We have cloned from a Xenopus ovary cDNA library a novel protein kinase gene whose expression peaks in the oocyte and unfertilized egg, begins to decrease gradually after fertilization, and disappears during the gastrulation stage of embryogenesis. The cloned gene, termed XEEK1 (for Xenopus egg and embryo kinase), encodes a protein with a predicted molecular mass of 49 kDa. Bacterially expressed XEEK1 migrates at 57 kDa upon polyacrylamide gel electrophoresis analysis, and a XEEK1-specific antibody recognizes a protein of 57 kDa in Xenopus oocyte and egg extracts. The XEEK1 kinase domain shares 35% identity (~65% similarity) with the yeast SNF1 kinase and related kinases. However, expression of XEEK1 does not complement a snf1 deletion mutation in yeast, which suggests that it is probably not a Xenopus homolog of SNF1. Recombinant XEEK1 kinase autophosphorylates on threonine residues in vitro in a reaction that prefers Mg$_2^+$ ions. Site-directed mutagenesis of the conserved lysine residue (Lys-81) within the kinase domain to isoleucine totally abolishes kinase activity, and threonine 192 has been identified as the autophosphorylation site. This site is distinct from the conserved threonine (Thr-215 in XEEK1) present in the protein kinase activation loop that is the site of autophosphorylation for many protein kinases. XEEK1 is a substrate for the cyclic AMP-dependent protein kinase both in vitro and in vivo, suggesting a possible mode of regulation of XEEK1. An immunoprecipitate of oocyte/egg extracts with anti-XEEK1 serum contains a protein of ~155 kDa that may be a substrate and/or a regulatory component of the kinase.

One of the main mechanisms that govern cell cycle progression in eukaryotic cells is the sequential activation and deactivation of Cdk2 kinase. Several lines of evidence have shown that the Wee1 protein kinase and its related kinases, Myt1 and Myt1, negatively regulate Cdk2 kinase activity by phosphorylating a conserved tyrosine residue in the Cdk2 kinase (Russell and Nurse, 1987; Gould and Nurse, 1989; Lundgren et al., 1991; Mueller et al., 1995b). This negative regulation is believed to be a major control mechanism that prevents the onset of mitosis until the completion of DNA synthesis (Enoch and Nurse, 1991). Because of the central importance of Wee1-related kinases in cell cycle control, we undertook a polymerase chain reaction strategy to identify and characterize such kinases in Xenopus, where the availability of cycling egg extracts provides a system for functional analysis of cell cycle biochemistry. We report here the analysis of one protein kinase, XEEK1, identified in this manner, that is likely to play a role in early embryonic development.

EXPERIMENTAL PROCEDURES

Materials—The catalytic subunit of type II bovine heart cAMP-dependent protein kinase (PKA) was purified by the triple-column procedure described by Beavo et al. (1974). The heat-stable inhibitor of PKA (PKI) was prepared from bacteria producing recombinant rabbit muscle PKI (Thomas et al., 1991).

PCR Amplification of Protein Kinase Sequences—In an effort to clone novel Xenopus Wee1-related protein kinase genes by PCR amplification, we designed degenerate oligonucleotides of conserved protein kinase domains, e.g. kinase domains VIIb, VII, VIII, and IX, as defined by Hanks and Quinn (1991): primer V1b-5', 5'-GGCA TTG GCC GTA TGG AAAC-3', corresponding to amino acid sequence DL(IV)KPLILSF(ED)AE(DN); primer V1c-5', 5'-GGCA TTG ACA MZ TZZGZG GAN TTZ GG-3', corresponding to amino acid sequence KL(IV)GDGD(FL); primer V1c3-5', 5'-GGC GTA GAC ZAR TZX YXAZLZX MTC-3', corresponding to amino acid sequence DWWV(V)WYF(A)-3'. The code for nucleotide mixtures is as follows: K = (A, T), L = (A, C), M = (A, G), N = (C, T), P = (C, G), Q = (G, T), R = (G, T, C), X = (A, T, C), Y = (G, A, C), Z = (G, A, T, C), BamHI and SalI restriction sites, underlined in the primer sequences, were generated at the 5'- and 3'-ends of the PCR products, respectively. Total Xenopus ovary cDNA was prepared as described previously (Su and Maller, 1995), except that synthesized cDNA was used directly as template for PCRs. For the first PCR, 10 ng of cDNA, 50 pmol each of primers V1b-5' and V1c-5', dNTPs, and Taq polymerase (Boehringer Mannheim) in a final volume of 100 μl were subjected to five cycles of 94°C for 1 min, 37°C for 2 min, and 72°C for 1 min, followed by 30 cycles of 94°C for 1 min, 52°C for 30 s, and 72°C for 1 min. Products from the first PCR were purified by a QIAquick spin PCR purification kit (QIAGEN Inc.) and used as template for the second PCR. The second PCR, containing 1 μl of the first PCR products, 50 pmol each of primers VII-5' and VIII-3', dNTPs, and Taq polymerase, was subjected to 40 cycles of 94°C for 1 min, 52°C for 30 s, and 72°C for 1 min. Amplified fragments of ~100–300 bp were purified from a 2% agarose gel and cloned into the pCRII/Script SK+ vector (Stratagene) following the manufacturer's suggestions. Cloned DNA was recovered and subjected to DNA sequencing analysis on both DNA strands by the dyeoxynucleotide chain termination method with the Sequenase version 2 kit (U. S. Biochemical Corp.). The DNA sequence data were analyzed with a MacVector 3.5 program (IBI) on a Macintosh Quadra 800 and through
The NCBI BLAST E-mail Server. From initial screening of 10 clones, six protein kinase-related sequences were obtained. Among them, five clones belonged to the known protein kinase families of XMEK2, PKC, and CDK2 in Xenopus. However, one novel protein kinase sequence, designated XEEK1, was also identified and chosen for further study.

