

c-Jun N-terminal Kinase Activation in *Xenopus laevis* Eggs and Embryos

A POSSIBLE NON-GENOMIC ROLE FOR THE JNK SIGNALING PATHWAY*

Received for publication, September 1, 2000, and in revised form, October 5, 2000
Published, JBC Papers in Press, October 11, 2000, DOI 10.1074/jbc.M008050200

Christoph P. Bagowski, Wen Xiong, and James E. Ferrell, Jr.‡

From the Department of Molecular Pharmacology, Stanford University, Stanford, California 94305-5174

The c-Jun N-terminal kinases (JNKs) are members of the mitogen-activated protein kinase family that play critical roles in stress responses and apoptosis. We have discovered that JNK is present in *Xenopus* oocytes, an experimental system that offers a variety of powerful experimental approaches to questions of protein function and regulation. Like ERK2/p42 MAPK, JNK is activated just prior to germinal vesicle breakdown during *Xenopus* oocyte maturation and remains active throughout meiosis I and II. However, unlike p42 MAPK, which is inactivated about 30 min after eggs are fertilized or parthenogenetically activated, JNK stays constitutively active until the early gastrula stage of embryogenesis. These findings suggest that the JNK pathway may play a role in oocyte maturation and embryogenesis. JNK was activated by microinjection of Mos, by activation of an estrogen-inducible form of Raf, and by a constitutively active MEK-1 (MEK R4F), indicating that the p42 MAPK cascade can trigger JNK activation. However, the MEK inhibitor U0126 blocked progesterone-induced p42 MAPK activation but not progesterone-induced JNK activation. Thus, progesterone can stimulate JNK activation both through the MEK/p42 MAPK pathway and through MEK/p42 MAPK-independent pathways. Many of the key substrates of JNKs identified to date are transcriptional regulators. However, since transcription is not required for germinal vesicle breakdown in progesterone-treated oocytes or for the early embryonic cell cycles, our findings suggest that in these contexts the JNK pathway exerts nongenomic effects.

The Jun N-terminal kinases (JNKs),¹ also termed stress-activated protein kinases, are a subfamily of MAP kinases implicated in cytokine and stress responses (1–3). In mammals there are three JNK genes, the ubiquitously expressed *jnk1* and *jnk2* genes and the brain-, heart-, and testis-specific *jnk3* gene (4). Each of

these genes can give rise to 46- and 55-kDa forms of the JNK protein through alternative mRNA splicing (4).

Like the ERK1/2 and p38 subfamilies of MAP kinases, the JNKs are components of a modular MAP kinase cascade (5, 6). At the top of the cascade is a MAP kinase kinase kinase. The MAPKKK (JNKKK) phosphorylates and activates a MAP kinase kinase. The MAPKK (JNKK) in turn phosphorylates and activates the MAPK (JNK). Two JNK-activating MAPKKs have been identified, MKK4 and MKK7. These dual-specificity protein kinases activate JNK by phosphorylating conserved Thr and Tyr residues (Thr-Pro-Tyr) situated in the kinase activation loop. MKK4 and MKK7 in turn can be activated by numerous MAPKKKs. The MKK4/MKK7-activating MAPKKKs include MEKK1, MEKK2, MEKK3, MEKK4/MTK1, MEKK5/MAPKKK5, MST/MLK2, MUK/DLK, MLK3/SPRK, TAK1, Tpl-1/2, and ASK1 (3). In addition, Ste20-related protein kinases, calmodulin kinases, and phosphatidylinositol 3-kinases can all bring about JNK activation (7–9). Thus, many different signaling proteins are able to feed into the activation of the p46 JNK and p55 JNK proteins.

JNK cascades have long been suspected of mediating pro-inflammatory and pro-apoptotic responses. Recently these ideas have been tested through the analysis of transgenic mice with disruptions in their *jnk1*, *jnk2*, and/or *jnk3* genes or in upstream activators (10–12). In addition, a JNK homolog has been identified in *Drosophila*, allowing genetic analysis of JNK function. These studies have provided important insights into JNK function in morphogenesis (3, 13–16).

Here we have begun examining JNK regulation in another powerful experimental system, *Xenopus laevis* oocytes, eggs, and embryos. By virtue of their large size and natural cell cycle synchrony, these cells have been fruitful systems for the identification of cell cycle regulators and inducers of early development, exploration of apoptotic pathways, and examination of quantitative aspects of signal transduction systems. We set out to determine whether *Xenopus* oocytes possess a JNK-like protein and whether any such JNK protein was activated during oocyte maturation or early embryogenesis. Here we show that *Xenopus* oocytes express p40 JNK and p49 JNK isoforms. JNK activity increases abruptly just prior to germinal vesicle breakdown during *Xenopus* oocyte maturation, at the boundary between G₂-phase and meiotic M-phase, and remains high throughout early embryogenesis until early gastrulation. It is possible that JNK plays an important role in these processes and exerts its function by regulating targets other than transcriptional regulators.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-JNK antibodies used were mouse monoclonal anti-JNK2 (D2; Santa Cruz Biotechnology), rabbit polyclonal anti-JNK1 (SC571; Santa Cruz Biotechnology), and anti-JNK2 (SC572; Santa Cruz Biotechnology) as well as anti-phospho-JNK (G7; Santa Cruz Biotech-

* This work was supported by National Institutes of Health Grant GM46383 and a Deutsche Forschungsgemeinschaft Postdoctoral Fellowship. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Molecular Pharmacology, Stanford University, 269 West Campus Dr., Stanford, CA 94305-5174. Tel.: 650-725-0765; Fax: 650-723-2253; E-mail: ferrell@cmgm.stanford.edu.

¹ The abbreviations used are: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; MAP, mitogen-activated protein; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; GVBD, germinal vesicle breakdown; MBP, myelin basic protein.

nology). The X15 polyclonal anti-p42 MAPK antiserum was used for immune complex kinase assays (17). Polyclonal anti-estrogen receptor antibodies (HC-20) were obtained from Santa Cruz Biotechnology.

Recombinant Proteins—The cDNAs for a constitutively active, His₆-tagged version of human MEK-1 (with Ser-218 replaced by Glu, Ser-222 replaced by Asp, and a deletion of amino acids 32–51, hereafter referred to as MEK R4F) and catalytically inactive MEK-1 (with Lys-97 replaced by Met) were provided by Natalie Ahn (University of Colorado, Boulder, CO) (18, 19). MEK proteins were expressed in *Escherichia coli* and purified to homogeneity by nickel-chelate chromatography. The cDNA for a male-Mos fusion protein was provided by Monica Murakami (NCI-Frederick Cancer Research and Development Center) and George Vande Woude (Van Andel Research Institute, Grand Rapids, MI). male-Mos was expressed in *E. coli* and purified as described (20).

