obtained (Vacquier and Moy, 1978). Complementary DNA from the mRNA encoding bindin was isolated from a testis cDNA library using an oligonucleotide mix derived from a portion of the amino acid sequence. The nucleotide sequence for the complete probindin precursor was reported by Gao *et al.* (1986; reviewed in Minor *et al.*, 1989). Gao *et al.* (1986) showed that the mRNA for bindin is found exclusively in the testis. In *S. purpuratus* there is a unique form of histone, called H2b-1, the transcript of which is testis specific. As revealed by *in situ* hybridization, the transcripts of H2b-1 accumulate in the basal germinal layer of the testis within replicating cells (Poccia *et al.*, 1989).

The interval between the first postmetamorphic steps in testis formation and the dynamics of differentiation and gene activity in the mature testis remain incompletely described. In the mature testis of adult male sea urchins the rapid time course of spermatogenesis results in groups of cells at different stages which physi-

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cally overlap. Observations on testes undergoing the first wave of spermatogenesis may illustrate previously unseen details of the differentiation process. To clarify the sequential stages with respect to gene expression, we have reared purple sea urchins, *S. purpuratus*, through first maturity and correlated the locale and level of the mRNA for the bindin with the histological appearance of the testis.

MATERIALS AND METHODS

Culture experiments. Embryos from a single fertilization of the purple sea urchin, *S. purpuratus*, were reared by standard methods (Leahy, 1987). On the basis of evidence that short-day conditions maintain gametogenic activity in purple sea urchins (Pearse *et al.*, 1986; Bay-Schmith and Pearse, 1987), postmetamorphic juveniles were reared under short-day conditions of 8 hr light and 16 hr dark or under constant light. At intervals, gonads were dissected from five individuals. The majority of the gonad was frozen in liquid nitrogen and stored at −70°C. A small fragment of the isolated gonad was immediately processed for histological examination and *in situ* hybridization (see below).

Histology. Gonad fragments were fixed in 3% paraformaldehyde, 0.3% glutaraldehyde, and 50 m*M* sodium phosphate buffer, pH 7.5 (Poccia *et al.*, 1989). After dehydration in ethanol and embedding in Paraplast, 10-μm sections were cut and mounted on polylysine-coated slides. Sections were stained with a standard recipe of hematoxylin and eosin before viewing for stage of maturation. About 80 individuals were scored over the course of this study.

Morphometric analysis of selected sections was performed from photographs of microscope fields at 400× using a bit pad and SigmaScan (Jandel Scientific, Inc.). Three areas from each testes were counted. Field sizes were calibrated with photographs of a hemocytometer grid.

In situ hybridization. *In situ* hybridizations were carried out using previously described methods (Hough-Evans *et al.*, 1987) based on the procedures of Angerer and Angerer (1981) and Cox and co-workers (1984, 1986). Five-micrometer sections from each sample previously prepared for histological examination were mounted on the same slide. The sections were deproteinized with proteinase K, treated with acetic anhydride, and hybridized with ^{35}S probes. In order to reduce background binding associated with ^{35}S probes, reducing agents (5 m*M* dithiothreitol and 1% β-mercaptoethanol) were incorporated into the hybridization solution and posthybridization washes included 1% β-mercaptoethanol (Hough-Evans *et al.*, in press).

RNA isolation. Frozen gonads were individually ground into a powder with an equal volume of dry ice. The powder was transferred to 10–20 vol of guanidinium lysis buffer (5 *M* guanidinium thiocyanate, 50 m*M* EDTA, 50 m*M* Tris-HCl, pH 7.0, 5% β-mercaptoethanol) without allowing it to thaw. After the suspended powder had thawed and bubbling had ceased, the lysate was sonicated for 30 sec. Debris was removed by centrifugation at 4000 rpm for 30 min. The supernatants were layered over a cesium chloride cushion (5.7 *M* CsCl, 10 m*M* Na acetate, pH 5.5) and centrifuged at 40,000 rpm, 20°C overnight in a Beckman SW 55 rotor (Glisin *et al.*, 1974). Pelleted RNA was washed with 70% ethanol and dissolved in water, and the total mass recovered was determined by absorption at 260 nm. RNA from this solution was precipitated overnight from 0.3 *M* sodium acetate with 2 vol of ethanol. The ethanol pellet was dissolved in water and precipitated overnight with 4.5 *M* Na acetate. This pellet was dissolved in water, brought to 0.3 *M* Na acetate, and precipitated with 2 vol of ethanol. Transcript titrations were performed on this purified material.

Probe. The probe used for the transcript titrations was prepared from a *SalI* restriction fragment of the bindin cDNA recombinant clone isolated by Gao *et al.* (1986). This 438-bp fragment was cloned into the *SalI* site of pBluescript KS M13+ (Stratagene) in an orientation which yields an antisense RNA product from the T7 promoter after linearization with *XhoI*. Labeled RNA for transcript titrations was prepared using this template, T7 RNA polymerase and [^{32}P]UTP. These reactions were carried out under conditions that routinely yielded about 6×10^8 dpm/μg. For *in situ* hybridization probes were made with ^{35}S to a specific activity of 1×10^9 dpm/μg.

Transcript titration. The estimation of transcript number by single-stranded probe excess titration has been described (Lee *et al.*, 1986). Briefly, probe excess RNA-RNA solution hybridizations at a criterion of 0.4 *M* Na^+, 50% formamide, 50°C were carried out with a probe derived from the isolated cDNA clone of the bindin gene (Gao *et al.*, 1986, see above). After overnight incubation, the unhybridized probe was removed by RNase A and RNase T_1 treatment. Hybrid molecules were collected on glass fiber filters after TCA precipitation and subsequently counted. The number of transcripts was calculated from the specific activity of the probe and the amount of probe protected.

RESULTS

Timing of the Onset of Gametogenesis

Because the reproductive periodicity of purple sea urchins is regulated by seasonal changes in photoperiod (Pearse *et al.*, 1986) and individuals cultured on fixed *short-day* (8L:16D) cycles maintain ripe gonads (Bay-

Schmith and Pearse, 1987), we reared postmetamorphic sea urchins under either constant light or short-day conditions in an attempt to measure the effect of photoperiod on the onset of gametogenesis. To identify the first cascade of differentiation in the testis, gonads were scored histologically for the presence of differentiated sperm at intervals. Over the 11-month interval following metamorphosis, neither the juveniles reared under constant light conditions nor those reared under short-day conditions possessed gonads with sperm. Even in young, relatively undifferentiated gonads, ovaries could be excluded from the analysis on the basis of the presence of very distinctive germinal vesicles in the small primary oocytes. Thus animals with neither germinal vesicles nor differentiated sperm were considered to be immature males. At 11 months when a large proportion of the animals were over 20 mm in test diameter, there were two males in the five animal short-day sample and one contained a few recognizable sperm heads. No constant light animals were sacrificed for histology at 11 months. At 12 months both constant light and short-day animals contained sperm although some testes were still negative; i.e., immature males were identified by the above criteria. At 13 and 14 months after metamorphosis all testes in the samples contained sperm. Because of the small sample size and the infrequent sampling interval described here, no effect of photoperiod on the onset of spermatogenesis could be demonstrated. Mature sperm appeared in the testes of juveniles reared under either short-day conditions or under constant light conditions at the same time, about 11 months after metamorphosis. All assayed individuals from both light treatments were producing sperm by 14 months after metamorphosis.

