Phosphorylation of Conserved Serine Residues Does Not Regulate the Ability of *mos*^{xe} Protein Kinase to Induce Oocyte Maturation or Function as Cytostatic Factor

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Abstract. Expression of the *mos*^{xe} protein kinase is required for the normal meiotic maturation of *Xenopus* oocytes and overexpression induces maturation in the absence of other stimuli. In addition, *mos*^{xe} functions as a component of cytostatic factor (CSF), an activity responsible for arrest of the mature egg at metaphase II. After microinjection of *Xenopus* oocytes with in vitro synthesized RNA encoding either wild-type *mos*^{xe} or kinase-inactive *mos*$^{xe(R90)}$, both proteins are phosphorylated exclusively on serine residues and exhibit essentially identical chymotryptic maps. Since the phosphorylated kinase-inactive *mos*$^{xe(R90)}$ protein was recovered from resting oocytes that have not yet begun to translate endogenous *mos*^{xe}, this indicates that the major phosphopeptides of *mos*$^{xe(R90)}$ are phosphorylated by a preexisting protein kinase present in resting oocytes, and are not the result of autophosphorylation. The results presented here also indicate that the *mos*^{xe} protein does not undergo significant phosphorylation at unique sites during oocyte maturation.

If the biological activity of *mos*^{xe} were regulated by phosphorylation, a site of regulatory phosphorylation would most likely be conserved among *mos* proteins of different species. Site-directed mutagenesis was used to construct 13 individual serine→alanine mutations at conserved residues (3, 16, 18, 25, 26, 57, 71, 76, 102, 105, 127, 211, and 258). These 13 mutants were analyzed for their abilities to induce oocyte maturation and to function as CSF. Results obtained with the *mos*$^{xe(A105)}$ mutant revealed that serine-105 is required for both maturation induction and CSF activity, even though serine-105 does not represent a major site of phosphorylation. All of the remaining serine→alanine *mos*^{xe} mutants induced oocyte maturation and exhibited CSF activity comparable with the wild type. These results demonstrate that none of the conserved serines examined in this study function as regulatory phosphorylation sites for these biological activities. Peptide mapping of the remaining *mos*^{xe} mutants identified serine-3 as a major phosphorylation site in vivo, which is contained within the chymotryptic peptide MPSPIPVERF.

THE reversible phosphorylation of proteins is a key regulatory mechanism involved in biological processes such as growth factor signal transduction and cell cycle control. The consequences of inappropriate phosphorylation are exemplified by the many oncogenes that encode protein kinases (for reviews see Hunter and Cooper, 1986; Cantley et al., 1991; Hunter, 1991; Freeman and Donoghue, 1991). The v-*mos* oncogene and its cellular homolog, c-*mos*, encode proteins with intrinsic serine/threonine protein kinase activity (Maxwell and Arlinghaus, 1985; Hannink and Donoghue, 1985; Singh et al., 1986; Yew et al., 1991).

Several observations demonstrate that the protein kinase encoded by the *Xenopus* c-*mos* gene (*mos*^{xe}) plays an obligatory role in the meiotic maturation of oocytes. By blocking synthesis of the *mos*^{xe} protein with antisense oligonucleotides, Sagata et al. (1988) showed that expression of *mos*^{xe} was required for normal oocyte maturation induced by progesterone. Other experiments demonstrated that microinjection of in vitro synthesized *mos*^{xe} RNA into immature oocytes could induce maturation in the absence of progesterone (Freeman et al., 1989; Sagata et al., 1989*a*). Translation of *mos* is also required for progression from meiosis I to meiosis II, both in murine oocytes (O'Keefe et al., 1989) and also *Xenopus* oocytes (Kanki and Donoghue, 1991). Furthermore, the *mos*^{xe} protein constitutes an essential component of cytostatic factor (CSF)[1] (Watanabe et al., 1989; Sagata et al., 1989*b*), which is responsible for maintaining unfertil-

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1. *Abbreviations used in this paper*: CSF, cytostatic factor; GVBD, germinal vesicle breakdown; MBS-H, modified Barth's solution.

ized eggs in a state of metaphase II arrest (Meyerhof and Masui, 1979).

Xenopus oocyte maturation is accompanied by a two- to threefold increase in protein phosphorylation (Maller et al., 1977). Recently, several protein kinases have been shown to be activated during maturation. For example, the $p34^{cdc2}$ kinase (Gautier et al., 1989; Dunphy and Newport, 1989), S6 kinase (Erikson et al., 1987), and microtubule-associated protein kinase (Gotoh et al., 1991) increase in activity during maturation. Moreover, each of these enzymes is itself regulated by phosphorylation by other protein kinases. Phosphorylation of $p34^{cdc2}$ occurs on threonine and tyrosine residues and appears to exert both activating and inhibitory effects on its kinase activity (Dunphy and Newport, 1989; Gautier et al., 1989; Gould and Nurse, 1989; Labbe et al., 1989; Morla et al., 1989; Solomon et al., 1990; Lee et al., 1991). S6 kinase II is known to be activated via phosphorylation by microtubule-associated protein kinase in vitro (Sturgill et al., 1988), and phosphorylation of threonine and tyrosine residues in microtubule-associated protein kinase correlates with its activation in vivo (Ray and Sturgill, 1988; Anderson et al., 1990).

The mos^{xe} protein kinase induces oocyte maturation through an unknown mechanism that involves the activation of maturation promoting factor (Sagata et al., 1989*a*; Freeman et al., 1991). Activation of maturation promoting factor is known to involve changes in the phosphorylation state of its two subunits, the $p34^{cdc2}$ protein kinase and the cyclin B proteins (for review see Pines and Hunter, 1990; Maller, 1990; Freeman and Donoghue, 1991). The mos^{xe} protein itself undergoes phosphorylation during oocyte maturation (Sagata et al., 1988; Watanabe et al., 1989). In addition, the kinase activity of the mos^{xe} protein is required for both maturation-promoting activity and CSF activity as demonstrated by the absence of these activities in the kinase-inactive mutant $mos^{xe(R90)}$, in which the lysine residue in the ATP binding site has been substituted with arginine (Freeman et al., 1989, 1990). Hyperphosphorylated forms of the mos^{xe} protein have been observed in mature eggs (Watanabe et al., 1989; Sagata et al., 1989*b*), and dephosphorylation of mos^{xe} was recently shown to precede the degradation of mos^{xe} that occurs shortly after egg activation (Watanabe et al., 1991).

In this work we have used peptide mapping to characterize the phosphorylation state of the mos^{xe} protein. In addition to examining the wild-type mos^{xe} and kinase-inactive $mos^{xe(R90)}$ proteins, we have also examined 13 individual serine→alanine mutants affecting potential phosphorylation sites. Our results suggest that the mos^{xe} protein does not undergo significant phosphorylation at unique sites during oocyte maturation. Moreover, our results demonstrate that none of the conserved serines examined in this study function as regulatory phosphorylation sites for either the induction of oocyte maturation or its ability to function as CSF. Peptide mapping of the various serine→alanine mos^{xe} mutants also identified serine-3 as a major phosphorylation site in vivo.

