A Sperm Chemoattractant Is Released from Xenopus Egg Jelly during Spawning

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Although the jelly layers surrounding amphibian eggs are known to be essential to fertilization, no biological activities have been reported for jelly macromolecules in any vertebrate. Here we provide evidence that the jelly surrounding *Xenopus laevis* eggs releases a small diffusible protein into the surrounding media that serve as sperm chemoattractant. Using video microscopy we find that *Xenopus* sperm will preferentially turn toward and contact a glass capillary filled with egg jelly extract. In experiments using a two-chamber bioassay device we find that egg jelly extracts are capable of stimulating sperm movement across a membrane barrier sixfold over controls. This activity is not observed in materials unrelated to egg jelly and the response of sperm to egg jelly extract is clearly chemotactic rather than chemokinetic. A concentration gradient of the extract is absolutely necessary, and the chemotactic activity of the extract exhibits a biphasic dose dependence similar to that of chemotactic agents in other systems. We have been able to characterize the factor as being a heat-stable protein about 10 kDa in size. This study, therefore, provides the first clear evidence for a diffusible sperm chemoattractant in a nonhuman vertebrate, as well as the first demonstration of a physiological role for egg jelly macromolecules in *Xenopus* fertilization. © 1998 Academic Press

Key Words: chemotaxis; egg jelly; fertilization; sperm motility; *Xenopus laevis*.

INTRODUCTION

Sperm are prepared for fertilization before they reach the egg surface by a series of interactions with the egg extracellular matrix [Katagiri, 1987; Hedrick and Nishihara, 1991]. One would likely include among these interactions activation of sperm motility, chemotaxis toward the egg, penetration of the outer extracellular layers of the egg, induction of an acrosome reaction or a sperm surface reorganization, binding to and penetration of the inner egg envelope, and finally binding to and fusion with the egg plasma membrane. Each of the individual steps have been elucidated partially or fully in echinoderms, amphibians, or mammals and there a numerous instances of mechanistic similarities between different phyla [Ward and Kopf, 1993]. Recently, for example, it has been shown that the inner egg envelope of anurans—the vitelline envelope—is a homologue of the mammalian zona pellucida and like the zona pellucida contains specific sperm binding proteins [Omata and Kata-giri, 1996; Yang and Hedrick, 1997: Kubo et al., 1997; Tian et al., 1997a,b]. However, in many amphibians such as *Xenopus laevis*, the mechanisms or even the occurrence of sperm chemotaxis, an acrosome reaction, or other steps that would prepare the sperm for binding to the egg are unknown. These steps would logically be mediated by the jelly layers.

Recently, we have studied the three jelly layers surrounding *Xenopus* eggs using a combination of gel electrophoresis, column chromatography, and electron microscopy and have found that each layer consists of a fibrillogranular network [Bonnell et al., 1996; Bonnell and Chandler, 1996]. The fibrous network itself is composed of high-molecular-weight proteoglycans (450 to 900 kDa) containing large amounts of acidic and neutral sugars. Bound to or included within these networks are low-molecular-weight (10 to 200 kDa) globular proteins, some of which are capable of diffusing rapidly into the surrounding medium. Based on these results we consider these outer extracellular matrices to be composed of a stable, fibrous network of sugar and protein polymers to which are attached "diffusible" proteins or factors.

Earlier studies in a number of amphibian species provided evidence that egg jelly components play a role in preparing sperm for fertilization of the egg. In *Bufo arenarum* and *Bufo japonicus*, diffusible components either alone (Barbieri and Villeco, 1966; Barbieri and Reisman, 1969; Barbieri...
and Oterno, 1972; Katagiri, 1973) or in combination with "stable" components [Katagiri, 1966; Barbieri and Oterno, 1972] facilitate fertilization. Likewise, both stable and fusible components were shown to support fertilizability of Rana pipiens eggs [Elison, 1971, 1974]. In X. laevis eggs, Oliphant et al. (1970) reported a component of the outermost [3] layer which facilitated fertilization, and Wolf and Hedrick (1971) found that the fertilizability of jellyless eggs was markedly increased in the presence of jellied eggs. In none of these studies, however, were the fertilization-enhancing constituents of jelly characterized as to their mode of action.