Histology and Morphometrics

In testes at 12 months of age, the accessory cells fill the testis tubule and the germ line cells lie in small, widely separated clusters only a few cell diameters thick at the periphery. As maturation of the testes into fully cycling gonads proceeds, the numbers of differentiating cells increase via two paths: (1) The number of pyramid-shaped clusters increases as additional foci of spermatogonial proliferation develop filling the tubule margin with closely apposed pyramids. (2) Within each pyramid more spermatocytes appear, which subsequently increases the number of differentiating cells extending toward the lumen of the tubule. The lumen is expanded by the pool of differentiated sperm.

In testes from individuals at 12 months of age, the spermatocytes did not extend very far into the lumen of the tubule. The number of spermatocyte nuclei varied from 60 (1 SD = 24)/10^4 μm^2 in one short-day treatment to 13 (1 SD = 2)/10^4 μm^2 in another (Table 1). Testes from a mature male possessed pyramids in contact all around the periphery of the gonad tubule and 52 (1 SD = 10) spermatocyte nuclei/10^4 μm^2. The mean number of sperm varied from 73 (1 SD = 23)/10^4 μm^2 in the mature male to 2 (1 SD = 1)/10^4 μm^2 in maturing testes samples.

In Situ Hybridization

Tissue from animals which exhibited sperm in sections was selected for *in situ* hybridization and the titration of the bindin gene transcripts. Previously fixed and embedded tissue was used for the *in situ* procedure and total RNA was isolated from the frozen testes.

In sections from testes of the mature male the pyramids of differentiating cells are fully formed and closely apposed. When these sections are hybridized *in situ* with antisense RNA to the message for bindin they exhibit silver grains densely clustered over a discrete band of cells displaced toward the lumen from the germinal epithelium of the gonad tubule (Fig. 1a). The dense band of exposed silver grains overlies the region of spermatocytes before condensation begins (Fig. 1c) and extends over all the pyramids of the tubule (Fig. 1d). Densities of silver grains above background did not appear over accessory cells, peripheral spermatogonia, condensed spermatids, or sperm.

In the developing gonads of the 12-month-old males, cells positive for bindin message are revealed in the same radial position in the emerging pyramids although the pyramids are proportionately smaller than those of the mature testis. Because the pyramids are fewer and more widely separated, silver grains are seen as widely separated dense clusters (Fig. 1b). At this time the pyramids are just extending toward the lumen and sperm are few.

The least developed testes in which only a few sperm were found possessed groups of differentiating spermatocytes which were only a few to 10 cells thick, usually with no apex to the pyramid. Furthermore, no positive signal for bindin message was found in this testis (data not shown).

Bindin Message Number

Total RNA was isolated from the frozen tissue corresponding to the testes sections found positive for sperm. The maturing testes yielded from 20 to 240 μg of total RNA. Several total RNA samples with adequate amounts were assayed for the mass of bindin message present using an RNA protection technique. Likewise, the total RNA isolated from the testes of a mature animal was assayed (Table 1). The proportion of bindin message in total RNA from testes was lowest at 3×10^6 transcripts/μg total RNA for the 12-month-old constant

TABLE 1

Age	Transcript per μg total RNA	Mean no. nuclei/10,000 μm²*	Mean no. sperm/10,000 μm²*	*In situ* hybridization
12-Month constant light male	3×10^6	19 ± 5	2 ± 1	Negative
12-Month short-day male	1×10^7	60 ± 24	40 ± 18	Positive
12-Month short-day male	1.5×10^7	13 ± 2	9 ± 9	Positive
14-Month short-day male	4×10^7	ND	ND	ND
Mature male	8×10^7	52 ± 10	73 ± 23	Positive

* The number of nuclei or sperm is expressed as a mean of three measurements ± one standard deviation.

light-reared individual and increased with age to a high value of 4.0×10^7 transcripts/μg total RNA for a 14-month-old male. The mature cycling testes yielded 8.0×10^7 transcripts/μg total RNA. These values were proportional to the relative number of differentiating cells in the gonad.

DISCUSSION

The Locale of Bindin Accumulation

Previously, bindin mRNA expression was demonstrated by RNA gel blot analysis to be restricted to the testes (Gao *et al.*, 1986). By *in situ* hybridization, we show that the expression of this message is restricted to a subset of germinal epithelial cells in the mature testes tubule. Within the characteristic pyramid of differentiating cells the positive cells form a group clearly displaced from the most basal portion which does not extend to the center of the tubule. The region of expressing cells corresponds to the "luminal germinal layer" (Poccia *et al.*, 1989) wherein lie late spermatocytes to early spermatids. This subset of cells forms a band in a mature testes because the tubule is filled with pyramids of differentiating cells, while in testes undergoing the first maturation only separated clusters of expressing cells are evident. These isolated clusters reflect the more sparse distribution of pyramids in maturing testes.

The bindin protein which is translated from the transcripts localized to late spermatocytes can be identified in these same late spermatogenic cells as a punctate immunofluorescence using a rhodamine-conjugated polyclonal antibody. Electron microscopy with immunogold-labeled ultrathin sections reveal that this punctate pattern is a result of the localization of the bindin in intracellular vesicles which subsequently fuse to form the single acrosomal vesicle (Nishioka *et al.*, in press). Since the protein is found in the same cells where the message is localized, the bindin message must be processed and translated very soon after it is transcribed.

Comparing Measures of Spermatogenic Activity

The various measures of spermatogenic activity (transcript number, mean number of sperm per unit area, and a positive result with *in situ* hybridization) increase in a parallel fashion with respect to maturity. The testes with the fewest sperm/μm² also had the lowest number of bindin transcripts/μg total RNA and a negative *in situ* hybridization result. It possessed very poorly developed pyramids of differentiating cells which did not span the region of the gonad tubule in which bindin message was demonstrated in more mature preparations. More mature testes with greater numbers of sperm had higher proportions of bindin mRNA as well as better developed pyramids. The highest level of bindin mRNA was found in the mature cycling testes which also possessed the most sperm and pyramids closely packed around the tubule. Thus the bindin message accumulation appears to reflect an increase in the relative proportion of accumulating cells rather than an increased number of transcripts per cell.

The single morphometric measure which was not parallel across samples of increasing activity was the mean number of nuclei/μm². One intermediate sample appeared more like the least mature testis and one appeared like the mature testis. This may reflect a peculiarity of the kinetics of cell proliferation at first maturation in a testis. The intermediate sample with the higher number of nuclei may have undergone a burst of proliferation without reaching the level of differentiation seen in the mature testis, while the intermediate sample with the lower number may have begun to differentiate before the increased proliferation occurred.

