Phorbol Ester Treatment Stimulates Tyrosine Phosphorylation of a Sea Urchin Egg Cortex Protein

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Abstract. Fertilization of the sea urchin egg results in the phosphorylation, on tyrosine, of a high molecular weight protein localized in the egg cortex. In the present study, treatment of unfertilized eggs with the phorbol ester 12-0-tetradecanoylphorbol 13-acetate stimulated tyrosine phosphorylation of the high molecular weight cortical protein to levels three- to fivefold higher than that occurring in response to fertilization. Experiments using agents that inhibit the egg Na^+/H^+ exchange system or mimic the fertilization-induced shift in cytoplasmic pH_i, suggest a signal transduction pathway in which protein kinase C activates the egg Na^+/H^+ exchange system and the resultant cytoplasmic pH_i shift promotes tyrosine phosphorylation of the high molecular weight cortical protein.

FERTILIZATION involves a cell–cell interaction between the sperm and egg which both introduces the paternal genome into the egg and triggers a series of preprogrammed biochemical events that function to activate egg metabolism and induce cell division. The mechanism by which the initial sperm–egg interaction is transduced into various cellular responses is of interest since it represents an example in which a cell–cell interaction acts as a signal culminating in cell proliferation and execution of the developmental pathway.

Protein phosphorylation has been proposed as a regulatory mechanism that functions to activate various aspects of egg metabolism in response to fertilization (19). It is now known that fertilization results in activation of one or more tyrosine protein kinases (9, 25) resulting in increased phosphorylation of a 350-kD egg protein (23), which we now refer to as high molecular weight cortical protein (HMWCP).[1] The mechanism by which the phosphorylation of this protein is regulated is not known but previous studies raised the possibility that protein kinase C may control many of the biochemical events that normally occur in response to fertilization (5, 6, 20, 31). Protein kinase C has been shown to be an important signal-transducing enzyme in a number of systems. It is the receptor for the tumor-promoting phorbol ester 12-0-tetradecanoyl phorbol-13-acetate (TPA) as well as for diacylglycerol generated by phospholipase C hydrolysis of polyphosphoinositides. The phorbol ester TPA as well as several protein kinase C inhibitors have been used to probe the role of protein kinase C in various cellular responses. H7 is an isoquinolinesulfonamide derivative that inhibits cyclic nucleotide-dependent protein kinases as well as protein kinase C. N-(2-guanidineoethyl)-5-isoquinoline-sulfonamide hydrochloride is a similar compound which preferentially inhibits the cyclic nucleotide-dependent kinases and has a much lower affinity for protein kinase C (17). CKS-17-BSA is a heptadecapeptide conjugated to BSA that specifically inhibits protein kinase C in mammalian systems (4, 14).

In the present study, we have found that treatment of unfertilized eggs with the phorbol ester TPA induced an increase in the phosphotyrosine content of the HMWCP to levels three- to fivefold higher than that occurring in response to fertilization. The effect of TPA was specific for the β phorbol isomer and was inhibited by 1(5-isoquinolinesulfonyl)-2-methylpiperizine as well as the synthetic peptide CKS-17-BSA suggesting that the effect was mediated through protein kinase C. TPA stimulation of tyrosine phosphorylation was found to require sodium in the extracellular medium indicating that protein kinase C activation of the egg Na^+/H^+ exchange system might lead to increased phosphorylation of the HMWCP. Subsequent experiments using amiloride to inhibit the Na^+/H^+ exchange system and NH_4Cl to artificially induce an alkaline shift in cytoplasmic pH_i supported this model. The results of this study are consistent with a signal transduction system in which protein kinase C stimulates the egg's Na^+/H^+ exchange system and the resultant alkaline shift in cytoplasmic pH_i activates an egg tyrosine protein kinase which phosphorylates the HMWCP.