Cloning of the Full-length Protein Kinase cDNA—We adopted, with slight modification, a recent protocol to isolate full-length cDNA clones from Xenopus oocytes (Pippin et al., 1994). A DNA fragment (~150 bp) containing the novel kinase sequence was excised from the pCR-Script plasmid and purified. Ten micrograms of this purified DNA fragment was dissolved in 20 μl of H2O, and 2 μl was spotted onto each of 10-cm2 nylons membranes (Boehringer Mannheim). After washing the membranes were dried overnight at 80°C, then immersed in 1 M NaOH, 1.5 M NaCl-saturated Whatman No. 3MM filter paper for 30 min; transferred to dry filter paper; and UV-cross-linked with a Stratalinker UV cross-linker (Stratagene). DNA-bound membranes were neutralized in 10 ml of 1× Tris-HCl, 1.5 M NaCl, pH 8.0, for 5 min and rinsed in 10 ml of 5× SSC (Sambrook et al., 1989) for another 5 min. Prehybridization was then performed with 10 ml of 1× prehybridization solution from a μWAVE screening kit (Invitrogen) at 65°C for 1 h. A microcentrifuge tube containing 200 μg of plasmid DNA from a Xenopus ovary cDNA library (Su and Muller, 1995) was prepared as follows. DNA was denatured by the addition of NaOH to a final concentration of 0.2 M and incubation at room temperature for 5 min. Next, 0.1 volume of 3× sodium acetate, pH 4.8, was added along with 2 volumes of 70% ethanol. Precipitates were washed with 1× SSC, digested with 13,000 × g for 15 min, the DNA was resuspended in 100 μl of H2O and placed in a 15-ml tube. Five milliliters of 1× hybridization solution (μWAVE screening kit) that had been prewarmed to 65°C was added immediately, and the solution was boiled for 2 min. The DNA-bound membranes were removed from the prehybridization solution, added to the tube, and incubated at 65°C o/Night. The membranes were washed for 5 min twice with 50 ml of 1× Tris-HCl, 1 M EDTA, 0.5% SDS, pH 7.6, and once with 50 ml of 10× Tris-HCl, 1 M EDTA, pH 7.6. Each individual membrane was then placed in a microcentrifuge tube containing 300 μl of H2O and 30 μg of yeast tRNA. DNA was eluted from the membranes by incubation at 95°C for 2 min, mixed briefly by vortexing, and then snap-frozen in dry ice. The samples were allowed to thaw slowly on ice and mixed. DNA eluted from the membranes was recovered by alcohol precipitation, resuspended in 50 μl of H2O, and used to transform Escherichia coli DH5α Electromax competent cells (Life Technologies, Inc.). A few hundred bacterial colonies were obtained on the selective plates. The original DNA fragment (~150 bp) containing the novel kinase sequence was radiolabeled and used as a probe to screen these colonies with the μWAVE screening kit according to the manufacturer’s instructions. Several positive colonies were covered and used to prepare plasmid DNA for further analysis.