Gene Activity during Spermatogenesis

Like bindin, the mRNAs coding for unique H1 and H2b histone protein subtypes and their mRNAs can be found exclusively in testis (Lai and Childs, 1986; Lieber *et al.*, 1986; Lai and Childs, 1988). These histone variants appear early in spermatogenesis, well before spermatid differentiation, as the predominant representatives of

FIG. 1. *In situ* hybridizations in testes sections with a probe for bindin mRNA. (a) Dark-field illuminated section of the testis of a mature male. Overexposed to show the band of silver grains some distance from the margin of the tubule. The bar indicates the region of the proximal germinal layer. Bar = 75 μm. (b) Dark-field illuminated section of a testis from a 12-month-old maturing sea urchin. Overexposed to show the isolated clusters of positive cells scattered around the tubule. Bar = 75 μm. (c) Bright-field detail of a single pyramid in the testis tubule of a mature male demonstrating silver grains over uncondensed nuclei in the luminal germinal epithelium. Arrowhead indicates tubule margin. Bar = 8 μm. (d) Bright-field photomicrograph of a mature testis tubule with positive cells in the luminal germinal layer of many pyramids. Arrow indicates tubule margin and bar indicates the proximal germinal layer. Bar = 19 μm.

their class (Poccia *et al.*, 1987). The dephosphorylation of these sperm-specific histones may act to stabilize the chromatin of the spermatozoan or aid in the shaping of the sperm nucleus (Poccia *et al.*, 1987). In contrast to bindin which accumulates in the luminal germinal layer consisting of late spermatocytes and early spermatids, the testis-specific histone, H2B-1, is expressed exclusively in a subset of male germ line cells which lie in the basal germinal layer and are morphologically identical to replicating cells pulse-labeled with [^{3}H]thymidine (Poccia *et al.*, 1989). Thus the accumulation of the sperm-specific histones precedes the condensation of the chromatin in the spermatids by a greater margin than the accumulation of bindin precedes the assembly of the terminal differentiation products such as the acrosomal granule. Histones probably are assembled into the chromatin at the last round of DNA replication while the terminal differentiation gene products are accumulated just before the assembly of organelles such as the acrosomal granule. Thus multiple, probably sequential, classes of unique gene activity contribute to the differentiation of sperm.

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APPENDIX C MOLECULAR REPRODUCTION AND DEVELOPMENT 27:181–190 (1990)

Localization of Bindin Expression During Sea Urchin Spermatogenesis

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ABSTRACT Expression of the bindin gene was examined in testicular cells of the sea urchin *Strongylocentrotus purpuratus*. In situ hybridization studies, using an ^{35}S-labeled antisense RNA probe transcribed from a bindin cDNA, reveal that bindin mRNAs are localized in spermatogenic cells displaced towards the lumens of maturing testicular acini. Little or no hybridization is observed in spermatogenic cells displaced towards the perivisceral epithelium or in somatic cells of the testis. A similar localization of the bindin protein itself is observed using a rhodamine-conjugated polyclonal antibody against bindin, which shows a punctate immunofluorescence pattern in late spermatogenic cells. Immunogold labeling of ultrathin sections and electron microscopy reveal that this punctate immunofluorescence is an apparent result of localized deposits of bindin in intracellular vesicles. Through the terminal stages of spermatogenesis, these bindin-containing vesicles apparently fuse to form the single acrosomal vesicle of the mature spermatozoon. These results indicate 1) that bindin mRNAs are transcribed relatively late in spermatogenesis, 2) that bindin is translated soon after production of its mRNA, 3) that bindin quickly associates with intracellular vesicles during or soon after its synthesis, and 4) that these vesicles fuse to form the single acrosomal vesicle during the terminal stage of spermatogenesis.

Key Words: Testis, Spermatogenic cells, Bindin mRNA, In situ hybridization, Immunocytochemistry, Electron microscopy

INTRODUCTION

During sea urchin fertilization, specific recognition between sperm and egg is mediated by bindin, a 24 kDa protein located within the acrosomal vesicle of the mature spermatozoon (Vacquier and Moy, 1977; Gao et al., 1986). Contact of the spermatozoon with the jelly layer of the egg induces the acrosome reaction, in which the acrosomal vesicle undergoes exocytosis and an acrosomal process is elongated from the tip of the sperm head (reviewed by Tilney, 1985; Schackmann, 1989). Bindin is externalized during the acrosome reaction but remains closely associated with the plasma membrane over the acrosomal process where it can bind to complementary sperm receptors on the vitelline layer of the egg. Under natural conditions this complementary binding ensures the species specificity of fertilization for most species (Glabe and Vacquier, 1977; reviewed by Minor et al., 1989).

Adding to the many studies determining the role of bindin in sperm-egg interactions are more recent studies directed at the gene coding for this important protein. The cloning and sequencing of cDNAs for bindin (Gao et al., 1986) have elucidated the precursor structure of the probindin molecule and allowed for experiments demonstrating that the bindin mRNA is testis specific. It has also led to in situ hybridization studies determining when during postmetamorphic development the bindin gene first becomes transcriptionally active (Cameron et al., 1990). In the latter study, radioactively labelled antisense RNA transcribed from a 438 bp *SalI* fragment of the bindin gene was used to probe postmetamorphic and adult testis sections for the presence of bindin mRNAs. No bindin mRNA was detectable in early postmetamorphic juveniles but was detectable in late postmetamorphic and adult testes where it was most concentrated in spermatogenic cells displaced towards the lumens of testicular acini. These results suggested that the bindin gene was transcribed relatively late in spermatogenesis.

The present study was undertaken to provide additional information about bindin gene expression in spermatogenic cells of the sea urchin testis by determining wherein the testis and when during spermatogenesis the bindin protein itself becomes detectable. There are five testes in adult male sea urchins each radiating to a gonopore on the aboral surface and each of which is arborescent in form with thousands of acini branching from a central collecting duct. During a re-

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productive year, these testes undergo major structural reorganizations (Fuji, 1960; Holland and Giese, 1965; Longo and Anderson, 1969). Immature or unripe testes (between breeding seasons) are usually small and highly pigmented. Each acinus is composed of a somatic perivisceral epithelium monolayered over a basement membrane, a thin layer of smooth muscle, and a second basement membrane surrounding a central region consisting of nutritive phagocytes or supportive cells and a few spermatogenic precursor cells (stem cells or spermatogonia). The maturing testis (beginning of the breeding season) is characterized by the formation of a germinal epithelium, proliferation of spermatogonia, appearance of spermatocytes, and columns of cells with spermatogonia at the base of the epithelium and more advanced stages extending towards the lumen where spermatozoa begin to collect. In mature testes (end of the breeding season), the nutritive phagocytes recede to a diminishing germinal epithelium as spermatozoa fill the distended lumen. In this study we employ the techniques of in situ hybridization, indirect immunofluorescence, and immunogold labelling to determine 1) the location of bindin mRNAs in the maturing testis, and 2) the location of bindin proteins in the maturing testis at both the light and electron microscope levels.

MATERIALS AND METHODS

Animals and Testis Preparations

Sea urchins, *Strongylocentrotus purpuratus*, were purchased from Pacific Biomarine Laboratories, Inc. (Venice, CA) and maintained at 15°C in refrigerated aquaria containing Instant Ocean synthetic sea water. Males were induced to release semen by intracoelomic injection of 0.55 M KCl. When shedding was complete, the testes were excised, cut into 0.5–1.0 cm explants, and suspended in artificial sea water (ASW) (Harvey, 1956) overnight at 4°C to allow further release of semen. After several washings with ASW, the explants were fixed in ASW containing 3% paraformaldehyde and 0.1% glutaraldehyde.

Preparation of RNA Probes

A 438 bp *SalI* fragment of a bindin cDNA clone (Gao et al., 1986) was subcloned into Bluescript M13+ (Stratagene Cloning Systems, San Diego, CA) in both the sense and antisense orientations with respect to the T7 promoter. Prior to in vitro transcription, these plasmids were truncated with *XhoI* restriction endonuclease. Antisense riboprobes were prepared by in vitro transcription catalyzed by T7 RNA polymerase following the protocol of Promega Biotech (Madison, WI). The probes were labeled with [^{35}S]-uridine-5'-(α-thio)-triphosphate (850 Ci/mmole) (New England Nuclear, Boston, MA) to a specific activity of 10^9 dpm/μg RNA.