Materials and Methods

Eggs from the sea urchin *Lytechinus variegatus* were collected and washed

1. *Abbreviations used in this paper*: HMWCP, high molecular weight cortical protein; TAPS, tris(hydroxymethyl) methylaminopropane sulfonic acid; TPA, 12-0-tetradecanoylphorbol 13-acetate.

with acidified seawater (pH 5.5) to remove the jelly coat. Fertilization was accomplished by adding sperm to a 5% egg suspension in seawater buffered with 5 mM tris(hydroxymethyl)methylamino propane sulfonic (TAPS) at pH 8.3. Treatment with phorbol esters was done by incubating samples of eggs as a 2–10% vol/vol suspension in TAPS-buffered seawater containing the test compound. Samples to be treated with amiloride were washed into an artificial "low sodium" seawater containing 484 mM choline Cl, 25 mM NaCl, 10 mM KCl, 27 mM $MgCl_2$, 29 mM $MgSO_4$, 11 mM $CaCl_2$, 2.4 mM KH_2CO_3 at pH 8.3. Samples to be treated with NH_4Cl were washed into a "calcium-free" artificial seawater containing 425 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, 2 mM $NaHCO_3$, 25 mM EGTA, pH 8.3. At the end of the treatment period, the eggs were washed by centrifugation in ice-cold calcium-free PKME (8) buffer containing 425 mM KCl, 50 mM Pipes (pH 6.8), 10 mM $MgCl_2$, 10 mM EGTA, 10 μg/ml aprotinin, then homogenized in a potter-elvjeim homogenizer. The egg cortex fraction was collected by centrifugation at 1000 *g* for 1 min and solubilized in SDS gel sample buffer. The relative phosphotyrosine content of the HMWCP was determined by Western immunoblot analysis using an antibody specific for phosphotyrosine (23). Bound antibody was detected with ^{125}I-protein A (ICN K & K Laboratories, Inc., Plainview, NY) and autoradiographs were quantitated using a scanning densitometer.

Kinase C activity was assayed in a reaction mixture containing 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 660 μg/ml histone IIIs (Sigma Chemical Co., St. Louis, MO), and either 1 mM EGTA or 1 mM $CaCl_2$ and 80 μg/ml phosphatidylserine (Avanti Polar Lipids, Birmingham, AL). The reaction was started by addition of [γ-^{32}P] ATP (1.26 mCi/μmol) at a concentration of 80 μM. After incubation at 25°C for 5 min, the reaction was terminated by spotting the mixture on phosphocellulose paper and immediately washing in distilled water.

Results

Effect of TPA on HMWCP Phosphorylation

Fertilization of the sea urchin egg results in the phosphorylation, on tyrosine, of a high molecular weight protein of ~350 kD (23) which we refer to as the HMWCP. The phosphotyrosine content of this protein has been shown to increase several fold within the first 1–2 min after insemination, after which the level of phosphotyrosine returns to a value similar to that in the unfertilized egg. To determine whether HMWCP phosphorylation may be regulated by protein kinase C, we treated unfertilized eggs with the phorbol ester TPA, or with the inactive isomer 4α-phorbol 12,13-didecanoate, and measured the effect on the phosphotyrosine content of the HMWCP. Unfertilized eggs were incubated with the phorbol ester for 10 min, then washed with calcium-free PKME buffer, homogenized, and centrifuged to pellet the cell surface complex. The samples were then analyzed by SDS-PAGE and the phosphotyrosine content of the HMWCP was detected by Western blot analysis using an antibody specific for phosphotyrosine. Preparation of the cell surface complex fraction allows more sensitive detection of the phosphorylated HMWCP since the phosphotyrosine containing HMWCP is restricted to the egg cortex or cell surface complex (23). Western blot analysis of the low speed supernatant fractions confirmed that the phosphotyrosine-containing HMWCP was quantitatively recovered in the cell surface complex pellet. As seen in Fig. 1, treatment of unfertilized eggs with 100 nM TPA stimulated the phosphorylation of HMWCP to very high levels. The alpha isomer of TPA had only a small effect, suggesting that TPA was probably acting through its effect on protein kinase C. Concentrations of TPA greater than 25 nM were required to induce phosphorylation of HMWCP and exposure to 50 nM for 10 min elicited a maximal response (Fig. 2). The effect of TPA was largely reversible as seen in Fig. 3, where eggs exposed to TPA for 10 min were washed in regular seawater and allowed to develop further. The phosphotyrosine content of the HMWCP continued to increase for several minutes after removal of TPA, then rapidly declined although it did not return to the low level present in the untreated egg.