Expression and Purification of Recombinant Proteins—GST-XEEK1 was constructed by subcloning fragments amplified by PCR using the oligonucleotides 5′-GCC AGA TCT ATG CTG TGT CCT TCC AGT 9′ (designated K81I site upstream of the ATG initiation codon) and 5′-GCC GAA TTC TCA CTG TGT TTT GCA GGC GA-C-3′ (a 3′-primer introducing an EcoRI site downstream of the TGA termination codon). The 1299-bp BglII-EcoRI fragment containing the entire XEEK1 coding region was subcloned into the plasmid pGEX2-2 (Pharmacia Biotech Inc.) at BamHI and EcoRI sites to form pGEX2-XEEK1. The site-directed mutagenesis of pGEX2-XEEK1 was performed by a PCR method essentially as described previously (Landt et al., 1990), except that Taa polymerase was replaced by Pfu polymerase (Stratagene) in the PCR systems with the following oligonucleotides: K81I (5′-TTT TTT CCG TAT GAT TAT GAC AGC AGC GGC-3′; T192A, 5′-AGA ATT TTA GTG CCC GCT CTG TGG TGT GCT TCT C-3′; T212A, 5′-TGG TCC TTA CAT CGT TGT CCT GGC CAC C-3′; and T171C, 5′-GCC CCT ACA AGT ATC-3′). The lower-case letters underlined in the primer sequences indicate the introduced mutation sites, and mutations were confirmed by DNA sequencing. Wild-type or mutant clones of pGEX2-XEEK1 were transformed into E. coli strain BL21(DE3) (Novagen) for recombinant protein expression. Expression was induced by the addition of isopropyl-D-thiogalactopyranoside (2 mM) in response to glucose deprivation; were plated on LB agar plates; were grown for 16 h at 37°C, and were used for harvesting. The induced cultures were harvested by centrifugation at 10,000 g for 15 min, the pellets were washed with 0.5 ml of high and low salt buffers (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol, 0.1% Triton X-100), and washed with 0.5 ml of sodium acetate, 1 mM dithiothreitol, 30 mM p-nitrophenol phosphate, 10 μg/ml each leupeptin, pepstatin, and aprotinin) and centrifuged for 5 min in a microcentrifuge. The supernatants were used for analysis.

For embryos, eggs were fertilized in vitro; dejellied in 2% cysteine, pH 7.8; washed in 0.1× MMR buffer (10 mM NaCl, 0.2 mM KCl, 0.1 mM MgSO4, 0.2 mM CaCl2, and 0.5 mM Hepes, pH 7.8); and cultured at room temperature in 0.1× MMR buffer. The embryos were staged according to the NCBI BLASTe-mail Server. From initial screening of 10 clones, six with 75 units of pregnant mare’s serum gonadotropin 5 days prior to the experiment, and induced to ovulate by the injection of 550 units of human chorionic gonadotropin 14–16 h prior to the experiment. Metaphase II-arrested, cytosolic factor extracts were prepared from unfertilized eggs as described (Murray, 1991). For oocyte extracts, cleavage-stage embryos were manually dissected in isolation buffer and homogenized in 2 volumes of extraction buffer (80 mM β-glycerophosphate, 20 mM Hepes, pH 7.5, 15 mM MgCl2, 20 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 1 mM dithiothreitol, 30 mM p-nitrophenol phosphate, 10 μg/ml each leupeptin, pepstatin, and aprotinin), and centrifuged for 5 min in a microcentrifuge. The supernatants were used for immunoblotting.

Northern Analysis—Total RNA was isolated from staged embryos by homogenizing frozen samples in guanidinium isothiocyanate; extracting with acidic phenol, chloroform, and isomyl alcohol, and precipitating with isopropanol; resuspended in water; and quantitated by UV absorbance. The RNA (10 μg/lane) was fractionated by electrophoresis through a 1% formaldehyde–agarose gel and blotted under standard conditions. To determine the migration of the molecular mass markers, the portion of the gel with the RNA molecular mass markers (Life Technologies, Inc.) was stained with ethidium bromide (1 μg/ml) and illuminated by shortwave ultraviolet light. The RNA blot was probed with a 1300-bp EcoRI fragment of Xenopus src-1 cDNA kindly provided by Dr. R. Steele (University of California, Irvine, CA).

Antibody Preparation and Immunodetection—Antisera was raised in rabbits against purified recombinant XEEK1 protein expressed in bacteria. The antibody was affinity-purified on nitrocellulose blots of full-length recombinant XEEK1 as described (Olmsted, 1981). Immunoprecipitation of XEEK1 was performed as described (Harlow and Lane, 1988). Briefly, oocyte/egg extract (5–10 μl) was diluted in extraction buffer to a final volume of 150 μl, precleared with protein A-Sepharose (Sigma), incubated with 2.5 μl of affinity-purified antibody for 2 h on ice, mixed with 25 μl of 50% protein A-Sepharose for 1 h, washed with 0.5 ml of high and low salt buffers (20 mM Tris-HCl, pH 7.2, 200 μM EDTA, 0.1% Triton X-100, and 1% Triton X-100, respectively), and finally washed with buffer without any dication (20 mM Hepes, pH 7.2, 5 mM EGTA, 1 mM dithiothreitol, 0.1% Brij-35). Precipitates were resuspended in the same buffer and subjected to phosphorylation analysis.