In Situ Hybridization

The procedures of Poccia et al. (1989) as modified from Cox et al. (1984) were used with the following additional modifications. Paraffin sections were 4 μm thick. The protease pretreatment step was eliminated to preserve tissue structure. Hybridization was carried out at 45–47°C overnight in hybridization buffer containing 100 mM dithiothreitol (DTT). The 4× standard saline citrate (SSC) and RNase buffers contained 10 mM DTT. The slides were washed in hybridization buffer containing 10 mM DTT at 65°C for 10 min, twice in 2× SSC containing 10 mM DTT at room temperature, once in 0.1× SSC containing 10 mM DTT for 30 min at 50°C, before the final 0.1× SSC wash. Preparations were stained with Harris' hematoxylin and counterstained with Fast Green FCF in 95% ethanol. Probes were not hydrolyzed. Input RNA concentration was 0.1 μg/ml.

Photomicrographs were taken with a Zeiss MC-63 autocamera system using Kodak TMAX-100 film and a Zeiss Standard microscope equipped with bright-field optics and a green filter.

Immunocytology and Light Microscopy

Localization of bindin in paraffin-embedded thick sections of maturing testes was determined by indirect immunofluorescence. Four micrometer sections were deparaffinized with two washes of toluene and rehydrated through a 100% (2×), 95%, 80%, 70%, 50% distilled water series. Two drops cold bovine serum albumin (1 mg/ml in phosphate-buffered saline, PBS, pH 7.4) were added and the slides incubated for 60 min in a moist chamber. After three rinses with PBS, a drop of undiluted rhodamine-conjugated rabbit anti-bindin polyclonal antibody (Vacquier, 1984) was added and the slides incubated in a moist chamber for 60 min. The sections were then rinsed three times with PBS and labeled with a drop of Hoechst 33342 (10 μg/ml in dimethylsulfoxide) for 5 min. Following three final rinses with PBS, the sections were mounted in Fluorimount-8 (Fischer Scientific) and viewed with a Zeiss Standard microscope equipped for epifluorescence illumination.

Immunogold Labelling and Electron Microscopy

Fixed testis explants (see above) were postfixed in 1% OsO_4 in ASW and embedded in Epon 812 or Spurr's (Spurr, 1969) embedding media. Ultrathin sections were cut with a diamond knife on a Sorvall Porter-Blum ultramicrotome and collected on Formvar-coated, slotted nickel grids or on 200- or 300-mesh uncoated nickel grids. Immunogold labeling of the sections was performed as follows: the sections were pretreated for 30–60 min with 0.1 M sodium metaperiodate; washed with distilled water; incubated in blocking solution (1 mg/ml each bovine serum albumin and glycine in ASW) for 30 min; incubated for 2 hours in a 1/1,000 dilution of polyclonal antibody against bindin in blocking solution; washed with blocking solution for 15 min; incubated for 2 hours in a 1/40 dilution of 10 or 20 nm colloidal gold conjugated goat anti-rabbit IgG (E.Y. Laboratories, San Mateo, CA) in blocking solution; and

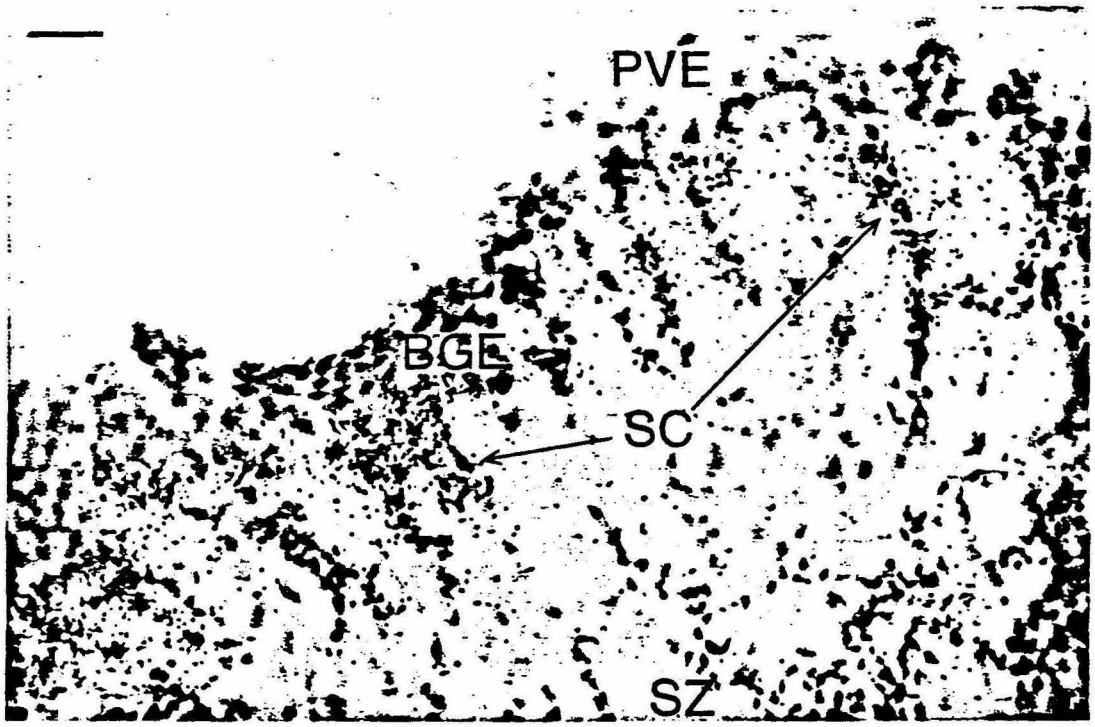

Fig. 1. In situ hybridization of ^{35}S-labeled bindin antisense RNA to a maturing sea urchin testicular acinus. PVE, perivisceral epithelium; BGE, basal germinal epithelium; SC, spermatogenic columns; SZ, spermatozoa. Bar = 10 μm.

finally washed with blocking solution and boiled distilled water. All incubations were performed at room temperature in an ASW-saturated chamber and all solutions were prepared fresh and filtered through 0.2 μm Millipore filters. The labelled sections were stained with 5% aqueous uranyl acetate for 4 min and lead citrate for 3 min and examined with a JEOL JEM-1200 EX election microscope operated at 80 kV.

RESULTS

Localization of Bindin mRNA

Figure 1 is an autoradiogram showing a portion of a maturing testicular acinus (see also Fig. 5 for more detail). The acinus is limited by a perivisceral epithelium that overlies a smooth muscle layer and several layers of spermatogenic cells. The most basal layer of the germinal epithelium contains mostly replicating cells, the spermatogonia and pre-meiotic spermatocytes (Poccia et al., 1989). More luminally the basal germinal epithelium contains spermatocytes and early spermatids and is continuous with columns of later stage spermatogenic cells that proceed between the nutritive phagocytes towards the lumen. Thus the spermatogenic columns may exhibit a gradient of differentiation from spermatocyte to mature spermatozoon. This peripheral-luminal gradient of differentiation observed in the sea urchin testicular acinus is similar to the gradient observed in the mammalian seminiferous tubule.

Autoradiography shows the in situ hybridization of ^{35}S-labeled antisense RNA transcribed from a 438 bp *Sal*I fragment of the bindin gene. The labeled RNA hybridizes most predominantly in the columns of spermatogenic cells between the nutritive phagocytes. Little or no hybridization is observed in the more basal spermatogenic cells or in the nutritive phagocytes. These results are shown at higher magnification in Figure 2 and would suggest that the bindin mRNA accumulates relatively late in spermatogenesis.