Isolation and Microinjection of Oocytes—*Xenopus* ovarian tissue was surgically removed, and oocytes were defolliculated for 1–1.5 h at room temperature with 2 mg/ml collagenase and 0.5 mg/ml polyvinylpyrrolidone in Ca²⁺-free modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 10 mM HEPES, pH 7.5). The oocytes were then washed four times with modified Barth's solution. Stage VI oocytes were sorted manually and incubated at 16 °C for at least 10 h in OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, pH 7.5) supplemented with 1 mg/ml bovine serum albumin and 50 µg/ml gentamicin. Immature oocytes were microinjected with MEK R4F or Mos protein and transferred to fresh OR2 for the duration of the time course. Ten oocytes were collected per time point, frozen on dry ice, and stored at –80 °C.

Isolation of Eggs, Parthenogenetic Activation, and in Vitro Fertilization—Female frogs were induced to lay eggs by injection with 500 units of human chorionic gonadotropin (Sigma) and kept in egg laying buffer (0.12 M NaCl, 10 mM KCl, 24 mM NaHCO₃, 8 mM MgSO₄, 30 mM Tris, pH 7.6, 0.7 mM CaCl₂) for 16 h. Eggs were collected by gentle squeezing and were immediately transferred to 0.1× MR buffer (10 mM NaCl, 0.18 mM KCl, 0.2 mM CaCl₂, 0.1 mM MgCl₂, 0.5 mM HEPES, pH 7.6). For parthenogenetic activation, eggs were dejellied for 5 min in 3% cysteine, pH 7.6, washed with 1× MR buffer (10× the concentrations described above), and given two 1-s electrical shocks (12 V, alternating current) (21). Testes were isolated, and *in vitro* fertilization was carried out as described (22). Fertilized eggs were dejellied in 3% cysteine, pH 7.6, for 4 min at room temperature, transferred to 0.1× MR, and lysed at the appropriate embryonic stages.

Lysis of Oocytes, Eggs, and Embryos—Frozen oocytes, eggs, and embryos were thawed rapidly and lysed by pipetting up and down in 60 µl of ice-cold extraction buffer (EB) (0.25 M sucrose, 0.1 M NaCl, 2.5 mM MgCl₂, 20 mM HEPES, pH 7.2) containing 10 mM EDTA, protease inhibitors (10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), and phosphatase inhibitors (50 mM 2-glycerophosphate, 1 mM sodium orthovanadate, 2 µM microcystin). Samples were clarified by centrifugation for 2.5 min in a Beckman E microcentrifuge with a right angle rotor. Crude cytoplasm was collected and processed for immunoblotting or kinase assays, as described below.

Immunoblotting—Aliquots of oocyte, egg, or embryo lysates were added to 0.2 volumes of 6× Laemmli sample buffer. Samples were separated on 10% SDS-polyacrylamide gels (bisacrylamide:acrylamide, 100:1) and the proteins transferred to PVDF blotting membrane (Amersham Pharmacia Biotech). The membrane was blocked with 3% non-fat milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris, pH 7.6) and incubated with primary antibodies. Blots were washed five times with TBS, 0.5% Tween 20 and probed with an peroxidase-conjugated secondary antibody for detection by enhanced chemiluminescence (ECL-Plus, Amersham Pharmacia Biotech). For reprobing, blots were stripped by incubation with 100 mM Tris-HCl, pH 7.4, 100 mM 2-mercaptoethanol, and 2% SDS at 70 °C for 40 min.

Jun Kinase Assay—Jun kinase assays were performed as described (23). Crude oocyte, egg, or embryo cytoplasm was diluted 1:1 in EB and pre-cleared with 20 µl of glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C with moderate shaking. Lysates were incubated with glutathione S-transferase GST-c-Jun-(1–79) fusion protein (hereafter denoted GST-Jun) immobilized on glutathione-Sepharose beads. After 3 h at 4 °C, the beads were washed three times with 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin (24) and once with 0.4 ml of kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 200 µM sodium orthovanadate). The bound JNK activity was detected by the addition of 1 µCi of [³²P]ATP. The reaction was terminated after 20

min at 30 °C, and the products were resolved by SDS-PAGE. The gels were transferred to PVDF membranes (Hybond; Amersham Pharmacia Biotech) and the incorporation of [³²P]phosphate into GST-Jun was visualized by autoradiography.

Immunocomplex in Vitro Kinase Assays—*Xenopus* p42 MAPK was immunoprecipitated from lysates using polyclonal antibody X15. Crude cytoplasmic lysates were diluted 1:1 in EB and pre-cleared with 20 ml of protein A-Sepharose (Sigma). After 3 h at 4 °C at moderate shaking, immunoprecipitates were washed three times with 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin and washed once with 1 ml of kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 200 µM sodium orthovanadate). Subsequently, kinase reactions were performed in 40 µl of kinase buffer supplemented with 4 mg/ml myelin basic protein (MBP) (Sigma), 50 µM ATP, and 1 mCi of [³²P]ATP for 20 min at 30 °C. Reactions were stopped by addition of 8 µl of 6× Laemmli buffer and subjected to gel electrophoresis on 12.5% gels. Gels were transferred to PVDF (Hybond; Amersham Pharmacia Biotech) membranes. Phosphorylated substrate was detected by autoradiography on Kodak Biomax MS film.

ΔRaf-DD:ER Expression, Immunoprecipitation, and Activity Assay—The cDNA for a human Raf-estrogen receptor chimera, designated ΔRaf-DD:ER, was a generous gift from M. McMahon. The Raf portion of the chimera has been activated by deletion of the N terminus and substitution of aspartate residues at Tyr-340 and -341 (25). The estrogen receptor portion of the chimera makes the Raf activity regulatable by estradiol. ΔRaf-DD:ER was cloned into pSP64 poly(A) (Promega), and mRNA was generated by *in vitro* transcription (Ambion). Stage VI oocytes were collected and injected with 50 ng of ΔRaf-DD:ER mRNA and incubated overnight to allow protein expression. Injected oocytes were incubated with 1 µM estradiol to activate the ΔRaf-DD:ER.

ΔRaf-DD:ER-expressing oocytes were lysed in 300 µl of 20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM aprotinin, 1 mM leupeptin, 1 µM pepstatin A, 1 mM EGTA, 10 mM NaF, and 100 µM β-glycerophosphate. Cleared lysates were incubated with 30 µl of protein A-Sepharose (precoated with anti-estrogen receptor antibody) at 4 °C for 2 h, and washed once with lysis buffer and once with kinase buffer (25 mM HEPES, pH 7.4, 10 mM MgCl₂, 1 mM MnCl₂, 1 mM dithiothreitol). Samples were then incubated at 30 °C for 30 min with kinase buffer supplemented with 1 µM ATP, 20 µCi of [³²P]ATP, and 50 ng of purified recombinant MEK protein. The reactions were denatured by boiling in sample buffer and electrophoresed on a 10% SDS-PAGE. The gel was transferred to a PVDF membrane and exposed to x-ray film (Kodak Biomax MS) to detect incorporated radioactivity.