For the experiments analyzing the phosphorylation of XEEK1 in vivo, immunoprecipitation was performed essentially as described previously with a slight modification (Erikson and Muller, 1989). Briefly, [γ-32P]ATP (20 μCi/ml) was added to oocyte extract in the presence or absence of PK1 and incubated at ambient temperature for 10 min. The
reaction was then diluted in 3 volumes of buffer containing 55 mM \(\beta\)-glyceraldehyde, pH 6.8, 5 mM EGTA, 5 mM MgCl\(_2,\) 50 mM NaF, 2 mM dithiothreitol, 100 \(\mu\)M phenylmethylsulfonyl fluoride, and 10 \(\mu\)g/ml each leupeptin, pepstatin A, and aprotinin. After centrifugation at 13,000 \(\times\) g for 10 min, 0.125 volume of 5 \(\times\) concentrated electrophoresis sample buffer was added to the supernatant, and the samples were boiled for 3 min. The samples were centrifuged again at 13,000 \(\times\) g for 10 min, and the supernatants were diluted and processed as described (Erikson and Maller, 1989).

**Kinase Assays and Phosphoamino Acid and Tryptic Phosphopeptide Analysis**—A sample of recombinant XEEK1 protein or immunoprecipitate was incubated at 30°C for 15 min in 30 \(\mu\)l of 20 mM Hepes, pH 7.2, 10 mM MgCl\(_2\) or MnCl\(_2\), 2 mM dithiothreitol, 0.1 mM EGTA, 0.1 mg/ml bovine serum albumin, 10 \(\mu\)M \(\gamma\)-[\(\beta\)-P]ATP (150 cpm/fmol). The reaction was stopped by the addition of 0.25 volume of 5 \(\times\) concentrated sample buffer and incubation at 95°C for 2 min. Products of the reaction were resolved by PAGE and visualized by staining and autoradiography. Phosphorylation reactions with PKA were done as described above, except that the reaction buffer comprised 25 mM MES, pH 6.5, 5.5 mM MgCl\(_2\), 3.75 mM \(\beta\)-mercaptoethanol, 50 \(\mu\)g/ml bovine serum albumin, and the concentration of \(\gamma\)-[\(\beta\)-P]ATP (1–5 cpm/fmol) was 100 \(\mu\)M. The products of the reaction were diluted with an equal volume of 50 mM CaCl\(_2\) digested with thrombin (16 \(\mu\)g/ml) for 1 h at ambient temperature, and analyzed as described above.

For phosphoamino acid analysis, radiolabeled proteins were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Immobi- lon-P, Millipore Corp.), visualized by autoradiography, and excised. The samples were hydrolyzed in 6 \(\mu\)l of 100°C for 1 h and lyophilized twice, and phosphoamino acids were separated by two-dimensional electrophoresis as described (Kamps and Sefton, 1989). For phosphopeptide mapping, proteins were transferred to a nitrocellulose membrane and digested with trypsin as described (Gabrielli et al., 1992). Tryptic phosphopeptides were separated by electrophoresis (pH 1.9, 1000 V, 30 min) in the first dimension and by chromatography (pyridinebutanol/acetic acid/water (3.3:5:1:4) in the second dimension.

### RESULTS AND DISCUSSION

Cloning of XEEK1 cDNA—During a search for kinases related to Wee1, we cloned a cDNA encoding a novel protein kinase, XEEK1, from a Xenopus ovary cDNA library using PCR technology. The cloning was performed in a two-step PCR in which primers corresponding to kinase domains VIb and IX were used in the first PCR to amplify total Xenopus ovary cDNA, and DNA products from the first reaction were subsequently amplified with a different set of primers corresponding to kinase domains VII and VIII to increase the specificity for related kinases from different organisms have a unique Glu-Gly-Asp triplet in this region, was thus chosen for this study. One of the cloned novel protein kinases, XEEK1, which contains a predicted open reading frame (1296 bp) encodes a protein of 432 amino acids with a predicted molecular mass of 49 kDa (Fig. 1A). Northern analysis of total RNA from Xenopus embryos using cloned XEEK1 cDNA as a probe identified a unique mRNA species of ~2.0 kilobases (see below), indicating that the cDNA analyzed was full-length. The predicted amino acid sequence of XEEK1 is most similar to the yeast SNF1 kinase (Booher et al., 1992). Toxicon phosphopeptides were separated by electrophoresis (pH 1.9, 1000 V, 30 min) in the first dimension and by chromatography (pyridinebutanol/acetic acid/water (3.3:5:1:4) in the second dimension.