Anti-Bindin Immunofluorescence

Figure 3 shows a paraffin section of a testicular acinus stained with Hoechst (Fig. 3a) and a rhodamine-conjugated anti- bindin polyclonal antibody (Fig. 3b). The Hoechst staining shows the same columns of spermatogenic cells shown for the in situ hybridization

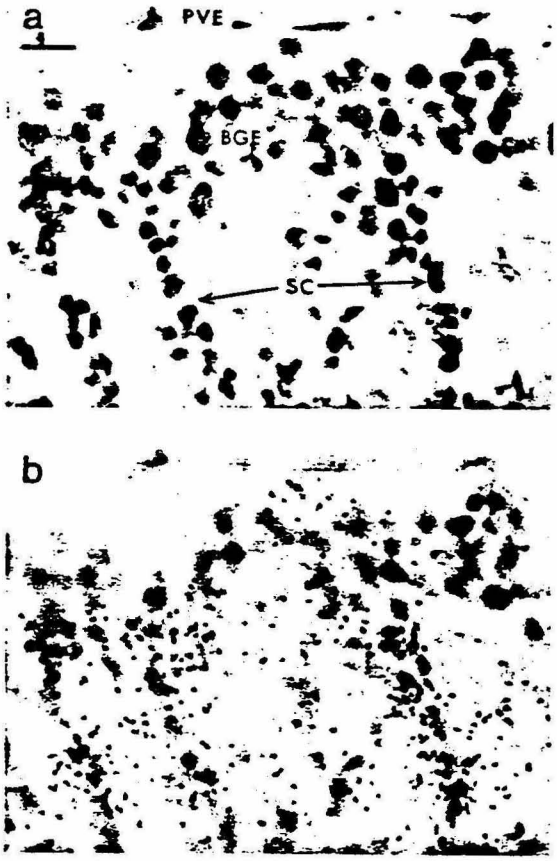

Fig. 2. In situ hybridization of ^{35}S-labeled bindin antisense RNA to a maturing sea urchin testicular acinus. a: Focus on hematoxylin stained nuclei. b: Same frame with focus on silver grains in photographic emulsion. PVE, perivisceral epithelium; BGE, basal germinal epithelium; SC, spermatogenic columns. Bar = 6 μm.

studies. In this case, however, since Hoechst is a fluorescent nuclear stain, the nutritive phagocytes appear as large spaces between the spermatogenic cells. Anti-bindin immunofluorescence staining reveals that, like its mRNA, bindin is localized most predominantly in the spermatogenic cells of the columns, and little or no immunofluorescence is observed in spermatogenic cells displaced towards the perivisceral epithelium or in nutritive phagocytes. On closer examination, it is further observed that within individual spermatogenic cells bindin is localized in one to several concentrated regions of the cells conferring a punctate immunofluorescence pattern to the acinus shown in Figure 3b. These results would suggest that bindin is synthesized soon after the appearance of its mRNA and that it quickly becomes localized in intracellular deposits.

Anti-Bindin Immunogold Labeling of Ultrathin Sections

To obtain a more detailed localization of bindin in testicular cells, the techniques of immunogold labeling and electron microscopy were employed. Figure 4 demonstrates the efficacy of the techniques devised for this study. A post-embed labeled thin section through the lumen of a mature testis is provided in Figure 4a and shows the expected acrosome-specific labeling of mature spermatozoa wherever the section is through an acrosomal vesicle. The electron micrograph provided in Figure 4b shows this acrosome-specific labelling in a single spermatozoon at higher magnification. Very little background labeling is observed in these micrographs.

Figure 5 is a photomontage of two low-magnification electron micrographs that shows the various cell types and their spatial relationships in the maturing testis. The perivisceral epithelium (PVE) is separated from the smooth muscle (SM) layer by one basal lamina while the muscle layer is separated from the basal germinal epithelium (BGE) by a second basal lamina and a layer of nutritive phagocytes (NP). Also shown in greater detail is the peripheral-luminal gradient of spermatogenic differentiation. Early spermatogenic cells, such as spermatogonia and 1°-spermatocytes, reside in the most basal region of the BGE while the later spermatogenic cells are found in the spermatogenic columns (SC) trailing between the nutritive phagocytes toward the lumen.

The higher magnification electron micrographs provided in Figure 6 correspond to the insets specified in Figure 5 and reveal where in the testis and in which cells bindin is localized. These immunogold localizations are consistent with the immunofluorescence localizations presented above in showing discrete intracellular deposits of bindin in later stage spermatogenic cells. Figure 6a shows two spermatids that are connected with one another by an intercellular bridge and one of which displays a small bindin-containing vesicle located in the peripheral cytoplasm. These cells have begun the process of spermiogenesis as indicated by the condensation of the nucleus, loss of cytoplasm, fusion of mitochondria, and formation of the tail. Figure 6b shows another spermatid in the same spermatogenic column at a later stage in spermiogenesis. This cell has yet to proceed through the final stages of spermiogenesis during which the subacrosomal fossa will be formed and the remaining cytoplasm will be shed. A labeled vesicle that will become the acrosome in the mature spermatozoon has migrated to the apical region of the cell. Spermatozoa in the same spermatogenic column and at the terminal stages of spermiogenesis are shown in Figure 6C with typical acrosome-specific labeling. In an adjacent spermatogenic column an earlier spermatid is shown with a bindin-containing vesicle (Fig. 6d). This cell appears to be undergoing the early

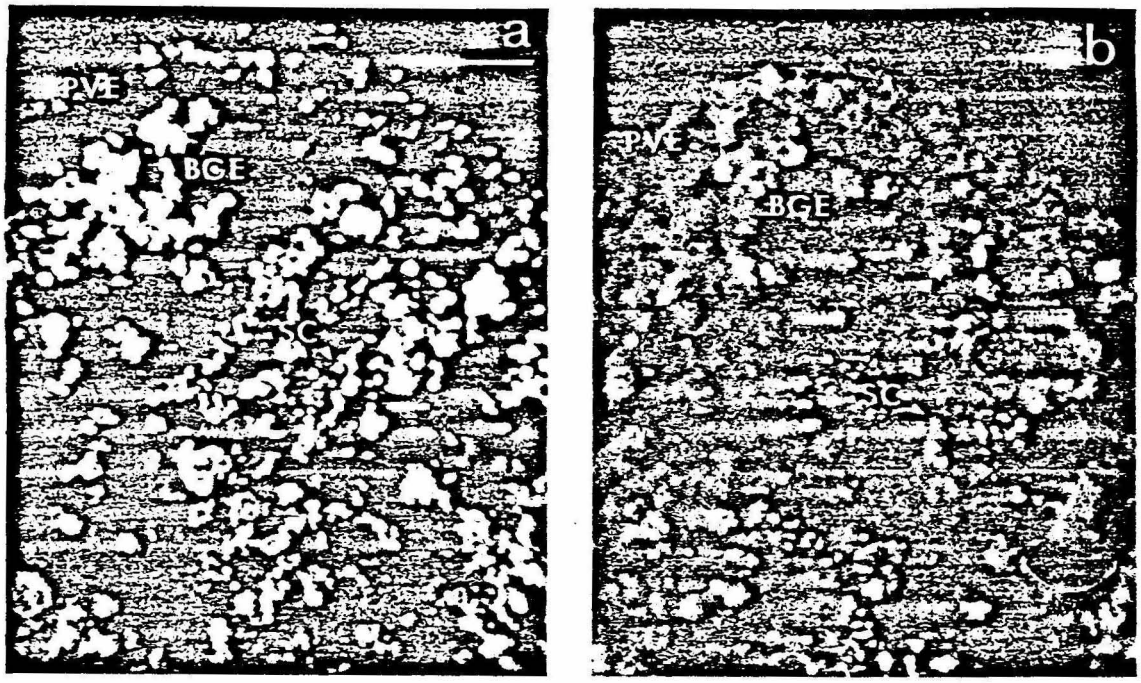

Fig. 3. Binding of rhodamine-conjugated anti-bindin antibodies to a maturing sea urchin testicular acinus. a: Hoechst 33342-stained nuclei. b: Same frame showing immunofluorescence. PVE, perivisceral epithelium; BGE, basal germinal epithelium; SC, spermatogenic columns. Bar = 6 μm.