Materials and Methods

Microinjection of RNA and Metabolic Labeling of Xenopus Oocytes

Fully grown (stage VI) oocytes were manually dissected from the ovaries of female *Xenopus* (obtained from *Xenopus* I, Ann Arbor, MI) and incubated at 18°C in modified Barth's solution (MBS-H; 10 mM Hepes, pH 7.4, 88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca[NO_3]_2$, 0.1 mg/ml each of penicillin and streptomycin). Healthy oocytes were microinjected with 50 nl of in vitro transcribed RNA (1–2 mg/ml) and then incubated at room temperature in either MBS-H alone, or MBS-H containing 0.5 mCi/ml [^{35}S]cysteine or 20 mCi/ml [^{32}P]orthophosphate. In some experiments, oocytes were labeled by microinjection with 50 nl of [^{32}P]orthophosphate (150–740 mCi/ml) (see Fig. 1). Oocytes were scored for germinal vesicle breakdown (GVBD) by the appearance of a white spot in the pigmented animal pole (Merriam, 1971). Oocytes were fixed in 5% TCA and dissected to confirm GVBD. For progesterone treatment, oocytes were incubated in MBS-H containing 15 μM progesterone.

Construction of mos^{xe} Mutants and In Vitro Transcription of RNA

The pSP64(polyA) vectors (Promega Biotec, Madison, WI) containing the mos^{xe} coding sequence and the $mos^{xe(R90)}$ sequence were described elsewhere (Freeman et al., 1989). Site-directed mutagenesis resulting in ser→ala substitutions at codons 3, 16, 18, 25, 26, 57, 71, 76, 102, 105, 127, 211, and 258 was performed as described (Zoller and Smith, 1983; Kunkel, 1985) using oligonucleotides ranging in size from 20 to 26 nucleotides. Restriction fragments containing the mutated DNAs were sequenced and then subcloned into the corresponding region of the wild-type mos^{xe}/pSP64-(polyA) plasmid. In vitro transcription reactions yielding 5′-capped and polyadenylated RNAs were carried out as described (Melton, 1987). The integrity of the RNAs was analyzed by electrophoresis and by in vitro translation using rabbit reticulocyte lysate (Amersham Corp., Arlington Heights, IL).

CSF Assays

CSF assays were performed essentially as described previously (Freeman et al., 1990). Ovulation was induced by injecting frogs with 200 IU of pregnant mare serum gonadotropin (Calbiochem-Behring Corp., San Diego, CA) 5–7 d before injection of 600 IU human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO). Eggs were collected into MR solution (5 mM Hepes, pH 7.8, 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2$) ~12 h after injection with human chorionic gonadotropin and fertilized in vitro. After the fertilized eggs rotated animal pole up, they were dejellied in 2% cysteine and cultured in MR containing 6% Ficoll before microinjection. Two-cell embryos were microinjected in the animal pole of one blastomere with 30 nl of RNA (1–2 mg/ml) just before completion of the first cleavage.

Immunoprecipitations

Metabolically labeled and ^{32}P-labeled oocytes were rinsed twice in MBS-H and then transferred to a 1.5-ml tube containing 3–5 μl/oocyte of ice-cold lysis buffer (8.5 mM Tris-HCl, pH 6.8, 1% NP-40, 150 mM NaCl, 50 mM β-glycerophosphate, 10 mM NaF, 5 mM EDTA, 2 mM DTT, 2 mM ATP, 2 mM sodium pyrophosphate, 2 mM EGTA, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μM pepstatin A). Oocytes were manually lysed, and the lysates were clarified by centrifugation at 10,000 *g* for 5 min. Cytosolic fractions were preadsorbed with 30–80 μl of a 50% (vol/vol) suspension of protein A–Sepharose/lysis buffer for 25 min at 4°C. After centrifugation in a microfuge for 3–5 min, the supernatants were collected and incubated with 5–10 μl of COOH-terminal antipeptide serum (anti-mos^{xe} serum) (Freeman et al., 1990) for 2 h at 4°C. Immune complexes were collected with protein A–Sepharose as described above, layered onto 1 ml of lysis buffer containing 10% sucrose, and then pelletted for 10 min at 2,500 *g*. The pellets were washed two–three times with lysis buffer and analyzed by 15% SDS-PAGE and autoradiography.

Peptide Mapping

Two-dimensional phosphopeptide mapping was performed as described by others (Hunter and Sefton, 1980). The mos^{xe} proteins were eluted from acrylamide gels and digested twice, each time with 10 μg α chymotrypsin (Worthington Biochemical Corp., Freehold, NJ), for 10–14 h and then 2–4 h. Peptides were separated in the horizontal direction by electrophoresis in pH 8.9 buffer (1% ammonium carbonate) for 23 min at 1 kV. Separation in the vertical direction was by chromatography in a system of *n*-butanol, water, pyridine, and acetic acid (75:60:50:15, [vol/vol]). Phosphoamino

acid analysis was performed by electrophoresis in two dimensions (pH 1.9 and 3.5) as previously described (Cooper et al., 1983).

Secondary protease digestions of chymotryptic phosphopeptides was performed as follows. Phosphopeptides were recovered from TLC plates and eluted from the cellulose with a solution of 88% formic acid, acetic acid, and water (5:16:180, [vol/vol]). The eluate was then lyophilized and the dry phosphopeptides were resuspended in 50 mM ammonium bicarbonate. Protease digestions were carried out for 4 h at 37°C with 5 μg proline-specific endopeptidase (ICN Biomedicals, Inc., Irvine, CA). The reaction products were analyzed by electrophoresis and chromatography as described above.

Results

Phosphorylation of the Kinase-inactive mos$^{xe(R90)}$ Protein Is Indistinguishable from that of the Wild-Type mos^{xe} Protein

Progesterone treatment of *Xenopus* oocytes stimulates the synthesis and phosphorylation of the *mos*^{xe} protein (Watanabe et al., 1989). Matured eggs contain additional phosphorylated forms of the *mos*^{xe} protein that are not present in maturing oocytes before GVBD (Watanabe et al., 1989; Sagata et al., 1989*b*; data not shown).

If some of the phosphate on *mos*^{xe} is the result of autophosphorylation, then a kinase-inactive mutant of *mos*^{xe} should lack these phosphorylations. To analyze the extent of *mos*^{xe} autophosphorylation, we compared the phosphorylation pattern of *mos*^{xe} to that of *mos*$^{xe(R90)}$, a mutant protein that contains a lysine-to-arginine substitution in the canonical ATP-binding domain. The *mos*$^{xe(R90)}$ protein is biologically inactive when expressed in oocytes (Freeman et al., 1989, 1990), and an analogous mutation in the v-*mos* protein abolishes its transforming and in vitro kinase activities (Hannink and Donoghue, 1985; Singh et al., 1986).