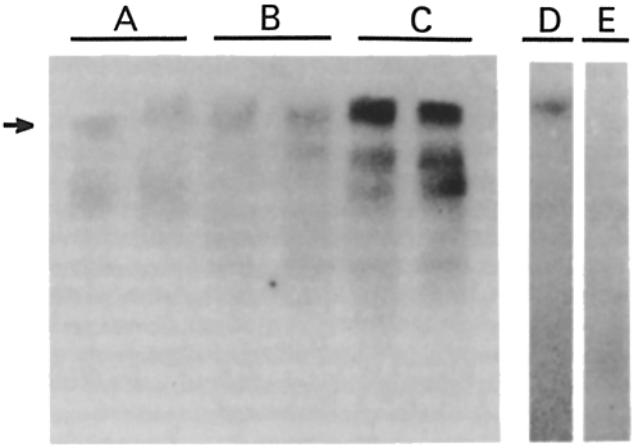

Figure 1. Effect of TPA on the phosphotyrosine content of an egg HMWCP. Suspensions of unfertilized eggs (2% vol/vol) were incubated with sea water containing no additions (*A*), 100 nM 4α-phorbol 12,13-didecanoate (*B*), or 100 nM TPA (*C*) for 10 min after which the cell surface complex was prepared, analyzed on 6.5% SDS polyacrylamide gel, and the phosphotyrosine content of the HMWCP was determined by Western immunoblot using an antibody specific for phosphotyrosine. Bound antibody was detected by incubation with ^{125}I-protein A and autoradiography. Samples A, B, and C show results from two separate batches of eggs. In a separate experiment, antibody specificity was tested by incubating blots prepared from TPA-treated eggs with antibody (*D*) or antibody + 5 mM phosphotyrosine as a competitive inhibitor (*E*).

Effect of Protein Kinase C Inhibition

The effects of phorbol ester treatment on HMWCP phosphotyrosine content suggest that protein kinase C is capable of stimulating the activity of a protein tyrosine kinase. We would therefore predict that inhibitors of protein kinase C

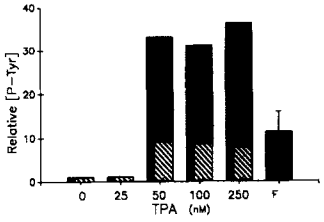

Figure 2. Effect of TPA concentration on the P-Tyr content of the HMWCP. Unfertilized eggs were incubated in seawater containing the indicated concentration of TPA (*solid bars*) or the inactive isomer 4α-phorbol 12,13-didecanoate (*crosshatched bars*) for 10 min, after which the eggs were homogenized and the cell surface complex was analyzed for phosphotyrosine content of the HMWCP by Western immunoblot. The relative phosphotyrosine content of the HMWCP was determined by densitometric scanning of the autoradiograph and the values are expressed relative to that obtained for the untreated eggs which was arbitrarily set as 1.0. For comparison, the relative phosphotyrosine content of the HMWCP in three samples of eggs taken 2 min after fertilization is also included (*F*).

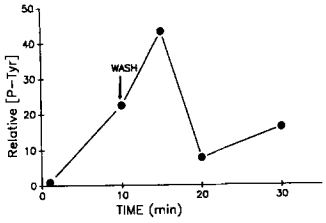

Figure 3. Reversibility of TPA effect. Unfertilized eggs (2% vol/vol) were incubated in seawater containing 100 nM TPA for 10 min then washed into regular seawater and incubated for an additional 20 min. Samples (90 mg protein) were taken at the indicated times and relative phosphotyrosine content of the HMWCP present in the egg cortex was determined as in Fig. 2.

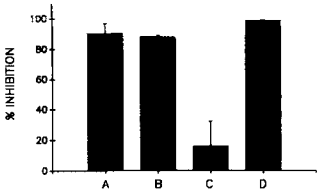

Figure 4. Inhibition of TPA-stimulated HMWCP phosphorylation. Unfertilized eggs (5% vol/vol) were preincubated with TAPS-SW (control), or 100 μg/ml BSA-CKS17 (*A*), 20 μm H7 (*B*), 20 μM HA1004 (*C*) for 30 min before treatment with 100 nM TPA for 10 min. To test the requirement for extracellular sodium, eggs were preincubated in sodium-free seawater and treated with TPA in sodium-free seawater (*D*). The cortex fraction was then prepared and the phosphotyrosine content of the HMWCP was determined by Western blot analysis and densitometric scanning. The basal level of TPA stimulation (x) was determined by subtracting the value from untreated eggs from that of eggs pretreated with TAPS SW, then incubated with TPA. The TPA stimulation in eggs pretreated with various inhibitors (y) was obtained by subtracting the value from untreated eggs from that of eggs pretreated with inhibitor and incubated with TPA. The inhibitory effect (*% inhibition*) of the various pretreatments was calculated by $(1 - (y/x))$ and is expressed ± SE.