In this report we demonstrate the presence of a sperm chemotactrant within egg jelly of X. laevis by using video microscopy and an in vitro assay for directed sperm movement. The chemotactrant appears to be a small, heat-stable protein that is released upon hydration of the jelly layers during spawning. The activity is specific to egg jelly and to our knowledge constitutes the first reported biological activity of any jelly component in vertebrate eggs and the first definitive evidence for a sperm chemotactic factor in a nonhuman vertebrate.

MATERIALS AND METHODS

Gametes

X. laevis females obtained commercially [NASCO or Xenopus One] and maintained on a 12 h light–12 h dark cycle were injected with 1000 IU human chorionic gonadotrophin into the dorsal lymph sac. Ten hours after injection, the eggs were stripped from the female. Male frogs were decapitated and their testes were separated by a polycarbonate membrane having 12-μm-diameter pores were used to quantify sperm chemotaxis. The bottom chamber (trans) was filled with F-1 buffer, and a 150-μl bolus of the chemotactrant was micropipetted onto the floor of the bottom chamber to bring the total volume to 1.5 ml. The upper chamber (cis), consisting of an insert with the membrane stretched across its base, was lowered into the bottom chamber, the chamber was filled with a 0.5-ml aliquot of a sperm suspension, and the system was incubated for 80 min at room temperature. The sperm suspension used for this assay was prepared by adding 50 μl of sperm in 1.5× OR2 to 500 μl of F-1 within 10 s before use. This dilution provided an osmotic shock so as to place the sperm in a highly motile state as described by Bernardini et al. (1988). Following incubation, the insert was removed, the lower chamber buffer was centrifuged, and the resuspended pellet was counted for sperm using a hemocytometer. All assay data in the figures represent means and standard errors of the mean for at least three similar experiments. Transwell assay data in all figures should be multiplied by a factor of 200 to obtain absolute values.

As shown in Fig. 2C addition of the chemotactrant as a bolus was necessary for a sperm response. By injecting a similar 150-μl bolus of lucifer yellow and monitoring fluorescence at the site of bolus injection in the lower chamber and in the center of the upper chamber versus time, we find that this protocol sets up a gradient of dye. At the site of bolus injection the initial dye concentration [designated 100%] decreases gradually, reaching the 50% level after 20 min. Sampling of the upper chamber shows that no dye is present initially but that dye concentration gradually rises over 10 min to 10% of the level found at zero time in the bottom chamber and holds steady at that level over the next 10 min. These dye studies suggest that our protocol quickly sets up a concentration gradient of chemotactrant extending between the floor of the bottom chamber and the upper chamber.

Preparation of Materials Tested for Chemotaxis

Solubilized fresh egg jelly was prepared by incubating 0.5 g of freshly spawned eggs in 2 ml of F-1 buffer [14.25 mM NaCl, 1.25 mM KCl, 0.25 mM CaCl₂, 0.06 mM MgCl₂, 0.5 mM Na₃HPO₄, 2.5 mM Heps, with pH adjusted to 7.8] containing 2% β-mercaptoethanol [BME] for 10 min with gentle swirling. The resulting solution was dialyzed against distilled H₂O and stored at −20°C. The diffusible components of jelly, referred to as egg water, were prepared by a modification of the method described by Heasman et al. (1991)]. One-half gram of fresh eggs was incubated in 2 ml of F-1 for 3 h at room temperature with gentle swirling followed by removal of the eggs. The resulting conditioned medium had a protein content of 18 to 30 μg (BCA assay, Pierce) and was stored until use at −20°C. The eggs which had been removed were washed three times with F-1 and their jelly [referred to as "structural jelly"] was solubilized with 2% BME, dialyzed, and stored as described for fresh egg jelly. Egg water was further concentrated for video microscopy experiments by storing in a dialysis sack overnight at 4°C in a bed of dry Sephadex. The resulting concentrated egg water contained 90 μg/ml protein and tested positive for chemotaxis activity in both video microscopy and transwell assays.