Endogenous *mos*^{xe} protein is translated from maternal mRNA during meiotic maturation and, therefore, exists at undetectable levels in stage VI oocytes in the absence of hormonal stimulation (Sagata et al., 1989*a*; Watanabe et al., 1989). Consequently, intermolecular phosphorylation of *mos*$^{xe(R90)}$ protein by endogenous *mos*^{xe} protein would not be expected to occur in resting stage VI oocytes.

Both *mos*^{xe} and *mos*$^{xe(R90)}$ proteins were phosphorylated in microinjected oocytes (Fig. 1 *a*); however, only oocytes injected with wild-type *mos*^{xe} RNA underwent meiotic maturation. Both proteins were phosphorylated to a similar extent during a 1-h labeling period (Fig. 1 *a*, lanes *1* and *3*). However, when oocytes were labeled for a longer time (corresponding to the time required for GVBD in 100% of the wild-type *mos*^{xe}-injected oocytes) the incorporation of radioactive phosphate into the wild-type *mos*^{xe} protein was much greater than the incorporation into the *mos*$^{xe(R90)}$ protein (Fig. 1 *a*, lanes *2* and *4*). This was probably not because of a change in the labeling efficiency during maturation since the pools of orthophosphate and nucleoside triphosphates have been shown to vary by <13% during meiotic maturation (Maller et al., 1977). Moreover, this was not because of an increase in the amount of *mos*^{xe} protein relative to *mos*$^{xe(R90)}$ protein as shown by [^{35}S]methionine labeling (data not shown).

Phosphoamino acid analysis of the *mos*$^{xe(R90)}$ and *mos*^{xe} proteins revealed only phosphoserine (Fig. 1 *b*). To compare the phosphorylation patterns in more detail, immunoprecipitated *mos*$^{xe(R90)}$ and *mos*^{xe} proteins were subjected to proteolysis with chymotrypsin and the resulting phosphopeptides were analyzed by two-dimensional phosphopeptide mapping (Fig. 2). All of the most highly phosphorylated peptides were present in both *mos*^{xe} and *mos*$^{xe(R90)}$ suggesting that

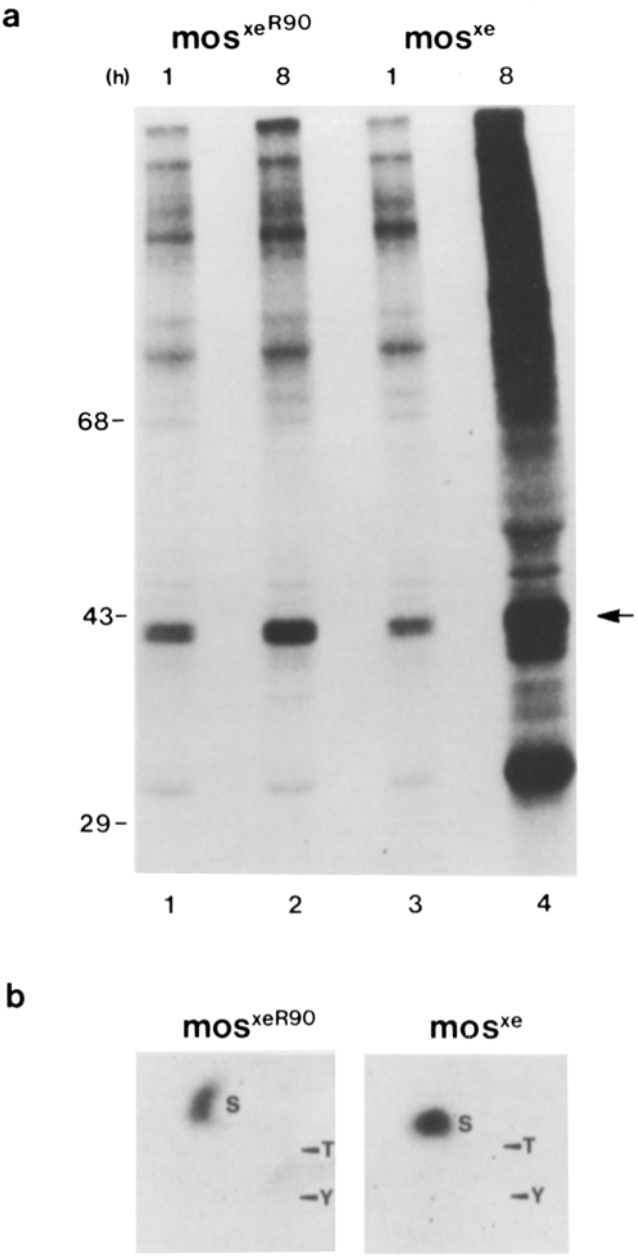

Figure 1. Comparison of *mos*^{xe} phosphorylation with that of *mos*$^{xe(R90)}$. (*a*) Oocytes were microinjected with *mos*^{xe} RNA or *mos*$^{xe(R90)}$ RNA. 2 h later, the oocytes were reinjected with [^{32}P]-orthophosphate (740 μCi/μl). Half of the injected oocytes were lysed 1 h after the [^{32}P]orthophosphate injection while the remaining oocytes were lysed after the *mos*^{xe} injected oocytes had undergone GVBD (8 h after the [^{32}P]orthophosphate injection). Immunoprecipitations were performed with anti-*mos*^{xe} serum and analyzed by 12.5% SDS-PAGE and autoradiography with an intensifying screen for 22 h. (*b*) The *mos*^{xe} and *mos*$^{xe(R90)}$ proteins shown above (labeled for 8 h) were eluted from the polyacrylamide gel. Phosphoamino acid analysis was performed and individual phosphoamino acids were separated by two-dimensional electrophoresis. The locations of unlabeled phosphoserine (*S*), phosphothreonine (*T*), and phosphotyrosine (*Y*) are indicated.