would prevent the effect of TPA and of fertilization on the phosphorylation of the HMWCP in vivo. We have used the protein kinase C inhibitor H7 as well as the synthetic peptide CKS17 conjugated to BSA which is known to inhibit phorbol ester stimulation of T-cells and can inhibit protein kinase C in extracts of these cells (4, 14). Both of these compounds were found to inhibit protein kinase C isolated from sea urchin eggs (Table I). As seen in Fig. 4, pretreatment of eggs for 30 min with CKS-17-BSA or with H7 largely prevented the TPA-dependent increase in HMWCP phosphorylation. No phosphotyrosine-containing HMWCP was detected in the low speed supernatant fraction from CKS-17-BSA–treated eggs, ruling out the possibility that CKS-17-BSA caused the phosphorylated HMWCP to become distributed in the cytosol. While CKS-17-BSA was very effective against TPA-stimulated HMWCP phosphorylation, it was less effective in inhibiting the fertilization-induced increase in HMWCP phosphorylation which was inhibited ~50% (not shown). This may be because the exocytosis of cortical secretory vesicles upon fertilization disrupts the association of CKS-17-BSA from the egg plasma membrane. These fertilized eggs were still capable of cleavage and successful development indicating that CKS-17-BSA inhibition was not sufficient to permanently interrupt development.

Role of the Na^+/H^+ Exchange System

The primary mechanism by which TPA activation of protein kinase C is thought to induce many aspects of egg activation is by stimulating the egg's Na^+/H^+ exchange system which is responsible for shifting the cytoplasmic pH_i from ~6.8 to 7.3 as occurs during fertilization. This cytoplasmic alkalinization requires extracellular sodium and can be prevented by treating the eggs with TPA in sodium-free seawater (31). When unfertilized eggs were suspended in sodium-free seawater before and during treatment with TPA, the stimulatory effect of TPA on HMWCP phosphorylation was almost completely inhibited (Fig. 4). This is consistent with the hypothesis that the stimulatory effect of TPA on the tyrosine phosphorylation of the HMWCP occurs through protein kinase C activation of the egg Na^+/H^+ antiporter (31), elevation of cytoplasmic pH_i, and subsequent stimulation of an egg protein tyrosine kinase. To more directly test this hypothesis, we examined the effect of amiloride, an inhibitor of the egg Na^+/H^+ exchange system (22, 30), on the fertilization-induced HMWCP phosphorylation. Amiloride has been shown to prevent the fertilization-dependent pH shift when fertilization is performed in a reduced concentration of sodium (25 mM instead of the usual 425 mM) (30). Therefore, eggs in low sodium seawater were mixed with sperm for 15 s, then amiloride was added and samples of eggs were taken at different time points for SDS gel electrophoresis and Western blot analysis. As seen in Fig. 5, the phosphotyrosine content of the HMWCP increased much more slowly when the eggs were treated with amiloride after fertilization.

In addition to the above experiments using inhibitors of Na^+/H^+ exchange, we used a low concentration of NH_4Cl in Ca^{++}-free seawater to artificially elevate the intracellular pH of the unfertilized egg. This procedure has been used extensively to parthenogenetically activate those components of the egg's metabolism that normally are stimulated by the fertilization-dependent pH_i increase (32). This procedure, when combined with exposure to the calcium ionophore A23187, can initiate successful parthenogenic development to the pluteus stage indicating that the $[NH_4^+]$ ion has no cytotoxic effects at these concentrations (2). Treatment of unfertilized eggs with 10 mM NH_4Cl in calcium-free seawater (Fig. 6) resulted in a sixfold increase in the phosphotyrosine content of the HMWCP. TPA, in this series of ex-