stages of nuclear condensation but still contains much of its cytoplasm and is closely associated with a nutritive phagocyte. A large labeled vesicle will presumably become the acrosomal vesicle in the mature spermatozoon.

Although labeled cells in earlier stages of spermatogenesis were not observed in the particular section shown in Figure 5, they were observed in other testis sections (Fig. 7). Figure 7a shows an earlier spermatogenic cell containing a labeled vesicle within its cytoplasm and closely apposed to the nucleus. Cells at this stage of spermatogenesis typically show vesicles in close association with the nucleus, whereas in more developed cells the vesicles tend to be more peripherally located. Figure 7b shows an early spermatid, in which the nucleus is condensing and much of the cytoplasm has been shed. This cell, like the spermatids shown in Figure 6a, 6b, and 6d, contains a labeled vesicle within its cytoplasm close to the periphery of the cell. Although very rarely, other testis sections revealed the presence of two labeled vesicles within the cytoplasm of one spermatogenic cell (results not shown).

In none of our immunogold studies did we observe labeling in earlier spermatogenic cells such as the spermatogonia and 1°-spermatocytes of the basal germinal epithelium, or in nutritive phagocytes that do not contain residual bodies. We did, however, occasionally detect bindin in the acrosomes of decomposing sperm in the residual bodies of nutritive phagocytes. In total, our anti-bindin immunogold results are consistent with our bindin mRNA localizations and our anti-bindin immunofluorescence localizations indicating that the bindin gene is expressed relatively late in spermatogenesis and in the spermatogenic cells themselves.

DISCUSSION

In the present study, we have further investigated bindin expression during spermatogenesis in sea urchins by 1) localizing bindin mRNA in the maturing testis and 2) localizing bindin proteins in the maturing testis at both the light and electron microscope levels. Our results indicate that the bindin gene is transcribed and the bindin mRNA is translated in spermatogenic cells of the testis during the later stages of spermatogenesis. There is no evidence that these syntheses occur in nutritive phagocytes or other somatic cells of the

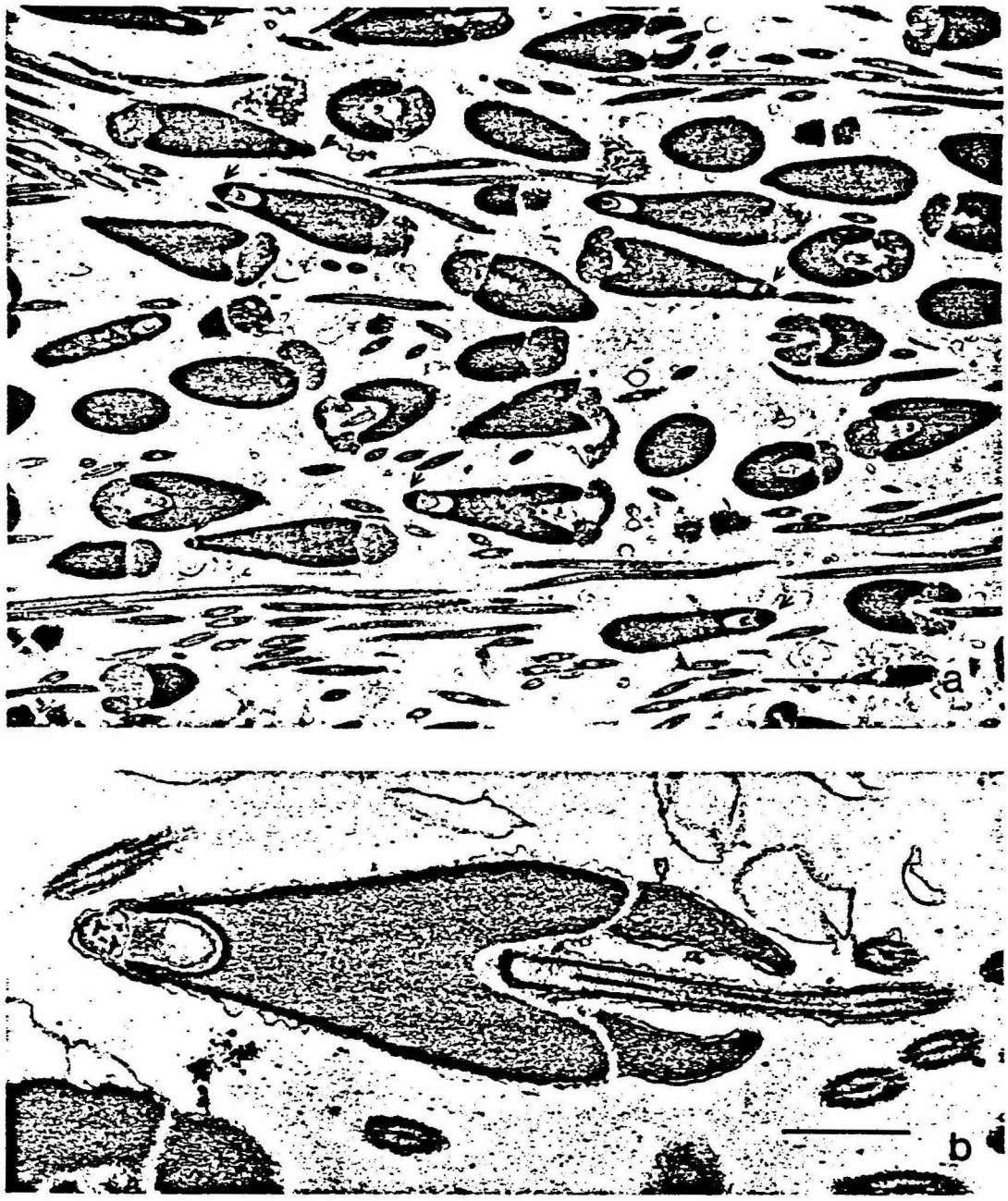

Fig. 4. Immunogold localization of bindin in mature (luminal) spermatozoa of a maturing sea urchin testis. a: Low-magnification electron micrograph showing acrosomal localizations (arrows) wherever the section is through an acrosome, bar = 2 μm. b: High-magnification electron micrograph showing specific labeling of the acrosome in a mature spermatozoon. Bar = 0.5 μm.