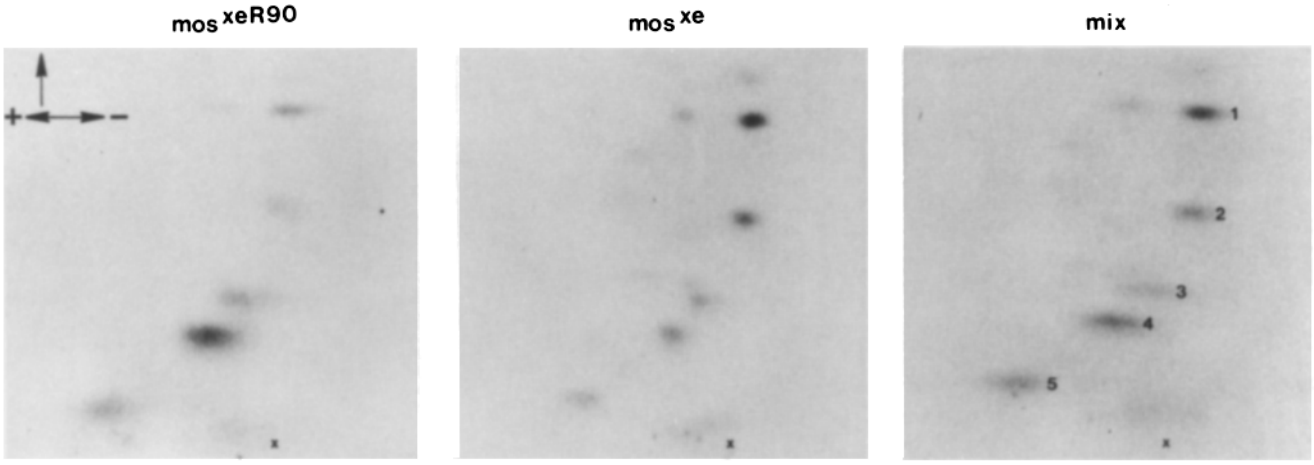

Figure 2. Chymotryptic phosphopeptide maps of $mos^{xe(R90)}$ and mos^{xe}. The mos^{xe} and $mos^{xe(R90)}$ proteins described in Fig. 1 (labeled for 8 h) were recovered from the polyacrylamide gel, oxidized with performic acid, and then digested with chymotrypsin. Phosphorylated peptides were separated on TLC plates by electrophoresis in the horizontal direction and by chromatography in the vertical direction, as indicated. In the mixed sample, an approximately equal number of cpm from the mutant and wild type were used. *x* indicates the location of the origin. Phosphopeptides were detected by autoradiography with an intensifying screen for 9 d.

they were not the result of autophosphorylation. These peptides are designated as chymotryptic peptides No. 1-5, as shown in the right panel of Fig. 2. The yield of peptide No. 5 was quite variable and usually low, although it is quite evident on the maps in Fig. 2.

Although trypsin is more often used for peptide mapping, our choice of chymotrypsin was influenced by previous peptide mapping experiments of the v-*mos* protein, in which chymotrypsin was found to yield superior maps to trypsin (Papkoff et al., 1982).

Mutagenesis of Conserved Serine Residues in mos^{xe}: Effects on Maturation-Inducing Activity

There are many examples of protein kinases that are regulated by phosphorylation, and the regulatory phosphorylation sites are often conserved. A well-studied example is the effect of phosphorylation on the kinase activity of the members of the *src* family of tyrosine protein kinases (Hunter and Cooper, 1986). Another example is the conservation of a tyrosine residue in $p34^{cdc2}$, that when phosphorylated inhibits kinase activity (Gould and Nurse, 1989). Since the *mos* proteins from several different species (*Xenopus*, human, mouse, and chicken) can all function to induce *Xenopus* oocyte maturation (Yew et al., 1991), we hypothesized that some of the biologically significant phosphorylation sites might be conserved.

In the mos^{xe} protein, there are 10-serine residues (S3, S16, S18, S25, S57, S102, S105, S127, S211, and S258) that are also conserved in the human, mouse, and chicken *mos* proteins (Fig. 3). To analyze the importance of these residues, we individually mutated the codon for each conserved serine to an alanine codon. We also constructed three additional serine→alanine mutations in mos^{xe} at positions where the serine was present in three out of four species of *mos* protein (S26, S71, and S76). To examine these mutants for biological activity, in vitro synthesized RNA encoding either a mutant mos^{xe} protein or the wild-type protein was microinjected into stage VI *Xenopus* oocytes (Table I). The injected oocytes were then monitored for GVBD. In these ex-

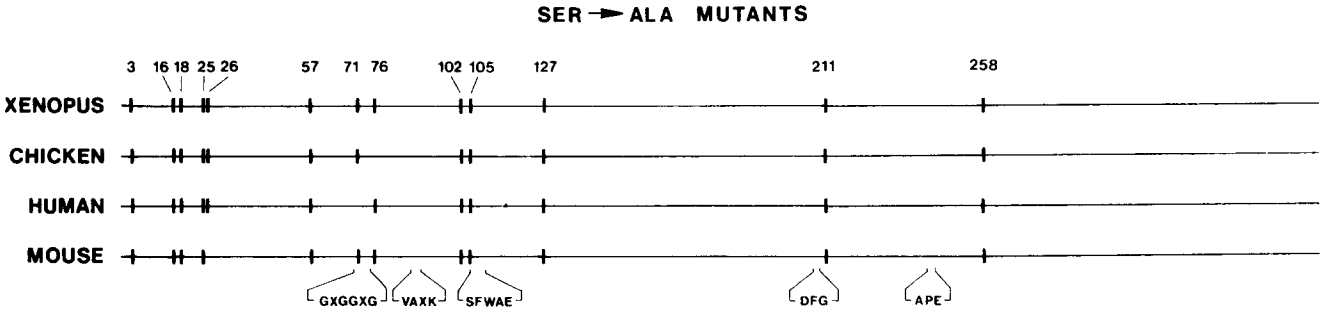

Figure 3. Location of the 13 most highly conserved serines in mos^{xe}. Serines 3, 16, 18, 25, 26, 57, 71, 76, 102, 105, 127, 211, and 258 in mos^{xe} were individually mutated to alanines by site-directed mutagenesis. The linear position of each of these residues is depicted in the *Xenopus*, chicken, human, and mouse *mos* proteins. For each sequence, the presence of a conserved serine is indicated by a vertical bar. These represent all serine residues that are conserved in at least three of the four species shown. Note that serines 26, 71, and 76 are present in only three species; the other serine residues are conserved among all four species. The locations of sequence motifs characteristic of protein kinases are shown at the bottom. (Amino acids indicated by single letter abbreviations.)

Table I. Biological Activity and Phosphopeptide Pattern of mos^{xe} ser→ala Mutants

mos^{xe} mutant	GVBD assays		CSF activity	Chymotryptic peptides
	Number of oocytes	GVBD		
		%		
wild type	124	90	+	wild type
A3	33	82	+	missing No. 1 and No. 2
A16	33	85	+	wild type
A18	33	91	+	wild type
A25	32	91	+	wild type
A26	36	97	+	wild type
A57	33	88	+	wild type
A71	35	86	+	wild type
A76	37	84	+	wild type
A102	35	91	+	wild type
A105	90	0	−	wild type
A127	36	100	+	wild type
A211	32	84	+	wild type
A258	31	94	+	wild type