Table I. Inhibition of Kinase C Activity by BSA-CKS17 and H7

Sample	Activity	% Inhibition
	fmol/min	
Ca^{++} only	0	–
Ca^{++}/PS	374	–
Ca^{++}/PS/BSA	386	0
Ca^{++}/PS/BSA-CKS17	91	78
Ca^{++}/PS/HA1004	272	26
Ca^{++}/PS/H7	43	89

Protein kinase C was partially purified from sea urchin eggs (28) and assayed as described in Materials and Methods. The effect of the BSA-CKS17 inhibitor was determined by preincubating the enzyme with 100 μg/ml of BSA (control) or BSA-CKS17. Other samples were incubated with the kinase C inhibitor H7 (20 μM) or the closely related but less active compound HA1004. Kinase C activity was determined by subtracting the level of histone phosphorylation in the presence of EGTA from that in the presence of Ca^{++}/PS. Values are the average of two experiments each done in duplicate.

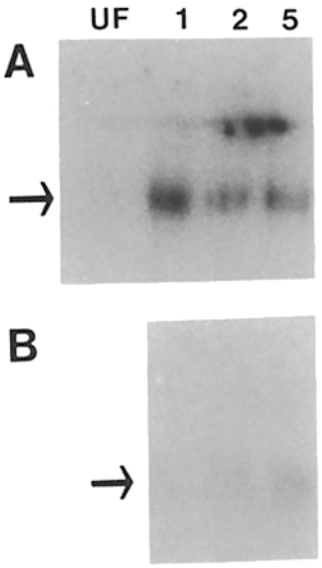

Figure 5. Effect of amiloride on the phosphorylation of HMWCP. Eggs were fertilized in artificial seawater containing 25 mM Na^+. After 15 s, an equal volume of low-sodium seawater (*A*) or low-sodium seawater with amiloride (*B*) (0.5 mM final concentration) was added to the eggs and samples were taken at different times after fertilization for analysis by SDS-PAGE and Western immunoblot analysis using the antiphosphotyrosine antibody. The time in minutes after fertilization is presented at top and the position of the HMWCP is indicated by the arrow. Antibody binding was inhibited by including L-phosphotyrosine (5 mM) during incubation with a duplicate sample (not shown).

periments, stimulated HMWCP phosphorylation ~15-fold. Taken together, these experiments have shown that conditions that block the Na^+/H^+ exchange-mediated pH_i shift inhibit HMWCP phosphorylation, and that parthenogenic elevation of cytoplasmic pH_i stimulates HMWCP phosphorylation. This suggests that TPA or fertilization-induced activation of protein kinase C stimulates tyrosine phosphorylation of the HMWCP by triggering the alkaline shift in cytoplasmic pH_i.

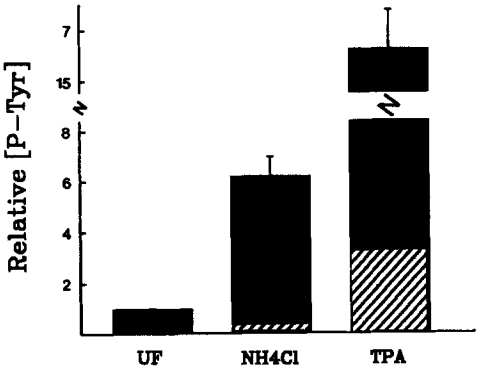

Figure 6. Effect of NH_4Cl on phosphorylation of HMWCP in vivo. Samples of eggs (0.5×10^6 cells) were either incubated with calcium-free artificial seawater containing 10 mM NH_4Cl or 100 nM TPA for 10 min, then the cell-surface complex was prepared and analyzed by SDS gel electrophoresis. The phosphotyrosine content of the HMWCP was detected by Western immunoblot analysis and quantitated by densitometric scanning of the autoradiographs under conditions where density was linearly dependent on the radioactivity. Values represent the average of three experiments ± S.E. and are expressed relative to the value of unfertilized eggs (*solid bars*). Duplicate blots were incubated with antiphosphotyrosine antibody in the presence of 5 mM phosphotyrosine as a competitive inhibitor (*crosshatched bars*).