### Figure 1. Sequence analysis of the XEEK1 gene.

A, nucleotide and predicted amino acid sequences of the XEEK1 gene. A predicted open reading frame of 432 amino acids is shown in single-letter code under the nucleotide sequence. The conserved amino acids are numbered, and the asterisk marks the termination codon. B, alignment and comparison of the amino acid sequences of the kinase domains from XEEK1, yeast SNF1 (Celenza and Carlson, 1986), and tobacco NPK5 (Muramaka et al., 1994). Amino acids are numbered on the right. Black boxes indicate identical residues shared by at least two sequences; dashes indicate gaps.

Sequence of XEEK1 exhibits the conserved elements of all other protein kinases within the 12 subdomains of the catalytic domain (Hanks and Quinn, 1991). Sequence analysis revealed that XEEK1 is most similar to the yeast SNF1 kinase, with ~35% identity (~65% similarity) within the catalytic domain. We have aligned the sequences of the catalytic domains from XEEK1, yeast SNF1 (Celenza and Carlson, 1986), and a to-
baclo SNF1-related kinase, NP5K (Muranaka et al., 1994). As shown in Fig. 1B, XEEK1 displays significant homology to these kinases, especially in the amino-terminal half of the catalytic domain. Yeast SNF1 is a protein serine/threonine kinase that has a major role in regulating glucose-repressed genes in response to glucose limitation (Carlson et al., 1981; Celenza and Carlson, 1986). Both mammalian and plant homologs of the SNF1 gene have been identified and shown to functionally complement a snf1 mutation in yeast (Alderson et al., 1991; Mitchelhill et al., 1994; Carling et al., 1994; Woods et al., 1994; Muranaka et al., 1994). To examine whether XEEK1 can complement the yeast snf1 mutation, XEEK1 was subcloned into a yeast expression vector, pMC944, in which expression is under the control of a constitutive mating factor promoter (MFα1) (Spevak et al., 1993). Because the yeast SNF1 function is required for expression of various glucose-repressible genes in response to glucose deprivation, snf1 mutants are unable to utilize sucrose, galactose, maltose, or nonfermentable carbon sources (Carlson et al., 1981). After pMC944-XEEK1 was transformed into a yeast snf1 truncation mutant strain, transformants were tested for their ability to grow in medium containing either glucose, galactose, or glycerol as the sole carbon source. Only those transformants growing in the presence of glucose were viable (data not shown). The expression of XEEK1 in yeast was confirmed by immunoblotting the yeast crude extract with anti-XEEK1 serum (Fig. 2). These results suggest that XEEK1 is not a homolog of SNF1. Furthermore, when expressed in two fission yeast mitotic catastrophe mutants using a fission yeast expression vector (Su and Maller, 1994, 1995), XEEK1 did not complement defects in Wee1 and Mik1 kinases (data not shown). This result suggests that XEEK1 is not functionally related to Wee1 or its related kinases.

Expression of XEEK1 during Early Xenopus Development—Expression of the XEEK1 gene was assessed by Northern blot analysis. Hybridization of total RNA prepared from staged embryos with a probe derived from the full-length XEEK1 cDNA clone revealed a single major species of ~2.0-kilobase mRNA (Fig. 3A). XEEK1 mRNA was detected in oocytes, eggs, and early embryos and therefore represents a maternal mRNA species. The level of XEEK1 mRNA changed significantly during early development (Fig. 3A). It was expressed at the highest level in the mature oocyte and unfertilized egg (Fig. 3A and data not shown) and declined gradually after fertilization, reaching a barely detectable level at stage 10 (gastrulation). Equal loading of RNA was judged by stripping the same blot and probing with a c-src clone, as shown in Fig. 3A (lower panel). This result clearly indicates that XEEK1 is expressed as an egg and early embryonic kinase.

To examine whether the protein level of XEEK1 correlates with the mRNA during the same developmental stages, extracts of eggs and embryos were analyzed by immunoblotting. As shown in Fig. 3B, consistent with the RNA result, the protein level of XEEK1 also peaked in the oocyte (Fig. 3C) and

**Fig. 2. Expression of XEEK1 in yeast.** The yeast expression construct of XEEK1, pMC944-XEEK1, or the expression vector, pMC944, alone was transformed into yeast strain MCY2916 (MATα snf1Δ his3 ura3 lys2 leu2). URA+ transformants were selected and grown to mid-log phase in minimal synthetic medium without uracil. Sixty micrograms of crude extract protein prepared from a fresh yeast culture was then resolved by SDS-PAGE, immunoblotted with anti-XEEK1 serum, and visualized by ECL. Molecular mass markers are indicated in kilodaltons.