Video Light Microscopy

Glass capillary tubes (160 μm inner diameter) were filled with a concentrated solution of 3 h egg water (90 μg/ml protein) and then placed on a glass slide and one end was surrounded by a circular dike of 1.5 Vasaline:paraffin. Sperm suspended in OR-2 buffer were diluted 1:10 with F-1 buffer to initiate motility and 250 μl was immediately added to the well. The final sperm concentration was approximately 2 × 10⁶ cells/ml. Specimens were then viewed at 25°C using a Zeiss Axioskop microscope equipped with 10 and 40× phase-contrast objectives, a Hamamatsu C2400-77 (CCD) video camera and controller, an Argus 10 image processor, and a Mitsubishi H5U-770 video cassette recorder. Reconstruction of sperm trajectories during the first 5 min after sperm addition was accomplished by tracing the paths of all motile sperm onto acetate sheets during frame-by-frame advancement of the videotape as viewed on a 21-in. monitor. Single frames from the videotape were digitized using the frame grabber of a Sony UP/9600MD color video/digital dye sublimation printer, the contrast and density of the digital file were adjusted using Adobe Photoshop on a 7100 PowerMac computer, and the processed file was printed using the Sony printer.

Chemotaxis Assay

Corning-Costar Transwell plates, consisting of two chambers separated by a polycarbonate membrane having 12-μm-diameter pores were used to quantify sperm chemotaxis. The bottom chamber (trans) was filled with F-1 buffer, and a 150-μl bolus of the chemotactrant was micropipetted onto the floor of the bottom chamber to bring the total volume to 1.5 ml. The upper chamber (cis), consisting of an insert with the membrane stretched across its base, was lowered into the bottom chamber, the chamber was filled with a 0.5-ml aliquot of a sperm suspension, and the system was incubated for 80 min at room temperature. The sperm suspension used for this assay was prepared by adding 50 μl of sperm in 1.5× OR2 to 500 μl of F-1 within 10 s before use. This dilution provided an osmotic shock so as to place the sperm in a highly motile state as described by Bernardini et al. (1988). Following incubation, the insert was removed, the lower chamber buffer was centrifuged, and the resuspended pellet was counted for sperm using a hemocytometer. All assay data in the figures represent means and standard errors of the mean for at least three similar experiments. Transwell assay data in all figures should be multiplied by a factor of 200 to obtain absolute values.

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Protease Digestion

Proteinase K and pronase E [Sigma, St. Louis, MO] were each mixed in F-1 to form a stock solution of 10 μg/ml, and sufficient stock solution was added to the egg water to bring the final protease concentration to 1 μg/ml. The mixture was incubated for 2 h at 37°C, the protease was deactivated by boiling for 45 min, and the digested egg water was stored at -20°C until assayed.

Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gradient gel electrophoresis (SDS-PAGE) was used to determine the protein composition of solubilized egg jelly and egg water. Samples were solubilized in sample buffer containing 0.125 M Tris-HCl, 4% SDS, 20% (v/v) glycerol, 0.2 M DTT, and 0.02% (w/v) bromophenol blue and heated for 5 min in a boiling water bath. The samples were then analyzed using a gradient polyacrylamide slab (7.5–20%). The gel was fixed and silver stained (Morrissey, 1981) and the wet gel image was captured and analyzed on a Sun densitometry workstation.

Determining the Molecular Weight Range of the Chemotactic Agent

Millipore Centricron devices were used to determine the molecular weight range of the chemotactic factor. The Centricron is composed of two removable chambers separated from one another by a porous filter. Two milliliters of egg water was added to the upper chamber and centrifuged (the force and time varied for different cutoff sizes as recommended by the manufacturer) to force the egg water through the filter into the lower chamber. Only the components of the egg water with a molecular radius less than the pore size of the filter were able to cross through into the lower chamber. Filters having pore sizes that pass 3-, 10-, 30-, and 50-kDa proteins as judged by the lack of yolk contamination.

RESULTS

We hypothesized that if a chemotactic factor were released from amphibian eggs it would logically be a diffusible component released from the jelly layers into the surrounding medium. These so-called diffusible components can be selectively isolated by soaking jellied eggs in an appropriate buffer for 3 h with gentle swirling and then removing the eggs. The resulting conditioned medium is referred to as egg water. SDS-polyacrylamide gel electrophoresis indicates that egg water contains about 17 major proteins having relative mobilities ranging from 10 to 180 kDa [data not shown]. The preparation does not contain egg proteins as judged by the lack of yolk contamination.