Discussion

Egg activation is known to involve stimulation of several classes of protein kinases (19, 24) including one or more tyrosine protein kinase(s) which are activated within the first few minutes after fertilization (9, 25). This results in increased phosphorylation (on tyrosine) of a high molecular weight (350–400 kD) egg protein (23). In investigating the factors that regulate the phosphorylation of this protein, we have found that the phorbol ester TPA, a potent activator of protein kinase C, increased the endogenous tyrosine protein kinase activity in egg homogenates and resulted in a 10–30-fold increase in the phosphotyrosine content of the HMWCP in unfertilized eggs, a response three to fivefold higher than that seen at fertilization. The stimulatory effect of TPA was specific for the β-isomer. TPA stimulation of tyrosine phosphorylation was inhibited by CKS-17-BSA as well as H7 but not by HA1004, a weak inhibitor of protein kinase C, providing additional evidence that protein kinase C functions in activating an egg protein tyrosine kinase.

Phorbol ester stimulation of tyrosine phosphorylation of specific endogenous protein substrates has been observed in several systems including fibroblasts (13) as well as various lymphoid cells including the LSTRA cell line (3), U937 monocytes (16), and normal B-cells (21). The phorbol ester, acting through protein kinase C, probably stimulates tyrosine protein kinases through an indirect mechanism since phosphorylation by protein kinase C usually has a negative effect on the specific activity of several tyrosine kinases (1, 3, 7, 15). In the sea urchin egg, TPA is known to stimulate a Na^+/H^+ exchange system resulting in a cytoplasmic alkaline shift similar to that which occurs in response to fertilization (20, 31). Our observation that TPA stimulation of HMWCP phosphorylation requires extracellular sodium, indicates that the Na^+/H^+ exchange system plays a role in regulating the phosphorylation of HMWCP. The fact that HMWCP phosphorylation is inhibited by amiloride treatment and is stimulated by an artificially induced alkaline shift in cytoplasmic pH_i, indicates that the Na^+/H^+ exchange-mediated pH_i shift stimulates HMWCP phosphorylation at fertilization. Taken together, these results are consistent with the following egg-activation pathway: sperm–egg interaction triggers the phosphatidylinositol cycle producing pools of Ins 1,4,5 P_3 and diacylglycerol (5) which allows protein kinase C to become active. Protein kinase C stimulates the egg Na^+/H^+ exchange system either by direct phosphorylation or by some other mechanism (20, 31). Once the Na^+/H^+ exchanger is activated, intracellular protons are exchanged for extracellular sodium causing the cytoplasmic pH_i to rise (10, 27, 29). The pH_i shift may directly increase the activity of a plasma membrane-associated protein tyrosine kinase, or stimulate it by some indirect mechanism resulting in increased phosphorylation of the HMWCP. The rapid nature of this response suggests that the tyrosine kinase undergoes positive regulation (reviewed in 18), perhaps through the action of a pH-sensitive protein kinase or phosphatase. While this hypothesis is consistent with our findings in TPA-stimulated eggs, the observation that BSA-CKS17 only partially inhibited HMWCP phosphorylation in fertilized eggs suggests that some other factors may be involved. A recent report has suggested that while the above protein kinase C pathway exists in eggs and can be activated by TPA, a second

calmodulin-mediated mechanism is also capable of stimulating the Na^+/H^+ exchange system (26). Other studies have proposed two independent Na^+/H^+ systems in the sea urchin egg (22), although the potential mechanisms by which each is regulated are unknown. The possibility that two pathways exist by which the fertilized egg can implement Na^+/H^+ exchange, would explain the observation that inhibitors of protein kinase C are only partially effective in inhibiting fertilization-induced phosphorylation of the HMWCP. In any case, it is clear that the Na^+/H^+ exchange-mediated pH_i shift is required for stimulation of HMWCP phosphorylation. In fact, the time course of HMWCP phosphorylation upon fertilization correlates with the "initial critical period" of egg activation during which the intracellular pH must increase to 7.3 if development is to proceed (10). Quite possibly, HMWCP phosphorylation could act as a pH sensitive "trigger" (11, 12) and function to activate various aspects of egg metabolism and growth control which themselves are not sensitive to such a small change in pH. Future studies using in vitro systems to study the interaction between protein kinase C, the egg Na^+/H^+ exchange protein, and plasma membrane tyrosine protein kinases may explain how phosphorylation of the high molecular weight egg cortical protein is controlled at fertilization.

This investigation was supported by National Institutes of Health Grant HD-14846 and Research Career Development Award HD-00620 to W. H. Kinsey.

Received for publication 30 October 1989.

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