RESULTS

***Xenopus* Oocytes Express p40 JNK and p49 JNK Isoforms**—We subjected lysates from G₂- and M-phase oocytes to immunoblotting with a JNK-specific monoclonal antibody. We detected two putative JNK bands, with apparent molecular masses of 40 and 49 kDa (Fig. 1A, left panel). Vertebrate JNKs bind tightly to the N terminus of c-Jun, which contains the residues (Ser-63 and Ser-73) that JNK phosphorylates (23). A fusion of GST with the first 79 amino acids of c-Jun can therefore be used as an affinity reagent to precipitate JNK protein and activity. We subjected *Xenopus* oocyte lysates to precipitation with GST-Jun, and we looked for JNK protein by immunoblotting. As shown in Fig. 1A (right panel), GST-Jun brought down the p40 and p49 bands seen in JNK immunoblots of whole lysates. Control GST beads did not bring down p40 or p49. This finding supports the identification of p40 and p49 as authentic JNKs, possibly corresponding to the p46 and p54 isoforms expressed in mammalian cells.

Immunoprecipitation of JNK Activity from M-phase Oocytes—Next we examined whether JNK activity differed in G₂-phase immature oocytes and M-phase mature oocytes. We subjected JNKs to precipitation with nonimmune serum, GST-Jun beads, or four different JNK antisera, one of which (G7) is known not to support immune complex kinase activity. As shown in Fig. 1B, two of the JNK antisera (SC571 and SC572) and the GST-Jun beads brought down substantial levels of Jun

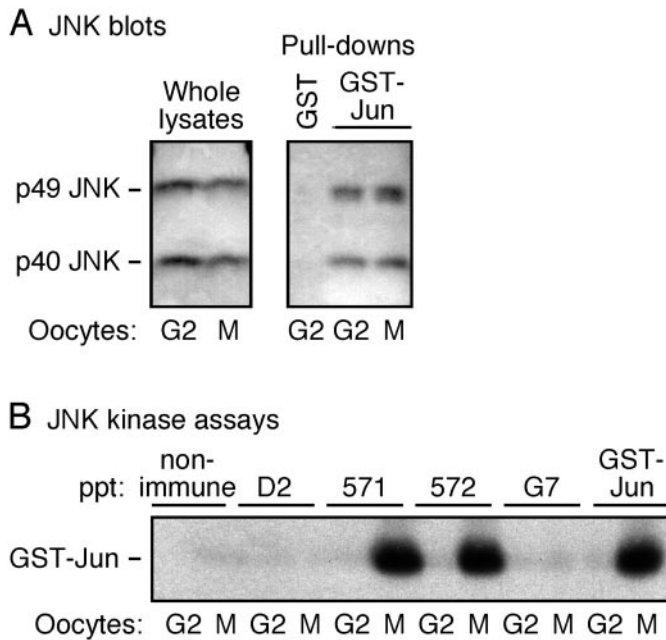


FIG. 1. JNK expression and activity in *Xenopus* oocytes. *A*, JNK immunoblots. Lysates of 10 unstimulated (G_2 -phase, Dumont Stage VI) or progesterone-stimulated (M-phase) oocytes were either directly separated on a 10% polyacrylamide gel (*left panel*) or were subjected to pull-down with control GST beads or GST-Jun followed by electrophoresis (*right panel*). Proteins were transferred to PVDF membranes and immunoblotted with monoclonal anti-JNK antibodies (D2; Santa Cruz Biotechnology). *B*, JNK precipitation and kinase assay. Lysates of G_2 - and M-phase oocytes (10 each) were subjected to immunoprecipitation with a nonimmune serum or an anti-JNK serum, as indicated. Immune complex kinase assays were performed using GST-Jun as a substrate. As a positive control, lysates were incubated with immobilized GST-Jun. Samples were separated on 12.5% polyacrylamide gels and transferred to PVDF membranes. Incorporated radioactivity was detected by autoradiography.

kinase activity from M-phase oocytes. Jun kinase activity was not detected in the nonimmune serum precipitates or in the D2 or G7 precipitates (Fig. 1*B*). None of the reagents brought down significant levels of Jun kinase activity from G_2 -phase oocytes (Fig. 1*B*). These findings further confirm that JNK is present in oocytes and indicate that JNK becomes activated during *Xenopus* oocyte maturation.

Time Course of JNK Activation during *Xenopus* Oocyte Maturation—Next we examined the time course of progesterone-induced JNK activation in detail. JNK activity increased markedly just prior to GVBD, as did p42 MAPK activity (Fig. 2). Like p42 MAPK activity, JNK activity remained high throughout meiosis I and meiosis II. This pattern of JNK activation suggests a possible role for the JNK signaling pathway in oocyte maturation and the G_2 /M cell cycle transition.

JNK Activity during the First Mitotic Cell Cycle—To examine JNK activity during the first mitotic cell cycle, we obtained dejellied, M-phase-arrested unfertilized *Xenopus* eggs and released them from their arrest with an electric shock. We then followed JNK activity and p42 MAPK activity. As shown in Fig. 3, p42 MAPK activity fell by 30 min post-shock and then remained low during the first mitotic cycle. In contrast, JNK activity was high throughout the entire time course (Fig. 3). Similar results were obtained with calcium ionophore-treated eggs (data not shown). These findings show that there are important differences between JNK regulation and p42 MAPK regulation in the *Xenopus* cell cycles.

JNK Activity during Embryogenesis—We fertilized *Xenopus* eggs *in vitro*, dejellied them, and then collected samples at various stages of embryogenesis. As shown in Fig. 4, JNK

activity was high during the rapid early embryonic cell cycles and the blastula stage and then decreased to basal levels at the early gastrula stage. In contrast, p42 MAPK activity fell before the first post-fertilization sample was taken (45 min after fertilization) and remained low throughout embryogenesis. These findings again point out the differences between JNK regulation and p42 MAPK regulation and suggest that JNK may play an important role in the pre-gastrula stage embryo.

Activation of *Xenopus* JNK by Mos, Δ Raf-DD:ER, and MEK R4F—As described above (see Fig. 2), *Xenopus* JNK and p42 MAPK are activated at about the same time during oocyte maturation. This raises the possibility that there may be some connection between the two pathways. To explore this possibility, we microinjected oocytes with various proteins or mRNAs that could activate the p42 MAPK cascade without triggering the most proximal events of progesterone signaling (such as the very rapid progesterone-induced inhibition of adenylyl cyclase) (26).

As shown in Fig. 5*A*, injection of Mos (yielding an estimated intra-oocyte concentration of 200 nM) brought about activation of both p42 MAPK and JNK and also induced GVBD. No JNK activation, p42 MAPK activation, or GVBD was seen in mock-injected controls (data not shown).

We also examined the effects of Raf activation on JNK activity. Although both Raf and Mos can function as MAPKKKs, their hierarchy within the p42 MAPK cascade is less clear, and the exact position of Raf within the p42 MAPK cascade in *Xenopus* oocytes is controversial. Nevertheless, active forms of Raf can be used as a way of activating the MAPK cascade and inducing GVBD in oocytes in the absence of progesterone. We therefore examined whether activation of Raf-1 causes JNK activation.