Video microscopy observations demonstrate that egg water is able to induce directed movement of Xenopus sperm toward a capillary filled with the concentrated extract. A narrow bore capillary filled with egg water (90 μg/ml protein) is laid flat on a glass slide, surrounded by a well of Vaseline:paraffin, and sperm suspended in F-1 buffer are introduced. Trajectories of motile sperm were recorded on videotape and traced onto acetate. One frame of such a video recording is shown in Fig. 1A with the capillary mouth being positioned at the upper left-hand corner of the field. As previously reported by Bernardini et al. (1988) only 40 to 50% of the sperm are motile; immotile sperm were ignored during data collection. In Fig. 1B, tracings for a 60-s period 3 min after insemination show evidence of sperm trajectories that bring sperm either in contact with or very close to the mouth of the capillary serving as a source of egg water proteins [solid arrows] and trajectories that do not [dotted arrows]. The incidence of these two types of trajectories is shown in Fig. 1C. If one assumes random movement of the sperm and takes into consideration the geometry of the capillary [which subtends about 45° of 360°] one would expect about 12% of the sperm to exit the field of view at the capillary position (none, Fig. 1C). Indeed, in control experiments, using capillaries filled only with buffer, we observed 13% of sperm trajectories to contact or approach the vicinity of the capillary. In contrast, use of capillaries filled with concentrated egg water increased the percentage of sperm reaching the capillary to 31%, almost a 2.5-fold increase over controls [egg water capillary, Fig. 1C]. This 2.5-fold increase in sperm reaching their target was accompanied by an increased number of trajectories that gently curved toward the capillary. If one calculates the sperm-turning preference by subtracting the percentage of sperm turning away from the capillary from the percentage of sperm turning toward the capillary [Fig. 1D], one observes a 33% turning preference toward capillaries filled with concentrated egg water. In contrast, this parameter for control capillaries filled with buffer is ~4% and is not significantly different [using the Student/Neuman/Keuls test] from 0%, the value expected for random movement. These values for the control and experimental conditions are significantly different at the P < 0.001 level.

The chemotactic activity of the egg water preparation was quantified in a transwell device by counting the number of sperm crossing a porous polycarbonate membrane separating the top [sperm-containing] chamber from the bottom [chemotactic agent-containing] chamber. As shown in Fig. 2A, the rate of sperm crossing the membrane barrier in response to egg water is high during the first 20 min of the assay and is considerably less at times greater than 40 min. These data correspond well with the earlier observations of Bernardini et al. (1988) indicating that Xenopus sperm exhibit exponentially decreasing motility during the first 30 min after dilution into low-salt buffers.

Using this assay we have obtained data that support the “diffusible factor” hypothesis. As shown in Fig. 2B, both egg water [composed solely of diffusible components] and solubilized whole egg jelly [composed of diffusible proteins and structural proteoglycans] exhibit a robust, sixfold stimulation of sperm movement over that seen in controls in which only F-1 buffer is present in the bottom well. In contrast, if jelly is solubilized from eggs that have been soaked in F-1 buffer for 3 h so as to partially wash out diffusible factors [referred to as structural jelly], its chemotactic activity is reduced by more than 50%. Thus, we chose to carry out our
further work with egg water rather than using solubilized egg jelly which is more complex in composition and structure.

Stimulation of sperm passage by egg water represents a bona fide example of chemotaxis. Our routine procedure was to introduce a bolus of egg water (or other material to be tested) onto the floor of the bottom well so as to allow a chemical gradient to form by diffusion within the bottom well itself. This procedure resulted in a reproducible stimulation of sperm movement from the top well to the bottom well (see Fig. 2B and Fig. 2C, second group, shaded bar). If, however, the egg water was premixed with the buffer used to fill the bottom well, thereby producing a uniform concentration rather than a chemical gradient, no stimulation of sperm movement is seen (Fig. 2C, second group, open bar). Furthermore, egg water, if placed in the upper chamber along with the sperm, does not stimulate sperm passage regardless of whether it is added as a bolus or is premixed (Fig. 2C, third group). These observations, coupled with the fact that sperm movement through the barrier is not stimulated even when premixed egg water is present in both chambers (Fig. 2C, fourth group, open bar), makes it highly unlikely that the chemoattractant we are dealing with is simply an activator of sperm motility. Rather, it would appear that sperm are stimulated to traverse the membrane only if there is a gradient set up in the bottom (trans) chamber. This conclusion is further supported by the observation that if the egg water is split into two halves and introduced as a bolus to both the upper and lower chambers (thereby creating lesser gradients in both chambers) sperm movement into the lower chamber is stimulated only half as much (threefold) as in normal assays in which the entire egg water aliquot is in the bottom (trans) chamber (Fig. 2C, fourth group, shaded bar).