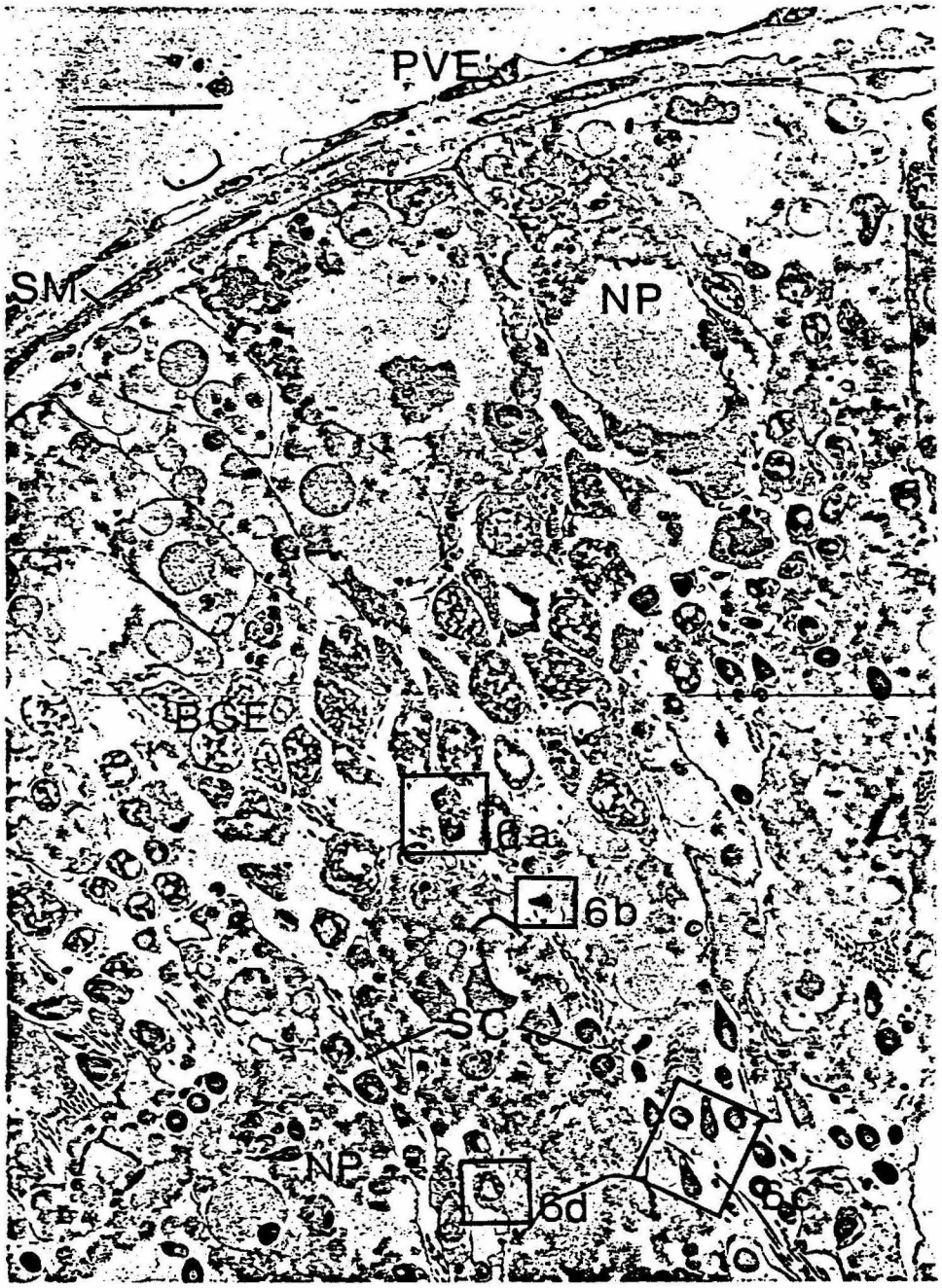

Fig. 5. Low-magnification electron micrographs (photomontage) of a maturing sea urchin testicular acinus. Lettered insets correlate with the high-magnification electron micrographs provided in Figure 6. PVE, perivisceral epithelium; SM, smooth muscle; NP, nutritive phagocyte; BGE, basal germinal epithelium; SC, spermatogenic column. Bar = 25 μm.

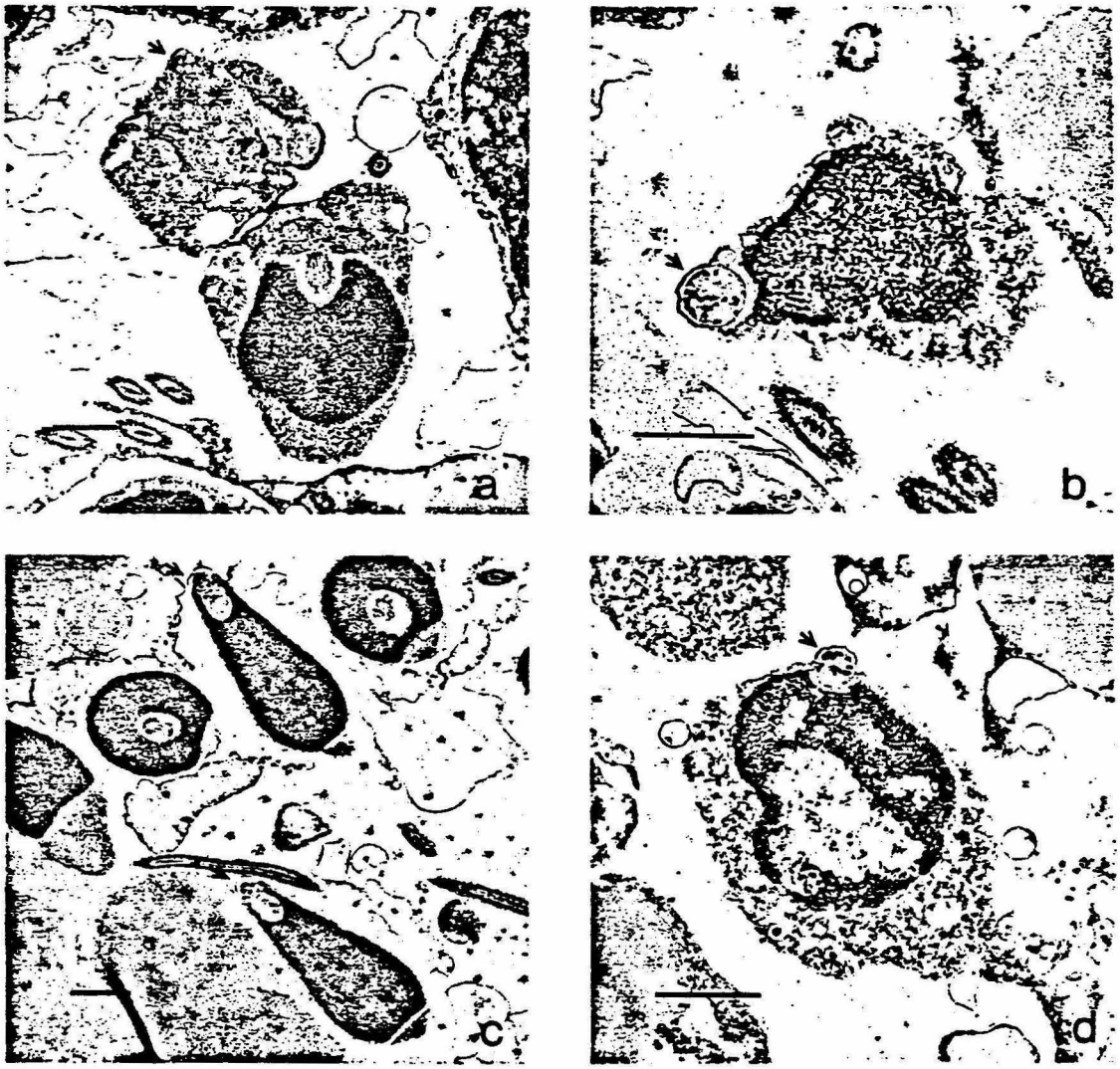

Fig. 6. High-magnification electron micrographs showing anti-bindin immunogold labeling of the spermatogenic cells within the lettered insets of Figure 5. Arrows indicate localized areas of concentrated labeling. Bars = 1 μm.

testis that might serve accessory functions. Our results also show that the bindin protein is first detectable in discrete cytoplasmic vesicles of spermatogenic cells before it is finally localized within the single acrosomal vesicle of the mature spermatozoon.