(*GVBD assays*) Oocytes were microinjected with 50 nl (1–2 mg/ml) of in vitro synthesized RNA encoding either the wild-type mos^{xe} protein or one of the 13 ser→ala mos^{xe} mutants. GVBD was scored by the appearance of a white spot at the animal pole and confirmed by fixation with 5% TCA and manual dissection. (*CSF activity*) One blastomere of cleaving two-cell embryos was injected with 30 nl (1–2 mg/ml) of in vitro synthesized RNA encoding either the wild-type mos^{xe} protein or one of the 13 ser→ala mos^{xe} mutants. For each RNA, 10 fertilized embryos were injected just as the first cleavage furrow was forming, ~1.3 h after fertilization. After ~4 h, embryos were scored for cleavage arrest of the injected blastomere. A + indicates that at least 7/10 injected blastomeres displayed at least partial cleavage arrest, and a − indicates that none of the injected blastomeres underwent cleavage arrest. See legend to Fig. 7 for further details. (*Chymotryptic peptides*) Chymotryptic phosphopeptide maps were prepared for each of the ser→ala mutant proteins as described in Materials and Methods. The peptide maps for each mutant were compared to those of the wild-type mos^{xe} protein.

periments, 12 of the 13 mutants induced GVBD with an efficiency and kinetics similar to the wild-type mos^{xe} protein. However, one of the mutants, mos$^{xe(A105)}$, was unable to induce GVBD. No sign of GVBD was detected in any of the mos$^{xe(A105)}$-injected oocytes even 6 h after the wild-type mos^{xe}-injected oocytes reached 100% GVBD. This was not because of a dramatically decreased half-life of the mutant protein, as the mos$^{xe(A105)}$ protein was translated and expressed at the same level as the wild-type mos^{xe} protein during labelings periods of varying duration after microinjection (data not shown).

Serine-105 Is Not a Major Site of Phosphorylation

The mos$^{xe(A105)}$ protein is phosphorylated to approximately the same level as the mos^{xe} protein in pre-GVBD oocytes. During longer labeling periods, the wild-type protein becomes phosphorylated to a greater extent than the mos$^{xe(A105)}$ protein (data not shown), because of maturation of the wild-type mos^{xe}-injected oocytes. To compare the phosphorylation pattern of mos$^{xe(A105)}$ to that of wild-type mos^{xe}, we prepared two-dimensional chymotryptic phosphopeptide maps of each protein (Fig. 4). Oocytes were microinjected with either mos$^{xe(A105)}$ or mos^{xe} RNA and labeled with [^{32}P]orthophosphate until the mos^{xe}-injected oocytes underwent GVBD. The wild-type and mutant proteins were purified by immunoprecipitation and digested with chymotrypsin. All of the major phosphopeptides in the wild-type mos^{xe} protein were also present in the mos$^{xe(A105)}$ protein. Thus, under these conditions, phosphorylation of the mos$^{xe(A105)}$ protein occurs at the same sites as in the wild-type mos^{xe} protein suggesting that serine-105 is not a significant phosphorylation site in mos^{xe}.

The chymotryptic peptides No. 1–4 are observed as in the

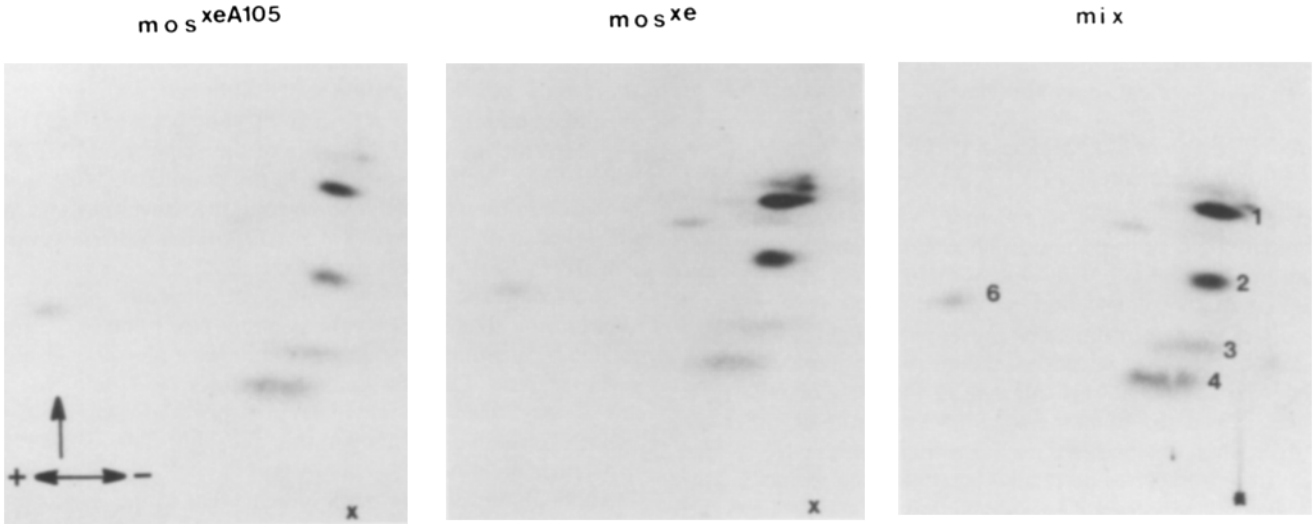

Figure 4. Chymotryptic phosphopeptide maps of mos$^{xe(A105)}$ and mos^{xe}. Oocytes miroinjected with the mos^{xe} and mos$^{xe(A105)}$ RNAs were incubated in MBS-H containing [^{32}P]orthophosphate until mos^{xe}-injected oocytes reached 100% GVBD. Note that the mos$^{xe(A105)}$-injected oocytes did not undergo maturation. The phosphorylated *mos* proteins were purified by immunoprecipitation with anti-mos^{xe} serum and 12.5% SDS-PAGE. The proteins were recovered from the gel and digested with chymotrypsin. The resultant phosphopeptides were separated on TLC plates by electrophoresis in the horizontal direction and by chromatography in the vertical direction, as indicated. In the mixed sample, an approximately equal number of cpm from the mutant and wild type were used. *x* indicates the location of the origin. Phosphopeptides were detected by autoradiography with an intensifying screen for 6 d.