The chemoattractant activity of egg water exhibits a complex dose-dependence (Fig. 3A, solid line). As the amount of egg water protein added to the bottom chamber is increased from 0 to 3 µg, a proportional increase in sperm

FIG. 1. (A) Single video frame of an experiment in which sperm trajectories near a capillary filled with concentrated egg water are observed. (B) Diagram of motile sperm trajectories during a 60-s period, 3 min after addition of sperm. Solid arrows denote trajectories by which sperm arrive at or near the target capillary; dotted arrows denote trajectories by which sperm do not approach the capillary. (C) Percentage of sperm trajectories that should on a theoretical basis approach the capillary (None) compared with the percentage actually observed for either control capillaries or capillaries filled with concentrated egg water. (D) Relative frequency of sperm trajectories that curve either toward or away from the capillary. Sperm-turning preference toward capillaries filled with concentrated egg water is highly statistically different (P < 0.001) from control capillaries filled only with buffer, the control value is not statistically different from 0%, the value expected for random movement.
FIG. 2. [A] Time course of sperm passage through the membrane of a transwell chemotaxis chamber in response to either 3 μg of egg water protein (open circles) or buffer alone (closed circles) in the lower chamber. [B] Sperm movement from the top to the bottom chamber of a transwell device is stimulated sixfold by solubilized whole egg jelly (3 μg protein) or by egg water (3 μg protein). Structural jelly, solubilized from eggs which have been depleted of diffusible jelly components, exhibits a greatly reduced ability to stimulate sperm movement. [C] Sperm movement depends on establishing a chemical gradient of egg water components. Egg water (3 μg protein) was either uniformly premixed with the buffer chemotaxis is observed. Maximal stimulation of chemotaxis occurs between 3 and 6 μg of egg water protein, while further increases in egg water protein result in a significant reduction in chemotactic activity. Such a biphasic dose-response curve is similar to that for leucocyte chemoattractants and it has been hypothesized that the inhibition of cell movement at high concentrations may play a role in preventing the chemoreponsive cells from moving further if they already have reached their target (Capsoni et al., 1989). In contrast, stimulation of sperm movement is minimal in response to human serum albumin over a wide range of concentrations (Fig. 3A, dashed line). The linear portion of the dose-response curve (from 0 to 3 μg egg water protein) was used subsequently to assay chemotactic activity of egg water in a quantitative manner.

The chemotactic activity is specific to egg water. As shown in Fig. 3B, little or no activity is observed in either proteins (serum albumin and egg albumin) or carbohydrate polymers (10-kDa dextran) unrelated to egg jelly or in cartilage powder which is a rich source of varied proteins and proteoglycans.

The chemotactic agent of egg water does not appear to require calcium for activity. In Fig. 4 (open bars), sperm tested for chemotaxis in a transwell device filled with calcium-free F-1 buffer containing 0.75 mM EGTA exhibit a response to calcium-free egg water that is indistinguishable from the normal sixfold stimulation of sperm movement seen in normal F-1 buffer containing 0.25 mM calcium (shaded bars, Fig. 4). We also find no significant difference in the number of sperm crossing the barrier in calcium-free buffer controls compared to controls in normal F-1 buffer.

The chemotactic activity of egg water is very heat stable. As shown in Fig. 5A, there is no decrease in activity if egg water is boiled for 30 min. In contrast, this activity is sensitive to proteases; incubation of egg water with either proteinase K or pronase E for 2 h at 37°C completely abolishes activity (see Fig. 5B). This is not a direct effect of either protease on sperm in the assay since the proteases were inactivated by boiling for 45 min prior to the assay. This boiling step had no affect on the chemotactic activity of egg water but, if carried out before digestion, did completely block the ability of these proteases to digest and inactivate the egg water chemoattractant.(see Fig. 5B).

The approximate size of the chemoattractant protein was determined using Centricon filtration devices having a range of pore sizes (see Materials and Methods). As shown in Fig. 6, this protein can be centrifugally driven through a filter containing pores large enough to pass a 50- or 30-kDa in the lower or upper chambers (open bars) or pipetted as a bolus onto the floor of the lower or upper chamber so as to create a chemical gradient (shaded bars). Alternatively, 1.5 μg of egg water protein was premixed or pipetted into both the upper and lower chambers. Stimulation of sperm movement occurs only when a chemical gradient is set up in the bottom chamber.