Localization of Bindin mRNA in the Testis

Our in situ hybridization results add to those reported in another recent study. Cameron et al. (1990) have hybridized the same probe used in our studies to the testes of early and late postmetamorphic sea urchins in an attempt to determine when during juvenile development and where in the testis the sperm-specific bindin gene first begins to be transcribed. Their results indicate that bindin mRNA first becomes detectable in the testes of 11–12 month postmetamorphic sea urchins and that it localizes most predominantly in spermatogenic cells displaced towards the lumens of maturing

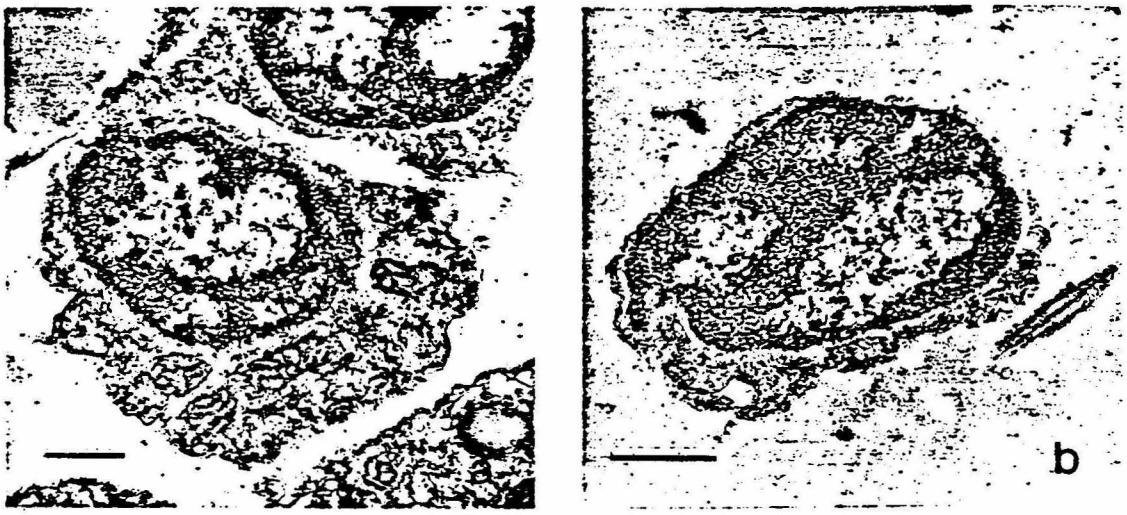

Fig. 7. High-magnification electron micrographs of representative spermatogenic cells showing anti-bindin immunogold labelling. Arrows indicate localized areas of concentrated vesicular labeling. Bars = 1 μm.

testicular acini. Their results with the testes of mature sea urchins, as further confirmed in this study, show a similar localization of bindin mRNA, indicating that it is synthesized relatively late in spermatogenesis.

Using the same techniques and under the same hybridization conditions, Poccia et al. (1989) have shown that transcription of the genes for the sperm-specific histones Sp H2B-1 of *S. purpuratus* and Sp H2B-2 of *Lytechinus pictus* occurs in early spermatogenic cells. The mRNAs for these histones localize in a subset of male germ line cells morphologically identical to replicating cells pulse-labeled with ^{3}H-thymidine. This localization within the most basal region of the germinal epithelium suggests that testis-specific histone gene expression is restricted to spermatogonia and premeiotic spermatocytes and that the resulting histone mRNAs are degraded rapidly before the early spermatid stage of spermatogenesis. These results are different from our results with the bindin gene, which suggest that transcription of this gene occurs after the replicative phase and that the bindin mRNAs persist well into the spermatid stage. Taken together, these results reveal that some sperm-specific genes may be transcribed early in spermatogenesis while others may be transcribed late. Likewise, they reveal that some sperm-specific mRNAs may be degraded early in spermatogenesis while others may be degraded late.

Localization of Bindin in the Testis

Both our anti-bindin immunofluorescence and immunogold labellings of testis sections suggest that bindin becomes localized in intracellular vesicles soon after its synthesis in spermatogenic cells of the maturing testis. The cells containing these vesicles correspond morphologically to those containing high concentrations of bindin mRNAs implying that these mRNAs are translated soon after their transcription. Our immunogold studies detect the earliest appearance of bindin-containing vesicles in 2°-spermatocytes, although our immunofluorescence studies, showing some labeling of the basal germinal epithelium, would suggest an even earlier appearance. As these cells progress through meiosis and spermiogenesis, the vesicles appear to fuse, migrate toward the apical end of the nucleus, and reach their final destination, the acrosome of the mature spermatozoon.

Labeled vesicles in earlier spermatogenic cells appear to be more centrally located than those in late spermatids. The close proximity of the vesicles to the plasma membrane in late spermatids may aid in the migration of the vesicles to their final destination. Although the mechanism of this migration is not clear, it is apparent that movement is occurring as the cell proceeds through spermiogenesis and may be facilitated by the cytoskeleton. An examination of the cytoskeletal properties of these cells might help to define a plausible mechanism.

The sperm acrosome has been shown to be derived from the Golgi apparatus in many mammalian species (Burgos and Fawcett, 1955; Clermont and Leblond, 1955; Clermont and Tang, 1985). In past studies of mammalian spermatogenesis as well as sea urchin

spermatogenesis, the Golgi apparatus is clearly evident in spermatocytes and early spermatids, but, as development proceeds, it becomes less regular and has usually disintegrated in late spermatids. A relationship that we cannot establish in our studies is one between definitive Golgi apparatuses with typical concentric saccules and the labeled vesicles. When we have observed definitive Golgi apparatuses, they were not labeled (results not shown), leaving open the question of whether the labelled vesicles are in fact Golgi derived. It is possible that bindin, a protein, is not processed through the Golgi apparatus but is synthesized on the endoplasmic reticulum and packaged into vesicles directly. In this scheme, the Golgi apparatus might still be involved in the production of these vesicles. Fawcett (1975) suggests that such a scheme may explain the appearance of vesicles that will contribute to the acrosome but are found at some distance from the Golgi apparatus and has provided some support for this view in a freeze-fracture study. In the future we will be using known Golgi-specific markers in an attempt to establish a relationship between the Golgi apparatus and the labelled vesicles. Additionally, we will be conducting similar experiments with a monoclonal antibody, mAb J18/2, that recognizes a 210 kDA plasma membrane glycoprotein and binds site-specifically over the acrosomal and tail regions of the mature spermatozoon (Nishioka et al., 1987, 1989; Trimmer and Vacquier, 1988; Ward et al., 1989). It will be interesting to determine if this glycoprotein, unlike the protein bindin, is processed through the Golgi apparatus.

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