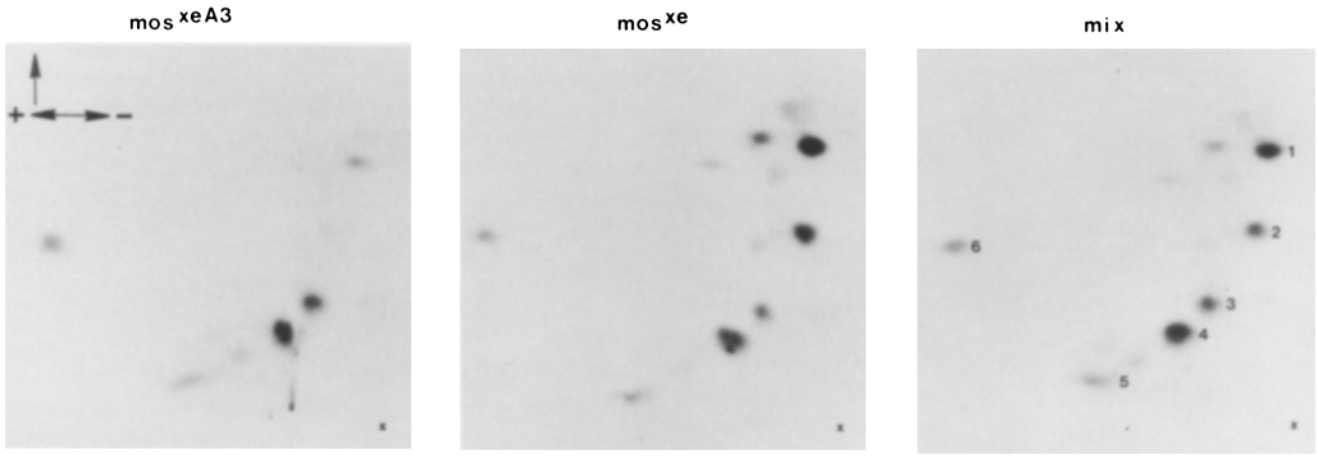

Figure 5. Chymotryptic phosphopeptide maps of $mos^{xe(A3)}$ and mos^{xe}. Oocytes microinjected with the mos^{xe} and $mos^{xe(A3)}$ RNAs were incubated in MBS-H containing [^{32}P]orthophosphate until the injected oocytes reached 100% GVBD. The phosphorylated *mos* proteins were purified by immunoprecipitation with anti-mos^{xe} serum and 12.5% SDS-PAGE. The proteins were recovered from the gel and digested with chymotrypsin. The resultant phosphopeptides were separated on TLC plates by electrophoresis in the horizontal direction and by chromatography in the vertical direction, as indicated. In the mixed sample, an approximately equal number of cpm from the mutant and wild type were used. *x* indicates the location of the origin. Phosphopeptides were detected by autoradiography with an intensifying screen for 7 d.

maps shown in Fig. 2. However, the yield of peptide No. 5 was very low, although it was faintly visible on all of the original autoradiograms of the maps shown in Fig. 4. The variable yield of peptide No. 5 is most easily explained as the consequence of chymotrypsin digestion at a secondary cleavage site. The maps in Fig. 4 also contain an additional labeled spot, No. 6, which is most likely because of contaminating [^{32}P]orthophosphate. It is possible that the chymotryptic peptide containing serine-105 was not resolved or recovered using the conditions employed here. However, this relatively hydrophilic chymotryptic peptide is predicted to be the only peptide that is positively charged, even when maximally phosphorylated. As further proof that serine-105 is not a major phosphorylation site, we found that tryptic phosphopeptide maps of the $mos^{xe(A105)}$ and mos^{xe} proteins were also identical (data not shown).

Identification of Serine-3 as a Major Site of Phosphorylation

To identify the origin of the mos^{xe} phosphopeptides, we compared chymotryptic phosphopeptide maps of each mutant to that of the wild-type protein. Only $mos^{xe(A3)}$ exhibited a phosphorylation pattern different from that of mos^{xe}, as shown in the maps presented in Fig. 5. The peptide map of $mos^{xe(A3)}$ lacked chymotryptic peptides No. 1 and No. 2, which were present in the map of wild-type mos^{xe}. Since the mutant and wild-type proteins differ by only a single residue, we reasoned that peptides No. 1 and No. 2 were likely to be related by containing serine-3. The predicted chymotryptic peptide containing serine-3 has the sequence MPSPIPVERF. The mobility of peptide No. 1 was altered after redigestion with trypsin (data not shown), as expected if trypsin cleaves after the arginine residue in chymotryptic peptide No. 1. The mobility of peptide No. 2 was not altered after redigestion with trypsin (data not shown), indicating that it arises from chymotrypsin cleavage at a secondary site within peptide No. 1. Since chymotrypsin cleaves with a probability of 4% after arginine (Keil, 1987), the most likely assignment of chymotryptic peptide No. 2 is MPSPIPVER. This assignment is also consistent with the relative mobilities of peptides No. 1 and No. 2 observed here.

To prove that both chymotryptic peptides No. 1 and No. 2 contain serine-3, we redigested the two peptides with proline-specific endopeptidase (Yoshimoto et al., 1980). As shown in Fig. 6, digestion of either chymotryptic peptide No. 1 (*B*) or peptide No. 2 (*F*) yielded two new phosphopeptides. Mixing of the secondary digestion products from peptides No. 1 and No. 2 revealed that the two sets of new peptides (labeled with *arrowheads A* and *B*) were identical (see panel *D*). Digestion of either chymotryptic peptide No. 1 or 2 with proline-specific endopeptidase should yield a peptide of the sequence MPSP, which most likely corresponds to peptide *B*. If only partial digestion were obtained with proline-specific peptidase, then both chymotryptic peptides No. 1 or No. 2 would also yield a peptide of the sequence MPSPIP, which most likely corresponds to the peptide *A*. Note that because proline-specific peptidase requires an amino acid at the −3 position (Yoshimoto et al., 1980), the cleavage products SP or SPIP are not expected.

The secondary digestions with proline-specific peptidase clearly show that chymotryptic peptides No. 1 and No. 2 are related by a common sequence containing a phosphorylated serine residue. The fact that both peptides No. 1 and No. 2 are absent from the chymotryptic peptide map of the $mox^{xe(A3)}$ protein confirms that this phosphorylated serine is in fact serine-3.

Although these data clearly indicate that serine-3 is a major phosphorylation site, mutation of this residue does not affect the ability of $mos^{xe(A3)}$ to induce oocyte maturation (Table I).

Cytostatic Factor Activity of mos^{xe} Serine→Alanine Mutants

The mos^{xe} protein has recently been identified as an essential component of cytostatic factor (Sagata et al., 1989*b*), an