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FIG. 3. [A] Sperm chemotaxis in response to egg water (solid line) exhibits a biphasic dose dependence. In contrast, similar doses of human serum albumin (dashed line) exhibit little stimulation of sperm movement. [B] Sperm chemotaxis is specifically stimulated by egg water (3 µg protein). Nonspecific proteins such as human serum albumin (3.5 µg) or egg albumin (3.5 µg), dextran polymer (10 kDa; 150 µg), or cartilage proteoglycans (150 µg) do not stimulate sperm movement above that seen in F-1 buffer controls.

protein but cannot be driven through a filter having pores so small as to pass only proteins 3 kDa or smaller. These data correlate well with dialysis data showing that no chemotactic activity is lost when the egg water is dialyzed against F-1 buffer for 24 h providing that the pore size of the dialysis membrane is no larger than the diameter of an 8-kDa
The egg water chemoattractant does not require calcium to stimulate sperm movement. Chemotaxis of sperm in response to 3 μg of egg water protein was measured in either normal F-1 buffer containing 0.25 mM calcium (shaded bars) or in calcium-free F-1 buffer containing 0.75 mM EGTA (open bars).

From these observations we conclude that the chemotactic factor is a small protein about 10 kDa in size.

DISCUSSION

We predicted that if there were a sperm chemoattractant involved in the fertilization of *X. laevis* the substance responsible would likely be a diffusible egg jelly component. Our data support this hypothesis by demonstrating that egg water, composed solely of diffusible components, contains substantial sperm attraction activity, while jelly from eggs that have been extensively washed to remove diffusible components (structural jelly) has lost much of its activity.

In addition, we have provided clear-cut evidence that our transwell assay is measuring chemotaxis, not simply motility activation (i.e., chemokinesis). Chemokinesis does not require a chemical gradient and therefore should be seen in response to a uniform concentration of egg water, while chemotaxis should not be seen under such conditions. Likewise, one would expect that stimulation of motility would be most effective if egg water were added to the same (cis) chamber as the sperm, whereas initiation of chemotaxis should not be seen unless egg water is added to the bottom (trans) chamber. Indeed, we found that the number of sperm crossing the membrane barrier was increased five- to eightfold only when the egg water was pipetted into the lower (trans) chamber and not if the egg water was pipetted into the upper (cis) chamber (Fig. 2B). Furthermore, egg water was effective in the lower chamber only when a concentration gradient was set up but not when the egg water was uniformly mixed. In fact, egg water, premixed so as to produce a uniform concentration, failed to stimulate sperm movement across the membrane regardless of whether the jelly extract was present in the top chamber, the bottom chamber, or both chambers. In addition, our video microscopy experiments suggest that egg water components actually induce *Xenopus* sperm to turn toward a capillary filled with egg water. These data show clearly that the sperm-attracting factor in egg water is a chemoattractant, that is, its effect on sperm is direction dependent and gradient dependent, the steeper the gradient the stronger the attraction. The *Xenopus* chemoattractant does not appear to be a sperm motility initiating factor such as with the 105-kDa chorion-associated protein reported in the Pacific herring (Pallai et al., 1993).

These results are not due to any of the common artifacts that might be encountered in the type of chemotaxis measuring device we have used. First, it is clear that the accumulation of sperm in the bottom well is not simply due to sperm being trapped by a viscous liquid. Structural egg jelly which contains all of the high-molecular-weight viscosity-generating proteoglycans that normal egg jelly does had only a weak (40% of normal) chemotactic activity. Furthermore, cartilage proteoglycan at a 50-fold higher concentration (based on protein) exhibited no sperm-attracting ability, while egg water, a very dilute protein solution containing no proteoglycans and having a viscosity similar to that of buffer, exhibited full activity. Second, while we set up the chemotaxis chamber so as to allow gravity to help sperm pass through the membrane barrier, this did not result in abnormally high controls. Indeed,
when only F-1 buffer was present in the bottom chamber the number of sperm entering the chamber whether due to gravity or random movement consisted of no more than 2% of the total sperm population in the upper chamber; this provided a low background that was only 10 to 20% of the signal.

Third, the effect of egg water chemoattractant on sperm is very specific and is not characteristic of macromolecules from other sources. This finding is important because in previous studies of human and bovine sperm a nonspecific sperm attraction was observed that was incorrectly interpreted as chemotaxis [Miller, 1982]. This attraction did not appear to be a matter of chemoattraction but rather an artifact produced by stickiness of the tested protein, inducing the sperm to stick either to one another or to the testing apparatus, thereby leading to aggregation and sperm accumulation. The results of our experiments clearly show that the sperm attraction recorded in our assay is very specific and occurs only in response to a component present in egg water.