We microinjected oocytes with synthetic mRNA encoding a chimera of an oncogenic form of human c-Raf-1 (Δ Raf-1 DD) fused to the hormone-binding domain of the human estrogen receptor (hereafter denoted Δ Raf-DD:ER) (25). After allowing protein expression overnight, Δ Raf-DD:ER was activated by the addition of estradiol. Note that unlike progesterone, estradiol does not initiate maturation by itself (27). Estradiol treatment caused the activation of Δ Raf-DD:ER, p42 MAPK, and JNK and brought about GVBD (Fig. 5*B*). The extent of p42 MAPK and JNK activation seen in the mature estradiol-treated oocytes was similar to that seen in progesterone-treated oocytes (Fig. 5*B*). Estradiol had no effect on JNK activity, p42 MAPK activity, or GVBD in oocytes that were not expressing Δ Raf-DD:ER (data not shown). Thus, both Mos and Raf are capable of activating JNK in *Xenopus* oocytes.

Finally, we examined the effects of constitutively active MEK-1 (MEK R4F) on JNK activity. Constitutively active forms of MEK-1 induce p42 MAPK activation and maturation when injected into oocytes (28, 29). As shown in Fig. 5*C*, concentrations of MEK R4F that were sufficient to cause p42 MAPK activation and GVBD also caused JNK activation. Microinjection of kinase-minus MEK-1 had no effect on JNK activity (data not shown).

In summary, activation of the p42 MAPK cascade by either Mos, Δ Raf-DD:ER, or MEK-R4F caused activation of JNK. Thus, in *Xenopus* oocytes, JNK can be activated downstream of the Mos/MEK/p42 MAPK cascade.

***Xenopus* JNK Activation in the Absence of p42 MAPK Activation**—By having shown that the JNK cascade can be activated downstream of the p42 MAPK cascade, we next examined whether activation of p42 MAPK was required for JNK activation. First we examined whether hyperosmolar stress, which is known to activate JNKs in other cell types, would activate JNK and p42 MAPK in oocytes. Hyperosmolar sorbitol (0.5 M) caused

FIG. 2. Time course of progesterone-induced JNK and p42 MAPK activation. Dumont Stage VI oocytes were stimulated with progesterone ($6 \mu\text{M}$) and frozen down at the times indicated. Lysates of 10 oocytes were split. A portion (75%) of the lysate was incubated with immobilized GST-Jun (upper panel) for JNK kinase assay, and the remainder (25%) was incubated with protein A-bound p42 MAPK antibodies ($15\times$) for p42 MAPK activity assay (middle panel). MBP was used as a substrate for the p42 MAPK kinase assay. Kinase reactions were separated by PAGE, transferred, and autoradiographed. GVBD was measured by appearance of a white dot and by manually dissecting the oocyte (lower panel). Similar results were obtained in three independent experiments.

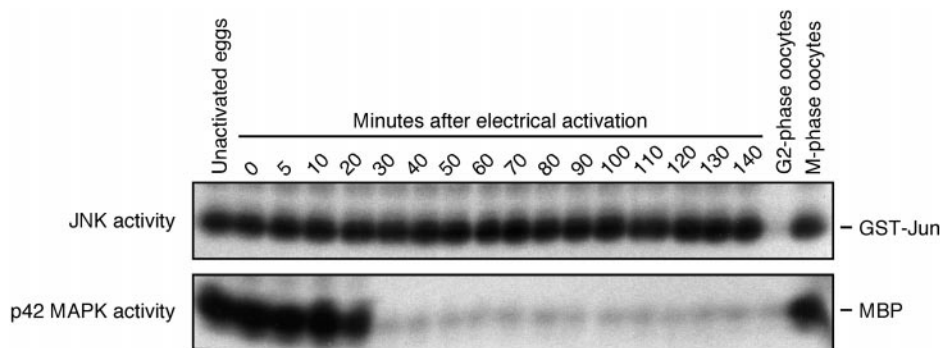
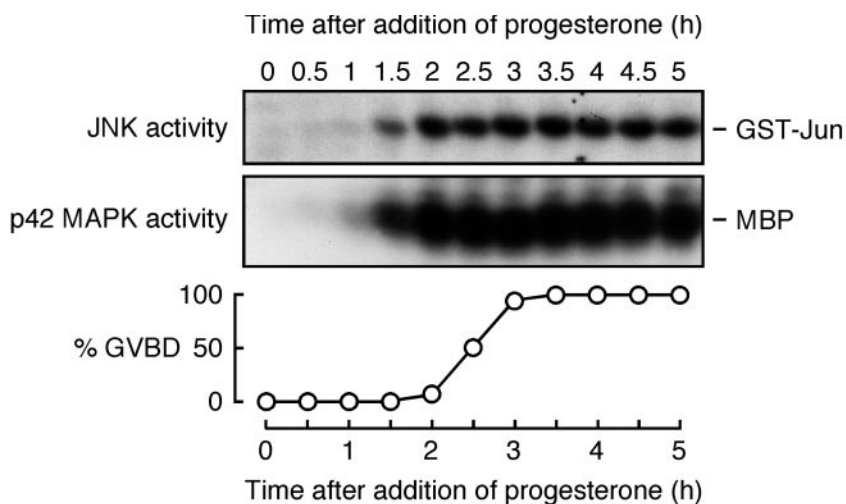
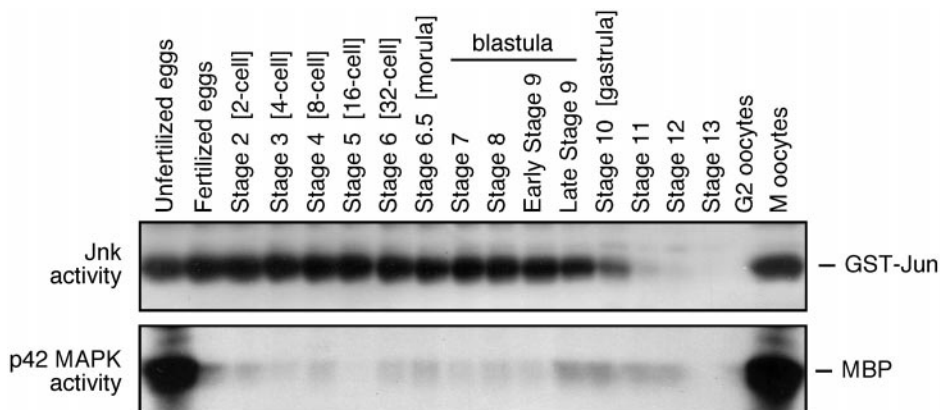


FIG. 3. JNK activity and p42 MAPK activity during the first mitotic cell cycle in parthenogenetically activated *Xenopus* eggs. Eggs were activated by electric shock and lysed at the indicated time points. Lysates split for JNK kinase assays and p42 MAPK assays, as described in Fig. 2. Phosphorylated substrates, GST-Jun and MBP, were detected by autoradiography. Similar results were obtained in three independent experiments.