**Fig. 3. Expression of XEEK1 during Xenopus development.** A, Northern analysis of XEEK1 mRNA. Total RNA was prepared from unfertilized eggs and staged embryos, resolved on a 1% denaturing agarose gel, transferred to nitrocellulose membrane, and probed with full-length XEEK1 cDNA (upper panel). The same blot was stripped of the probe and reprobed with the Xenopus src cDNA to serve as a control for equal RNA loading (lower panel). The numbers shown on top of the blot indicate the embryonic stages. B, protein level of XEEK1 during development. Protein from the equivalent of one egg or embryo was resolved by 12.5% SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with anti-XEEK1 serum, and visualized by ECL. C, subcellular localization of XEEK1. Xenopus oocyte nuclei (germline vesicles) were manually dissected from mature oocytes and separated from the cytoplasm. The oocyte membranes were prepared according to a previously described protocol (Sadler and Maller, 1982). Various protein extracts (equivalent of one oocyte, one cytoplasm, 15 nuclei, or five membranes) were resolved by 12.5% SDS-PAGE and subjected to immunoblotting analysis as described for B. Molecular mass markers are indicated in kilodaltons.
unfertilized egg and began to decrease gradually upon fertilization. However, unlike the RNA level, XEEK1 protein decreased at a much slower rate and remained detectable in the embryos even after gastrulation, at stages 13 and 25. This may be due to the higher sensitivity of immunodetection compared with Northern hybridization, or, alternatively, XEEK1 protein could be very stable and remain present after the disappearance of mRNA. In addition, the protein level of XEEK1 remained unchanged during the time course of progesterone-induced oocyte maturation and in in vitro cycling egg extracts (data not shown). This suggests that the level of XEEK1 protein is not regulated during cell cycle progression. To determine the subcellular localization of XEEK1, we analyzed cytoplasmic, nuclear (germinal vesicle), and membrane fractions prepared from Xenopus oocytes. As shown in Fig. 3C, XEEK1 is a cytoplasmic protein kinase.

Characterization of XEEK1 Protein Kinase Activity—To examine the function of XEEK1, we expressed it as a GST-tagged recombinant protein in bacteria and purified it to near homogeneity. Purified recombinant XEEK1 protein without the epitope tag displays an electrophoretic mobility of 57 kDa upon SDS-PAGE analysis, whereas the predicted molecular mass of XEEK1 within the coding region is 49 kDa (Fig. 4A). The electrophoretic mobility of recombinant XEEK1 is consistent with that of in vitro translated XEEK1 protein (Fig. 4B). The doublet protein bands shown in the in vitro translated product are apparently the result of two closely spaced initiation codons in XEEK1 since both protein bands were recognized by anti-XEEK1 serum (data not shown). Moreover, the same antiserum specifically recognized a 57-kDa protein from either Xenopus oocyte or oocyte extracts by immunoblotting (Fig. 3, B and C), suggesting that the authentic XEEK1 gene product also migrates at 57 kDa.

To ascertain whether XEEK1 has protein kinase activity, purified GST-XEEK1 protein was incubated in a kinase buffer containing various divalent cations and radiolabeled ATP. As shown in Fig. 5A (lane 1), XEEK1 was capable of autophosphorylation in the presence of Mg$^{2+}$. Interestingly, autophosphorylation increased almost 50-fold when Mn$^{2+}$ was used (lane 2), whereas no phosphorylation was observed when either Ca$^{2+}$ or Zn$^{2+}$ was present in the reaction (data not shown). Autophosphorylation of GST-XEEK1 occurred on the XEEK1 portion of the fusion protein and not on the GST portion (data not shown). A fusion protein of GST-XEEK1 in which the lysine residue at position 81 in the ATP-binding site was replaced by isoleucine had no kinase activity in the same phosphorylation assay, confirming that XEEK1 is a protein kinase (Fig. 5C). The preference of Mn$^{2+}$ over Mg$^{2+}$ for XEEK1 activity is similar to that described for tobacco NPK5 kinase (Muranaka et al., 1994). The preference of Mn$^{2+}$ for activity in some protein kinases has been suggested to reflect participation of the enzyme in a complex for full activity as a kinase. Analysis of the secondary structure of XEEK1 with a computer program (MacVector 3.5) revealed that XEEK1 contains a preferred small helix region within subdomain VIII of the kinase activation loop (data not shown). This is in contrast to the structure of PKA, which begins with a short $\beta$-strand in the same region (Knighton et al., 1991; Bossemeyer et al., 1993). The presence of a helix structure in this region is believed to have an autoinhibitory effect on the kinase activity, as described in the recently resolved structure of Cdk2, which requires the binding of a regulatory subunit (cyclin) in order to activate the kinase (De Bondt et al., 1993; Jeffreys et al., 1995). It is likely that XEEK1 also requires additional regulatory subunit(s) to fully express its kinase activity.