Further work demonstrated that the chemotactic factor in the jelly does not require Ca\textsuperscript{2+} in order to be effective. This was a surprise for two reasons. First, the work of Ishihara et al. (1984) in B. japonicus suggested that the main role of egg jelly in fertilization is to function as a store for ions, especially Ca\textsuperscript{2+} and Mg\textsuperscript{2+}. Thus, we were concerned that the attraction we were measuring was not toward a unique component of the egg jelly, but rather toward Ca\textsuperscript{2+} released by the jelly into the surrounding medium. Second, we wanted to determine whether extra-
cellular Ca\(^{2+}\) is important for sperm chemotaxis in *X. laevis*, since sperm chemotaxis in a number of organisms (sea urchins, hydrozoa, and ascidia) is dependent on extracellular Ca\(^{2+}\) [Miller, 1982b; Cosson et al., 1984; Ward et al., 1985]. However, all of these species are marine and therefore are bathed in a Ca\(^{2+}\)-rich environment, in contrast to the low Ca\(^{2+}\) environment in which *X. laevis* fertilization takes place. In fact, Bernardini et al. (1988) have shown that the motility of Xenopus sperm is relatively normal in the absence of calcium. Indeed, we did not observe any significant difference in sperm attraction when we compared normal egg water in F-1 buffer (0.25 mM Ca\(^{2+}\)) and egg water in which free Ca\(^{2+}\) was reduced to a micromolar levels by EGTA chelation. This result not only demonstrates that Ca\(^{2+}\) is not the chemotactic agent but also indicates that extracellular Ca\(^{2+}\) is not critical for the function of the chemotactic agent in the egg water.

The chemotactic factor appears to be a small protein that is very heat stable. It retains activity even after boiling for 45 min, but is readily degraded by proteases. The size of the protein appears to be about 10 kDa based on centrifugation-assisted passage through a series of filters of defined pore sizes and diffusion through dialysis membranes of three different pore sizes. The protein appears to be released quickly upon hydration of the egg jelly during spawning since its chemotactic activity can be detected in egg water prepared by only a 30-min egg incubation (Al-Anzi and Chandler, unpublished results). This is not surprising since Bonnell et al. [1996] found that as much as 30% of the diffusible protein in egg jelly is released into the medium within 5 min of spawning. Therefore, we suggest that the small chemotactrant protein detected in this study is likely released within minutes and likely plays an important biological role in guiding to the egg mass sperm that are very short lived in their motility [Bernardini et al., 1988].

Whether this activity is required *in vivo* for efficient fertilization is not clear. It has been observed that hydration of jelly layers or removal of jelly layers leads to loss of fertilizability in many amphibian eggs and that readdition of egg water to jellyless eggs restores their fertilizability [Barbieri and Villeco, 1966; Barbieri and Reisman, 1969; Barbieri and Otemo, 1972; Katagiri, 1973, 1987]. However, in preliminary experiments [Olson and Chandler, unpublished observations] we have found that fertilization rescue of these eggs appears to be mediated by a second diffusible jelly component rather than the chemotactic factor itself. Further experiments will be required to resolve this issue.

Earlier demonstration of a chemotactic factor in human follicular fluid [Falcone et al., 1991; Ralt et al., 1991, 1994] and now in this study of a diffusible chemotactic protein in amphibian egg jelly suggests that sperm chemotaxis may in future years be looked on as a general feature of vertebrate reproduction. Indeed, we believe that our experiments here open up a path by which vertebrate chemoattractants can be identified and characterized. The high stability of the activity we have described makes it likely that this activity will be purified and sequenced and that the sequence will be used to construct primers that can be used in PCR amplification techniques to identify and sequence the Xenopus gene(s) coding for this factor. Such primers could also be used to identify homologous gene(s) coding for putative sperm chemoattractants from mammalian genomes including the human genome. Indeed, the sperm chemotactic activity of human follicular fluid has been known for more than 8 years and yet no factor has been isolated, possibly due to its instability. Thus, by conclusively demonstrating the existence of a frog sperm chemotactic factor that is
extremely stable we have opened new doors of identifying such factors from all vertebrate species including humans.

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