FIG. 4. JNK and p42 MAPK activities during embryogenesis in *X. laevis*. Eggs were fertilized *in vitro* and lysed at the indicated stages of development. Lysates were split for JNK and p42 MAPK activity assays, as described in Fig. 2. Kinase reactions were performed, separated on 12.5% polyacrylamide gels, and transferred to PVDF membranes. Phosphorylated substrates were detected by autoradiography. Results represent data from three independent experiments and three different frogs.



marked activation of JNK (Fig. 6A) but no detectable activation of p42 MAPK (Fig. 6B). Thus p42 MAPK activation is not an absolute prerequisite for JNK activation in oocytes.

We next examined whether p42 MAPK activation was required for progesterone-induced JNK activation. We treated oocytes with progesterone in the presence or absence of U0126, an inhibitor of MEK-1. As shown in Fig. 7, U0126 effectively blocked progesterone-induced p42 MAPK activation but had no apparent effect on progesterone-induced JNK activation. Thus, although activation of the p42 MAPK cascade can bring about JNK activation, p42 MAPK activation is not required for JNK activation. Because the accumulation of Mos not only stimulates p42 MAPK activation, but also depends upon p42 MAPK activation (29–31), U0126 blocks Mos accumulation as well as MEK-1 and p42 MAPK activation (data not shown). Thus, there must be a pathway from the progesterone receptor to the JNK cascade that requires little or no support from Mos, MEK-1, or p42 MAPK.

Inhibition of the Mos/MEK-1/p42 MAPK cascade has been reported to either block maturation or delay it (29, 32–37). In the experiment shown in Fig. 7, none of the U0126-treated oocytes matured, even after 20 h of progesterone treatment, despite the fact that their JNK was fully activated. This finding demonstrates that full JNK activation is not sufficient to ensure maturation when p42 MAPK activation is blocked.

JNK Activation in “Unhealthy” Oocytes—Oocytes in culture sometimes degenerate, developing some mottling of the pigment in the animal hemisphere (Fig. 8, A and B). Ultimately these oocytes adopt the “gray puffball” phenotype and die. Since JNKs have been implicated in apoptosis in other systems, we examined JNK activity in mottled, unhealthy oocytes. As shown in Fig. 8C, unhealthy oocytes showed an elevated basal JNK activity but not an elevated basal p42 MAPK activity. Their JNK and p42 MAPK activities increased in response to progesterone, although the p42 MAPK activation was smaller than that seen in healthy oocytes (Fig. 8C). These findings

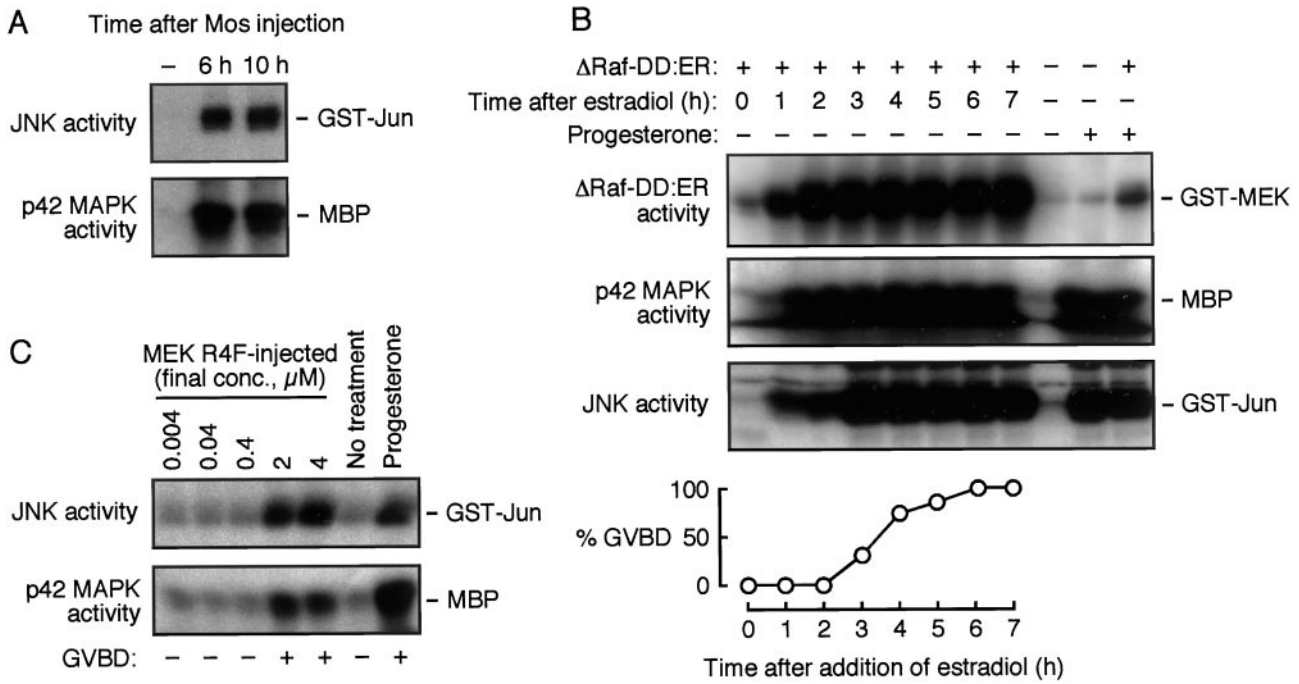


FIG. 5. Activation of *Xenopus* JNK and p42 MAPK by Mos, ΔRaf-DD:ER, and MEK R4F. A, activation of JNK and p42 MAPK by Mos. Stage VI oocytes were microinjected with 50 nl of Mos protein. Assuming the volume of an oocyte to be 1 μl, the final concentration of Mos was 200 nM. Oocytes were lysed either immediately or after 6 or 10 h. Lysates were subjected to JNK kinase and p42 MAPK assays as described in Fig. 2. Reactions were loaded on 12.5% polyacrylamide gels and transferred to PVDF membranes. Phosphorylated GST-Jun and MBP were detected by autoradiography. B, activation of JNK and p42 MAPK by ΔRaf-DD:ER. Stage VI oocytes were injected with 50 ng of ΔRaf-DD:ER mRNA, and the oocytes were incubated overnight to allow protein expression. Raf was activated by addition of 1 μM estradiol. Lysates were subjected to immunoprecipitation with an anti-estrogen receptor antibody, and Raf activity was assessed by incubation with [γ -³²P]ATP and purified recombinant GST-MEK. Lysates were also subjected to JNK kinase and p42 MAPK assays as described in Fig. 2. Phosphorylated GST-MEK, GST-Jun, and MBP were detected by autoradiography. C, activation of JNK and p42 MAPK by MEK R4F. Oocytes were microinjected with 50 nl of constitutive active MEK (MEK R4F) protein at various dilutions. The final concentrations of MEK assume a volume of 1 μl/oocyte. Oocytes were lysed 4 h after injection. GVBD was measured by appearance of a white dot and was first observed 3 h after injection. Lysates were used for JNK kinase (upper panel) and p42 MAPK assays (lower panel), as described in Fig. 2. Results are representative of three independent experiments.

raise the possibility that the JNK pathway may contribute to apoptosis in *Xenopus* oocytes.

