The c-Jun N-terminal kinases (JNKs), also termed stress-activated protein kinases, are a subfamily of MAP kinases implicated in cytokine and stress responses (1–3). In mammals there are six JNKs that are components of a modular MAP kinase cascade (5, 6). At the top of the cascade is a MAP kinase kinase kinase (MAPKKK) that phosphorylates and activates a MAP kinase kinase (MAPKK). These dual-specificity protein kinases activate JNK by phosphorylating conserved Thr and Tyr residues (Thr-Pro-Tyr) situated in the activation loop (5, 6). 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, X. 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 X. laevis oocytes possess a JNK-like protein and whether any such JNK protein was activated during oocyte maturation or early embryogenesis. Here we show that X. laevis oocytes express p40 JNK and p49 JNK isoforms. JNK activity increases abruptly just prior to germinal vesicle breakdown during X. laevis 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 Biotechnological). The costs of publication of this article were defrayed in part by advertisement 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). The JNK pathway exerts nongenomic effects.
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. The X15 polyclonal anti-p42 MAPK antiserum was used for immunoblotting (17). Polyclonal anti-estrogen receptor antibodies (HC-20) were obtained from Santa Cruz Biotechnology. The cDNAs for a constitutively active, His6-tagged version of human MEK-1 (with Ser-218 replaced by Glu, Ser-222 replaced by Ala) 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 malf-Mos fusion protein was provided by Monica Murakami (NCRF-Frederick Cancer Research and Development Center) and George Vande Woude (Van Andel Research Institute, Grand Rapids, MI). malf-Mos was expressed in E. coli and purified as described (20).

**Immunocomplex in Vitro Assays—Xenopus p42 MAPK was immunoprecipitated from lysates using polyclonal antibody X15. Crude cytoplasmic lysates were diluted 1:1 in EB and precleared with 20 ml of protein A-Sepharose (Sigma). After 3 h at 4 °C at moderate shaking, immunoprecipitates were washed three times with 50 ml HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glyceral, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. The bound JNK activity was detected by autoradiography.**

**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 described (22), aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, 1 mM leupeptin, 1 mM pepstatin A, 1 mM EDTA, 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 [γ-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**

**Effect of Estradiol on JNK Activity**—JNK activity was detected by autoradiography on Kodak Biomax MS film.
Proteins were transferred to PVDF membranes and pull-down with control GST beads or GST-Jun followed by electrophoresed on a 10% polyacrylamide gel (left panel) or were subjected to or progesterone-stimulated (M-phase) oocytes were either directly separated on a 10% polyacrylamide gel (right panel). Proteins were transferred to PVDF membranes and immunoblotted with monoclonal anti-JNK antibodies (D2; Santa Cruz Biotechnology). A. JNK precipitation and kinase assay. Lysates of G2- 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.

JNK expression and activity in Xenopus oocytes—A, JNK immunoblots. Lysates of 10 unstimulated (G2-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 G2- 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.

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, ΔRaf-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 adenyl cyclase) (26).

As shown in Fig. 5A, 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 (ΔRaf-DD:ER) fused to the hormone-binding domain of the human estrogen receptor (hereafter denoted ΔRaf-DD:ER) (25). After allowing protein expression overnight, ΔRaf-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 ΔRaf-DD:ER, p42 MAPK, and JNK and brought about GVBD (Fig. 5B). 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. 5B). Estradiol had no effect on JNK activity, p42 MAPK activity, or GVBD in oocytes that were not expressing ΔRaf-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. 5C, 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, ΔRaf-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...
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 pigmentation 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).
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. Unlike 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 MAP kinase 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.

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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.

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 perturbations, 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 perturbations, rather than immediately committing to apoptosis, the post-mid-blastula transition embryo initiates a cell cycle arrest and tries to correct the damage. Thus, when the times that 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 30 min after fertilization. The second 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, but these efforts were thwarted by 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.