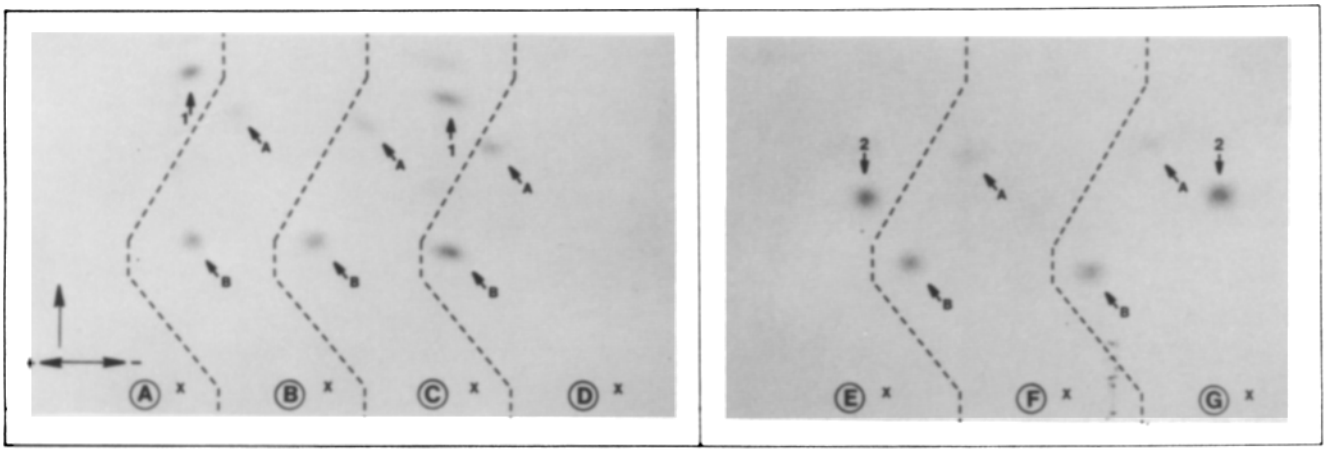

Figure 6. Secondary digestion of chymotryptic peptides No. 1 and No. 2. (*A*) Chymotryptic peptide No. 1, undigested. (*B*) Chymotryptic peptide No. 1, digested with proline-specific endopeptidase. (*C*) Chymotryptic peptide No. 1, mix of undigested plus digested with proline-specific endopeptidase. (*D*) Mix of chymotryptic peptides No. 1 and No. 2 each digested with proline-specific endopeptidase. (*E*) Chymotryptic peptide No. 2, undigested. (*F*) Chymotryptic peptide No. 2, digested with proline-specific endopeptidase. (*G*) Chymotryptic peptide No. 2, mix of undigested plus digested with proline-specific endopeptidase. Phosphopeptides corresponding to spots *1* and *2* in Fig. 5 were recovered from TLC plates and digested with proline-specific endopeptidase. The reaction products were then separated on TLC plates by electrophoresis in the horizontal direction and chromatography in the vertical direction, as indicated. Mixed samples contain an approximately equal number of cpm from each individual digest. *x* indicates the location of the origin for each sample. Phosphopeptides were detected by autoradiography with an intensifying screen for 10 d. Note that samples *A*, *B*, *C*, and *D* were spotted at intervals of 4 cm and analyzed together on one TLC plate. Samples *E*, *F*, and *G* were spotted at intervals of 5 cm and analyzed on a second TLC plate. Because of the relatively high electrophoretic mobility of the proline peptidase products labeled with arrowheads *A* and *B*, these peptides have moved leftward towards the origin of the adjacent map and, in some cases, have actually crossed over the origin. The dashed lines have been drawn to indicate this. As described in the text, the most probable assignments for these peptides are: chymotryptic peptide No. 1, MPSPIPVERF; chymotryptic peptide No. 2, MPSPIPVER; proline peptidase peptide *A*, MPSPIP; proline peptidase peptide *B*, MPSP. An additional faint peptide may be seen above chymotryptic peptide No. 1 in some of the maps. This peptide appeared to arise as a breakdown product from peptide No. 1 in repeated experiments and was not further characterized.

activity thought to be responsible for inducing metaphase arrest at meiosis II in a mature egg (Meyerhof and Masui, 1979). To test the serine→alanine mos^{xe} mutants for CSF activity, we microinjected in vitro synthesized RNA encoding wild-type mos^{xe} RNA, or RNAs encoding each of the 13 serine→alanine mutants. As shown in Table I, all but one of the serine→alanine mutants induced CSF arrest similar to wild type. The $mos^{xe(A105)}$ mutant failed to arrest cleavage of injected blastomeres. A similar result was observed when blastomeres were injected with RNA encoding the kinase-inactive mutant $mos^{xe(R90)}$. Representative photomicrographs of embryos ~4 h after microinjection are shown in Fig. 7. Although varying extents of CSF were observed, as shown in the two photomicrographs of embryos injected with wild-type mos^{xe} RNA, CSF arrest was reproducibly observed in all cases except for the $mos^{xe(R90)}$ mutant (Fig. 7, *R90*) and the $mos^{xe(A105)}$ mutant (Fig. 7, *A105*).

These results indicate that of the 13 most highly conserved serine residues in mos^{xe}, only serine-105 is essential for CSF activity. Since serine-105 was also essential for the induction of oocyte maturation, this indicates a complete correlation in these mutants between induction of oocyte maturation and CSF activity. In addition, these data indicate that although serine-3 represents a major phosphorylation site, phosphorylation at this residue is not required for the CSF activity of mos^{xe}.

Discussion

The mos^{xe} protein kinase is phosphorylated during oocyte maturation. Moreover, hyperphosphorylated forms of the mos^{xe} protein appear at or soon after GVBD (Watanabe et al., 1989; data not shown), as indicated by retarded electrophoretic mobility on SDS-PAGE. After egg activation, the mos^{xe} protein is first dephosphorylated and then rapidly degraded (Watanabe et al., 1989). Together, these results suggest the possibility that mos^{xe} activity may be regulated by phosphorylation.

In matured *Xenopus* eggs, the mos^{xe} protein constitutes only ~0.001% of the total soluble protein (Watanabe et al., 1989). Because of this low level of expression, we overexpressed the mos^{xe} protein by microinjecting oocytes with in vitro synthesized mos^{xe} RNA. Microinjection of ~100 ng of mos^{xe} RNA results in a 10–20-fold overexpression of the protein relative to the endogenous level found in progesterone matured oocytes (Sagata et al., 1989*a*; Freeman et al., 1990). When analyzed by SDS-PAGE, the overexpressed phosphorylated mos^{xe} protein comigrates with the phosphorylated species of the endogenous mos^{xe} protein (data not shown). Thus, although we have been unable to recover sufficient amounts of phosphorylated endogenous mos^{xe} protein for peptide mapping, it would appear that overexpressed mos^{xe} protein is phosphorylated similarly to endogenous mos^{xe} protein.

Phosphoamino acid analysis of the endogenous mos^{xe} protein from matured oocytes revealed only phosphoserine. This is consistent with the phosphoamino acid content of the v-*mos* protein isolated from *mos*-transformed NIH-3T3 cells (Papkoff et al., 1982).