DISCUSSION

In this study we have shown that *Xenopus* oocytes, eggs, and early embryos express 40- and 49-kDa isoforms of JNK, which we suspect correspond to the 46- and 54-kDa isoforms of mammalian JNKs. Both of the bands were recognized by anti-JNK1 and anti-JNK2 antibodies. Thus it is not certain whether *Xenopus* oocytes express four different JNK proteins (long and short splice variants of both JNK1 and JNK2) or just two JNK proteins (long and short) that cross-react with the anti-JNK1 and anti-JNK2 sera.

Xenopus JNK is activated just prior to GVBD during oocyte maturation, at about the same time as the activation of p42 MAPK. Like p42 MAPK, JNK stays active throughout meiosis I and meiosis II. But unlike p42 MAPK (and Cdc2), JNK remains active after fertilization. JNK activity is high throughout the period of rapid embryonic cleavages (fertilization through the midblastula transition) and then falls at the early gastrula stage. The decline in JNK activity occurs shortly after the commencement of zygotic transcription (38).

Stimuli that directly impinge upon the MAPK cascade, Mos, ΔRaf-DD:ER, and MEK R4F, cause activation of JNK. In principle this could be due to the direct activation of JNK by endogenous MEK-1 (in the case of Mos and ΔRaf-DD:ER) or by MEK R4F. However, in our hands MEK R4F does not phosphorylate immunoprecipitated JNK or GST-Jun-precipitated JNK *in vitro* (data not shown), and in general MEK-1 proteins appear to be highly specific for p42 and p44 MAPK. Therefore we favor the hypothesis that a more indirect pathway connects the MAP kinase cascade to the JNK cascade.

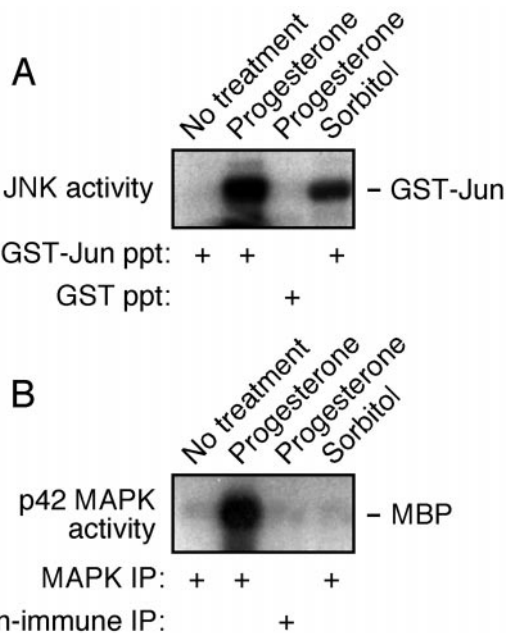


FIG. 6. Hyperosmolar stress induced by sorbitol activates JNK but not p42 MAPK. Dumont Stage VI oocytes were either left untreated, stimulated with progesterone (6 μM), or treated with 0.5 M sorbitol. Lysates were subjected to JNK (A) and p42 MAPK (B) assays, as described in Fig. 2. After transfer to PVDF membranes, incorporated radioactivity was detected by autoradiography. Results represent data of three independent experiments.

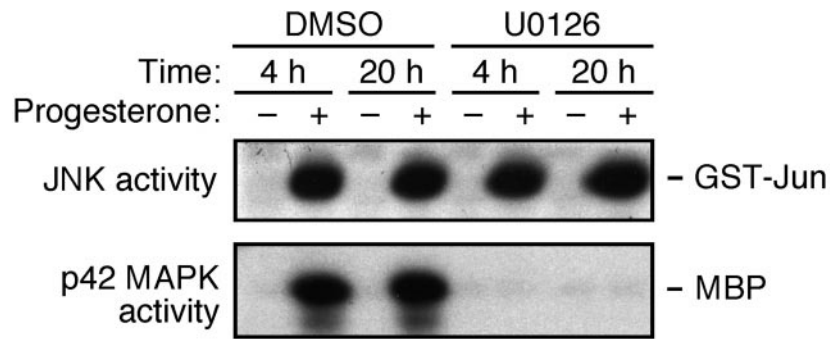


FIG. 7. **Inhibition of MEK-1 does not prevent progesterone-induced JNK activation.** Stage VI oocytes were pretreated for 1 h with the 50 μ M U0126 or with an equal volume of Me₂SO (DMSO) as a control. Unstimulated and progesterone-stimulated oocytes were lysed, split, and used for JNK and p42 MAPK assays as described in Fig. 2. Phosphorylated GST-Jun and MBP were detected by autoradiography. GVBD was observed only in the progesterone- and Me₂SO-treated control oocytes, and a *white dot* appeared 3 h after progesterone stimulation. U0126 completely blocked progesterone-induced GVBD in this experiment. Results represent similar data of three independent experiments.

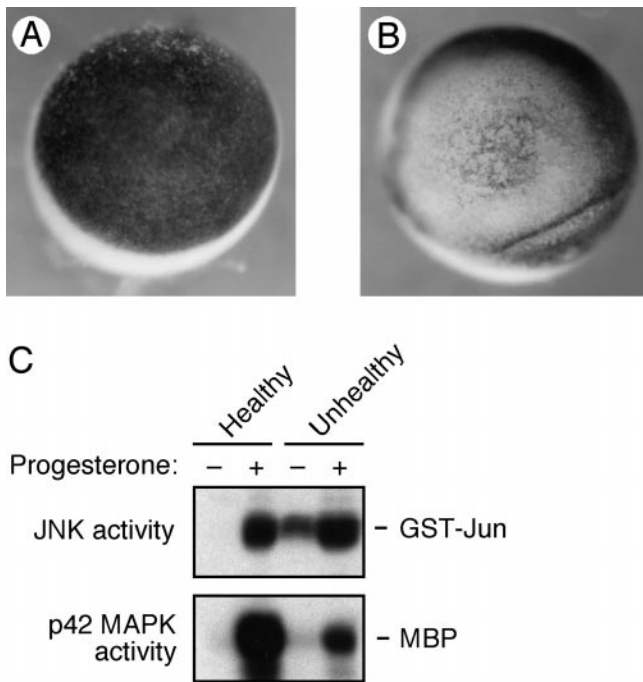


FIG. 8. **JNK activation in degenerating, unhealthy oocytes.** Regular, healthy, oocytes (A) were compared with unhealthy oocytes (B), which showed early signs of degeneration and which had a lighter color and mottling of the pigment in the animal hemisphere. Ten oocytes of either kind were stimulated with progesterone for 3 h or left untreated. After lysis, JNK kinase and p42 MAPK assays were performed as described in Fig. 2. After transfer to PVDF membranes, phosphorylated substrates were detected by autoradiography (C).