Phosphoamino acid analysis of autophosphorylated XEEK1 shows that the phosphorylation was exclusively on threonine residues (Fig. 5B). In the XEEK1 amino acid sequence, a well conserved threonine residue (Thr-215) within kinase subdomain VIII (Fig. 1B) is equivalent to Thr-197, the autophosphorylation site of PKA (Shoji et al., 1979; Steinberg et al., 1993). Thr-197 of PKA lies in a region of the consensus protein kinase core structure containing actual or potential sites for phosphorylation in most protein kinases (Hanks et al., 1988). To examine whether Thr-215 is the autophosphorylation site in XEEK1, site-directed mutagenesis was performed to construct various GST-XEEK1 mutant proteins, GST-XEEK1(T215A), GST-XEEK1(T212A), and GST-XEEK1(T192A), in which Thr-215, Thr-212, and Thr-192 were replaced with alanine residues, respectively. Thr-215 and Thr-212 are the only threonine residues located within subdomain VIII of XEEK1. Surprisingly, as shown in Fig. 5C, replacement of threonine at either position

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2. B. E. Kemp, personal communication.
215 or 212 with alanine did not affect the autophosphorylation of XEEK1. However, replacement of Thr-192 with alanine abolished the phosphorylation of XEEK1. Thr-192 is located within kinase subdomain VII and adjacent to a highly conserved Asp-197–Leu-198–Gly-199 triplet. This subdomain corresponds to Asp-184–Phe-185–Gly-186 in PKA, which lies in a loop that is stabilized by a hydrogen bond between Asp-184 and Gly-186 (Knighton et al., 1991; Bossemeyer et al., 1993). Asp-184 chelates the primary activating cations that bridge the β- and γ-phosphates of the ATP and thereby helps to orient the γ-phosphate for transfer. We conclude that Thr-192 is the autophosphorylation site of XEEK1.

In an effort to identify an in vitro substrate of XEEK1, many common protein kinase substrates available in this laboratory were tested, including histone H1, histone H2a, casein, myelin basic protein, protamine, lamin, tau protein, Kemptide, tyrosine hydroxylase, and various cardiac and skeletal muscle troponins. No phosphorylation of these proteins was observed, however. Furthermore, a screen of >40 peptides from a kinase substrate library appeared to be negative as well.3 These results suggest that XEEK1 is a very specific protein kinase, perhaps consistent with its restricted period of expression in early embryonic development.

Immunoprecipitation of XEEK1 from Xenopus Oocyte Extracts—To characterize the endogenous XEEK1 protein, oocyte extracts were immunoprecipitated with anti-XEEK1 serum. A

Fig. 5. Autophosphorylation of XEEK1. A, a sample of GST-XEEK1 was incubated in kinase reaction buffer in the presence of either MgCl2 (lane 1) or MnCl2 (lane 2) as described under “Experimental Procedures” and analyzed by SDS-PAGE. Molecular mass markers are indicated in kilodaltons. B, shown is the phosphoamino acid analysis of GST-XEEK1 phosphorylated in the presence of MnCl2. The positions of authentic phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y), determined by ninhydrin staining, are indicated. A similar result was obtained when GST-XEEK1 was incubated in the presence of MgCl2. C, samples of the various mutant constructs of GST-XEEK1, as indicated, were incubated in kinase reaction buffer in the presence of MnCl2 and analyzed as described for A. Only the pertinent region of the gel is shown. wt, wild type.

57-kDa XEEK1 protein was specifically immunoprecipitated, and the antiserum could remove 80% of the XEEK1 as judged by immunoblotting (data not shown). The purified immunocomplexes were subsequently used in a phosphorylation assay as described under “Experimental Procedures.” Surprisingly, no phosphorylation of endogenous XEEK1 was evident in this assay, but a band of ∼155 kDa was phosphorylated consistently (Fig. 6A, lane 2). Phosphorylation of this band was not seen when preimmune serum was used (lane 1). Two other higher molecular mass bands (∼195 and ∼190 kDa) with much less intensity in the autoradiograph are apparently the result of nonspecific phosphorylation since they were not reproducible in other experiments. Phosphoamino acid analysis of phosphorylated p155 indicates that the phosphorylation was on both serine and threonine residues (Fig. 6B). It is likely that p155 is a substrate of XEEK1 and that the phosphorylation of p155 by XEEK1 may compete for the ability of XEEK1 to autophosphorylate. The identification of p155 merits further investigation.