Progesterone can cause JNK activation even when Mos accumulation, MEK-1 activation, and p42 MAPK activation are inhibited by U0126. Thus, there is also a pathway from the progesterone receptor to the JNK cascade that does not depend upon input from Mos, MEK-1, or p42 MAPK. Likewise, hyperosmolar stress activates JNK without activating p42 MAPK. The *Xenopus* homologs of MKK4 or MKK7 could be likely participants in the p42 MAPK-independent JNK activation pathway.

Fig. 9 schematically summarizes our current understanding of the Mos/MEK-1/p42 MAPK pathway and the JNK cascade in oocytes. Important open questions include the exact identities of the p40 and p49 JNK proteins, the identities of the upstream kinases in the JNK cascade, the connections between the Mos/MEK-1/p42 MAPK cascade and the JNK cascade, and the connections between the progesterone receptor and the JNK cascade.

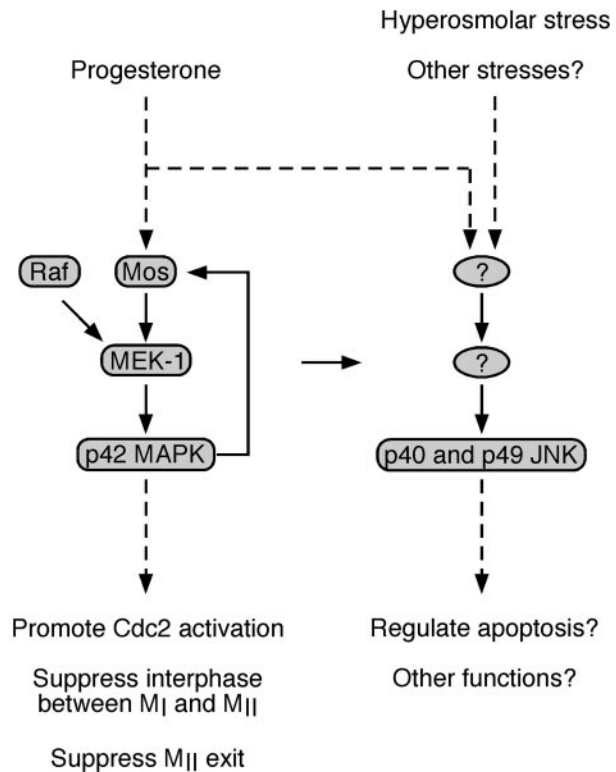


FIG. 9. **Model for the JNK signaling pathway, and its interplay with the Mos/MEK-1/p42 MAPK pathway, in *X. laevis* oocytes and eggs.**

Possible JNK Functions—Another important open question is what function the JNK pathway plays in oocyte maturation and embryogenesis. There are many possibilities; however, it seems likely that whatever the function, it will not depend upon the regulation of transcription factors by JNK, since oocyte maturation and early embryogenesis can proceed in the absence of transcription. Here we shall discuss two particular hypotheses that are suggested by the timing of JNK activation and inactivation.

Regulation of Apoptosis?—JNKs have been implicated in apoptosis in a variety of cell types. Analysis of hippocampal neurons from transgenic mice with disruptions of their *jnk3* genes has supported a role for JNK3 in excitotoxin-induced neuronal apoptosis (39). Similarly, analysis of embryonic fibroblasts from transgenic mice with disruptions of their *jnk1* and *jnk2* genes has supported a role for JNKs in UV-stimulated apoptosis upstream of cytochrome *c* release from the mitochondria (11). However, JNKs have also been suggested as inhibi-

tors of apoptosis. For example, MEKK1(-/-) embryonic stem cells fail to activate their JNK in response to hyperosmolarity and microtubule disruption, yet they exhibit a greater apoptotic response to these stresses (40). Further clarification of the roles of JNKs in apoptosis is clearly warranted.

Mature *Xenopus* oocytes and embryos up to the early gastrula stage, the stages when JNK is active, respond in an unusual way to cell cycle perturbations. Early embryos lack the normal checkpoints that make the initiation of mitosis contingent upon DNA replication and the metaphase/anaphase transition contingent upon successful alignment of the chromosomes at the metaphase plate (41, 42). The embryo has developed a simpler strategy for dealing with cell cycle perturbations; rather than arresting and trying to correct problems in response to cell cycle perturbants, the embryo commits to carrying out apoptosis (43–46). The apoptotic program is then executed just after the mid-blastula transition, at the early gastrula stage.

At the mid-blastula transition, the cell cycle slows down and gap phases begin to occur. Then, at the early gastrula stage, when JNK becomes inactive, the embryo switches strategies for responding to cell cycle perturbants, rather than immediately committing to apoptosis, the post-mid-blastula transition embryo initiates a cell cycle arrest and tries to correct the damage.

Thus, the times when JNK is active during oocyte maturation and embryogenesis are also the times when the embryo is particularly prone to undergoing apoptosis. It will be of interest to determine whether and how the JNK activation contributes to the apoptotic response.

Regulation of Cyclin B2 Localization?—Nearly 10 years ago, Hunt and coworkers (47) showed that cyclin B2 undergoes a mobility shift when oocytes undergo maturation and that this mobility shift depends upon the phosphorylation of Ser-90, which is situated next to a proline residue (SP). They also showed that at least two distinct kinase activities that can phosphorylate the N terminus of cyclin B2 become activated at about the time of germinal vesicle breakdown. The first kinase activity phosphorylated Ser-90; this kinase remained active until about 30 min after fertilization. The second kinase activity phosphorylated other sites in the N terminus of cyclin B2 and remained active throughout the first mitotic cell cycles. Neither of these activities appeared to be Cdc2 or p42 MAPK (47). Initial attempts were made to purify one or both of these activities. However, at the time it was unclear what the function of cyclin B2 phosphorylation might be, and so far none of these activities has been purified.

Subsequent work has shown that the N terminus of cyclin B1 contains a nuclear export sequence and that phosphorylation of residues near this nuclear export sequence is critical for nuclear accumulation of Cdc2/cyclin B1 and for the biological activity of Cdc2/cyclin B1 (48–50). The same appears to be true for cyclin B2 (51). Thus, the identification of the cyclin B2 N-terminal kinases is of high importance. Given the time course of JNK activity after parthenogenetic activation or fertilization of *Xenopus* eggs (Figs. 3 and 4), and given the fact that JNKs phosphorylate SP/TP residues, JNK is a candidate for the second cyclin kinase activity, the one that phosphorylates sites other than Ser-90 in the N terminus of cyclin B2. It will therefore be interesting to determine whether JNK can phosphorylate cyclin B2 *in vitro* and whether cyclin B2 phosphorylation in extracts depends upon JNK function.

The use of gain-of-function JNK mutations and JNK inhibitors will help to evaluate the role of the JNK signaling pathways in oocyte maturation, cell cycle progression, and embryogenesis. Moreover, these tools will clarify whether JNK plays a positive or negative role in apoptosis, the ultimate cell fate of many oocytes and eggs.

Acknowledgments—We are grateful to N. Ahn, M. Murakami, M. McMahon, A. Ullrich, and G. Vande Woude for providing plasmids; J. Baker for help with the embryos; R. Bhatt and J. Yoon for purification of MEK proteins; and M. Sohaskey, J. Besser, and D. Schechtman for discussions and critical reading of the manuscript.

REFERENCES

- Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) *Nature* **369**, 156–160
- Tibbles, L. A., and Woodgett, J. R. (1999) *Cell. Mol. Life Sci.* **55**, 1230–1254
- Ip, Y. T., and Davis, R. J. (1998) *Curr. Opin. Cell Biol.* **10**, 205–219
- Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derjard, B., and Davis, R. J. (1996) *EMBO J.* **15**, 2760–2770
- Robinson, M. J., and Cobb, M. H. (1997) *Curr. Opin. Cell Biol.* **9**, 180–186
- Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) *Adv. Cancer Res.* **74**, 49–139
- Polverino, A., Frost, J., Yang, P., Hutchison, M., Neiman, A. M., Cobb, M. H., and Marcus, S. (1995) *J. Biol. Chem.* **270**, 26067–26070
- Enslin, H., Tokumitsu, H., Stork, P. J., Davis, R. J., and Soderling, T. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 10803–10808
- Klippel, A., Reinhard, C., Kavanaugh, W. M., Apell, G., Escobedo, M. A., and Williams, L. T. (1996) *Mol. Cell. Biol.* **16**, 4117–4127
- Dong, C., Yang, D. D., Tournier, C., Whitmarsh, A. J., Xu, J., Davis, R. J., and Flavell, R. A. (2000) *Nature* **405**, 91–94
- Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288**, 870–874
- Lu, H. T., Yang, D. D., Wysz, M., Gatti, E., Mellman, I., Davis, R. J., and Flavell, R. A. (1999) *EMBO J.* **18**, 1845–1857
- Stronach, B. E., and Perrimon, N. (1999) *Oncogene* **18**, 6172–6182
- Noselli, S., and Agnes, F. (1999) *Curr. Opin. Genet. & Dev.* **9**, 466–472
- Kockel, L., Zeitlinger, J., Staszewski, L. M., Mlodzik, M., and Bohmann, D. (1997) *Genes Dev.* **11**, 1748–1758
- Goberdhan, D. C., and Wilson, C. (1998) *BioEssays* **20**, 1009–1019
- Hsiao, K.-M., Chou, S.-Y., Shih, S.-J., and Ferrell, J. E., Jr. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5480–5484
- Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994) *Science* **265**, 966–970
- Mansour, S. J., Candia, J. M., Matsuura, J. E., Manning, M. C., and Ahn, N. G. (1996) *Biochemistry* **35**, 15529–15536
- Yew, N., Mellini, M. L., and Vande Woude, G. F. (1992) *Nature* **355**, 649–652
- Murray, A. W. (1991) *Methods Cell Biol.* **36**, 581–605
- Newport, J., and Kirschner, M. (1982) *Cell* **30**, 675–686
- Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes Dev.* **7**, 2135–2148
- Bagowski, C. P., Stein-Gerlach, M., Choidas, A., and Ullrich, A. (1999) *EMBO J.* **18**, 5567–5576
- Bosch, E., Chervinski, H., Peterson, D., and McMahon, M. (1997) *Oncogene* **15**, 1021–1033
- Ferrell, J. E., Jr. (1999) *BioEssays* **21**, 833–842
- Baulieu, E. E., Godeau, F., Schorderet, M., and Schorderet-Slatkine, S. (1978) *Nature* **275**, 593–598
- Huang, W., Kessler, D. S., and Erikson, R. L. (1995) *Mol. Biol. Cell* **6**, 237–245
- Gotoh, Y., Masuyama, N., Dell, K., Shirakabe, K., and Nishida, E. (1995) *J. Biol. Chem.* **270**, 25898–25904
- Matten, W. T., Copeland, T. D., Ahn, N. G., and Vande Woude, G. F. (1996) *Dev. Biol.* **179**, 485–492
- Roy, L. M., Haccard, O., Izumi, T., Lattes, B. G., Lewellyn, A. L., and Maller, J. L. (1996) *Oncogene* **12**, 2203–2211
- Cross, D. A., and Smythe, C. (1998) *Exp. Cell Res.* **241**, 12–22
- Gross, S. D., Schwab, M. S., Taieb, F. E., Lewellyn, A. L., Qian, Y. W., and Maller, J. L. (2000) *Curr. Biol.* **10**, 430–438
- Fisher, D. L., Brassac, T., Galas, S., and Doree, M. (1999) *Development* **126**, 4537–4546
- Fabian, J. R., Morrison, D. K., and Daar, I. O. (1993) *J. Cell Biol.* **122**, 645–652
- Muslin, A. J., MacNicol, A. M., and Williams, L. T. (1993) *Mol. Cell. Biol.* **13**, 4197–4202
- Kosako, H., Gotoh, Y., and Nishida, E. (1994) *EMBO J.* **13**, 2131–2138
- Newport, J., and Kirschner, M. (1982) *Cell* **30**, 687–696
- Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) *Nature* **389**, 865–870
- Yujiri, T., Sather, S., Fanger, G. R., and Johnson, G. L. (1998) *Science* **282**, 1911–1914
- Dasso, M., and Newport, J. W. (1990) *Cell* **61**, 811–823
- Minshull, J., Sun, H., Tonks, N. K., and Murray, A. W. (1994) *Cell* **79**, 475–486
- Hensley, C., and Gautier, J. (1997) *Mech. Dev.* **69**, 183–195
- Stack, J. H., and Newport, J. W. (1997) *Development* **124**, 3185–3195
- Anderson, J. A., Lewellyn, A. L., and Maller, J. L. (1997) *Mol. Biol. Cell* **8**, 1195–1206
- Sible, J. C., Anderson, J. A., Lewellyn, A. L., and Maller, J. L. (1997) *Dev. Biol.* **189**, 335–346
- Kobayashi, H., Golsteyn, R., Poon, R., Stewart, E., Gannon, J., Minshull, J., Smith, R., and Hunt, T. (1991) *Cold Spring Harbor Symp. Quant. Biol.* **56**, 437–447
- Pines, J., and Hunter, T. (1994) *EMBO J.* **13**, 3772–3781
- Yang, J., Bardes, E. S. G., Moore, J. D., Brennan, J., Powers, M. A., and Kornbluth, S. (1998) *Genes Dev.* **12**, 2131–2143
- Hagting, A., Jackman, M., Simpson, K., and Pines, J. (1999) *Curr. Biol.* **9**, 680–689
- Yoshitome, S., Furuno, N., and Sagata, N. (1998) *Biol. Cell* **90**, 509–518