XEEK1 Is Phosphorylated by Cyclic AMP-dependent Kinase—XEEK1 protein contains several consensus sites for phosphorylation by a variety of protein kinases. To examine possible regulation of XEEK1 by phosphorylation, we immunoprecipitated [γ-32P]ATP. As shown in Fig. 7A (left panel), XEEK1 was phosphorylated in the oocyte extract. Moreover, when PKI was added to the extract before the labeling, the phosphorylation of XEEK1 was completely blocked (Fig. 7A, right panel). This result suggests that PKA could be responsible for the phosphorylation of XEEK1 in vivo. To ascertain whether XEEK1 is a substrate of PKA, we phosphorylated GST-XEEK1 with purified PKA in vitro (Fig. 7B). The phospho-

3 B. E. Kemp, J. L. Maller, and J.-Y. Su, unpublished results.
related GST-XEEK1 fusion protein was then cleaved by thrombin to remove the epitope tag and to verify that the phosphorylation was indeed on the XEEK1 protein. The phosphorylation was again specifically inhibited in the presence of PKI (Fig. 7B). XEEK1 phosphorylated by PKA in vitro and that phosphorylated in the extract were subsequently compared by phosphoamino acid and tryptic phosphopeptide analyses. As indicated in Fig. 7C, in both cases, XEEK1 was phosphorylated exclusively on serine residues. Finally, tryptic phosphopeptide analysis suggests that phosphorylation occurred at the same sites (Fig. 7D). We conclude that PKA is likely responsible for phosphorylating XEEK1 in vivo.

In summary, we have isolated a novel protein kinase that shares structural features with both the Wee1 and SNF1 kinases but is distinct from both of these protein kinase families. Analysis of the autophosphorylation of XEEK1 suggests that its mechanism of activation is distinct from that of PKA. The restricted period of expression and narrow substrate specificity of XEEK1 also suggest that it plays a role in early embryonic development. The phosphorylation of XEEK1 by PKA both in vitro and in vivo suggests that XEEK1 may participate in protein kinase cascades that are regulated by second messengers. Further work should clarify the role XEEK1 plays in the early embryo.

Acknowledgments—We thank Drs. M. Carlson, M. Castañón, R. Steele, and R. Maurer for providing the yeast snf1Δ strain, yeast expression vector, Xenopus c-src clone, and expression vector for PKI, respectively, and Dr. B. Kemp for analyzing phosphorylation of a peptide library with XEEK1. We thank J. Kyes for technical assistance and Dr. R. Hartley for help with embryo staging.

**FIG. 7. Phosphorylation of XEEK1 by the cAMP-dependent protein kinase.** A, phosphorylation of XEEK1 in an oocyte extract. Oocyte extracts were incubated with [γ-32P]ATP in the presence (+) or absence (−) of PKI and immunoprecipitated with either anti-XEEK1 or preimmune serum as indicated. Immunoprecipitation was performed under denaturing conditions to remove any nonspecific binding as described under "Experimental Procedures." Immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography. B, phosphorylation in vitro. A sample of GST-XEEK1 was incubated with PKA in PKA kinase buffer in the presence or absence of PKI, as indicated, and then digested with thrombin as described under "Experimental Procedures." The products of the reaction were resolved by SDS-PAGE and visualized by autoradiography. The positions of pGST-XEEK1, pXEEK1, and pGST were visualized by Coomassie blue staining. Molecular mass markers are indicated in kilodaltons. C, phosphoamino acid analysis of phosphorylated XEEK1. Phosphoamino acid analysis was done on XEEK1 phosphorylated in an extract, as shown in A (in vivo), and on XEEK1 phosphorylated by PKA and then digested with thrombin, as shown in B (in vitro). The positions of authentic phosphoamino acids are indicated (phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y)). D, phosphopeptide mapping of phosphorylated XEEK1 phosphorylated in an extract (in vivo) or in vitro by PKA (in vitro) were prepared and analyzed as described under "Experimental Procedures." The lower panel (mix) shows the analysis of a mixture of an equal number of counts from each preparation. The samples were analyzed by electrophoresis in the first dimension (left to right) and by chromatography in the second dimension (bottom to top). The x indicates the origin.
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