We have found that the kinase-inactive $mos^{xe(R90)}$ protein

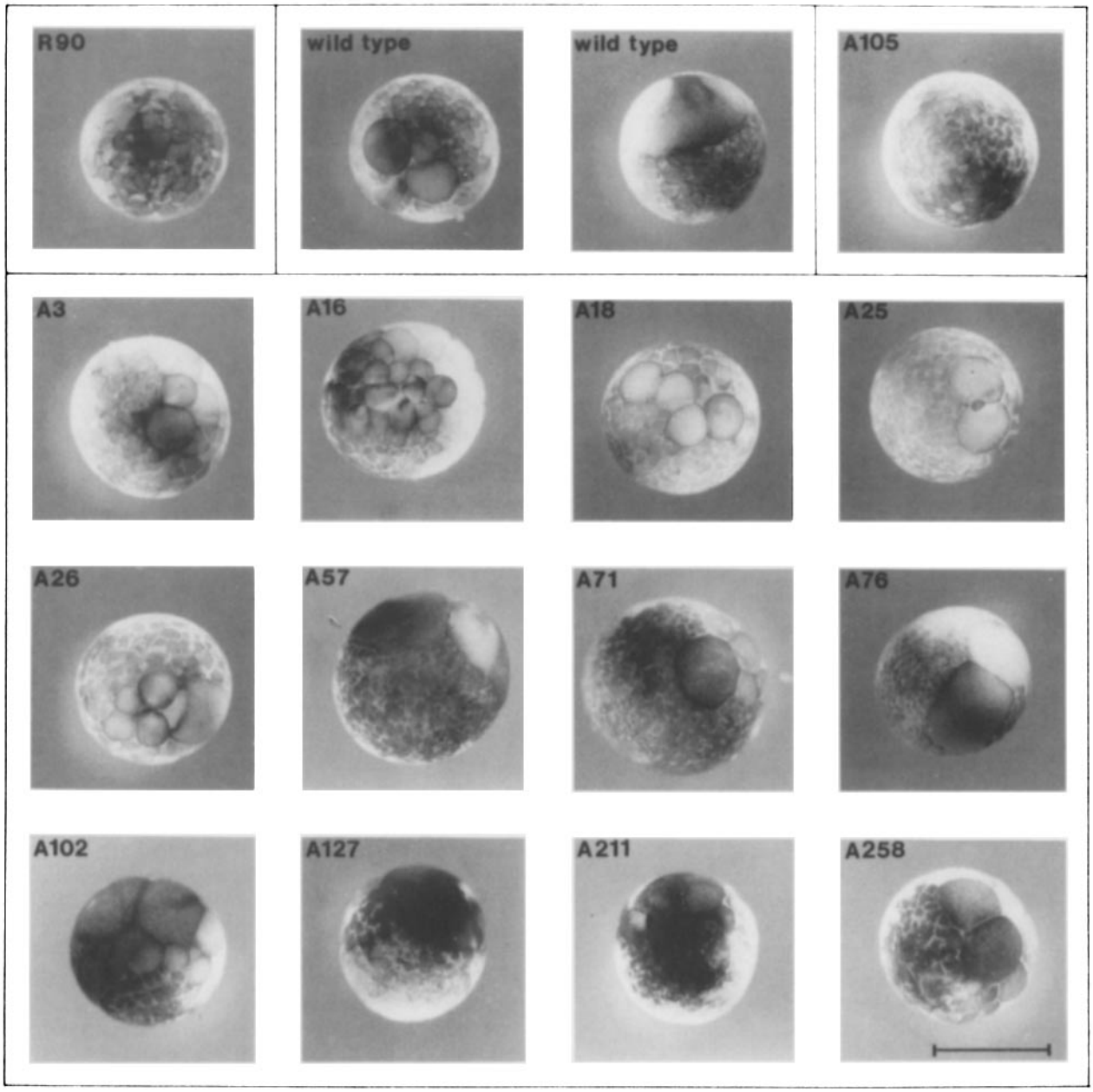

Figure 7. Assays of *mos*^{xe} mutants for CSF activity. CSF activity was assayed by microinjection of in vitro synthesized RNA into cleaving two-cell embryos. Photomicrographs of representative embryos are presented for each of the RNAs assayed. Additional experimental details are contained in Materials and Methods and the legend to Table I. Bar, (*R90*) As a negative control, RNA encoding the kinase inactive *mos*$^{xe(R90)}$ mutant was assayed, shown in the upper left photomicrograph. (*wild type*) As a positive control, RNA encoding wild-type *mos*^{xe} was assayed, shown in the middle two photomicrographs of the top row. These two photomicrographs were chosen to depict the range of CSF arrest that was observed. This ranged from arrest at the two-cell stage, resulting in noncleavage of half of the embryo, to arrest at the 16–32-cell stage, resulting in multiple large uncleaved blastomeres which are clearly visible. (*A105*) This was the only ser→ala mutant that was negative for CSF activity, as shown in the upper right photomicrograph. (Other ser→ala mutants) The remaining ser→ala mutants all displayed CSF activity, as shown in the three lower rows of photomicrographs. Bar, 1 mm.

is also phosphorylated on serine residues when expressed in oocytes. Two-dimensional phosphopeptide maps of the *mos*$^{xe(R90)}$ protein from resting oocytes and the *mos*$^{xe(R90)}$ protein from post-GVBD oocytes are essentially identical, demonstrating that the major phosphopeptides do not result from autophosphorylation. In addition, it is clear that the hyperphosphorylation of *mos*^{xe} at GVBD does not involve additional phosphorylations at new sites. However, we cannot rule out the possibility that the wild-type *mos*^{xe} protein could phosphorylate itself at the same sites phosphorylated on *mos*$^{xe(R90)}$ by other protein kinases. We have only recently detected a very low level of in vitro autophosphorylating activity in *mos*^{xe} immune complexes from mature oocytes consistent with the results of Yew et al. (1991). However, due to the inefficient labeling of *mos*^{xe} in vitro, we have not been able to compare the peptide maps of the in vitro and in vivo phosphorylated *mos*^{xe} proteins. We also cannot rule out the possibility that a small amount of endogenous *mos*^{xe} protein

is present in immature oocytes and is able to phosphorylate the *mos*$^{xe(R90)}$ protein in *trans*; however, we (unpublished data) and others (Sagata et al., 1989*a*; Watanabe et al., 1989) have been unable to detect *mos*^{xe} protein in oocytes before progesterone treatment.

Our results suggest that all of the major phosphorylations on *mos*^{xe} are catalyzed by an unidentified protein kinase which is active in immature oocytes. Since no new major sites of phosphorylation are used during maturation, this poses the question of why a slower electrophoretic species of hyperphosphorylated *mos*^{xe} should appear during oocyte maturation. The most likely explanation would be an increase in the stoichiometry of phosphorylation. For example, molecules which are singly phosphorylated at either serine-3 or an unidentified serine (corresponding to the unidentified chymotryptic peptides No. 3 or No. 4) may become doubly phosphorylated at two sites. However, since mutation of serine-3 does not inactivate the ability of the *mos*$^{xe(A3)}$ mutant to either induce oocyte maturation or function as CSF, the appearance of the hyperphosphorylated *mos*^{xe} species most likely has little functional significance except to reflect the overall increase in protein phosphorylation that accompanies oocyte maturation. A significant increase in protein phosphorylation is known to occur in maturing oocytes (Maller et al., 1977) and several proteins have been shown to undergo maturation-specific phosphorylations (Lohka et al., 1987; Cicirelli et al., 1988; Cooper, 1989). It should be noted that since no quantitative assays presently exist for *mos*^{xe} protein kinase activity, we cannot rule out the possibility that phosphorylation at serine-3 could affect its specific activity, leading to quantitative rather than qualitative effects on maturation induction or CSF activity.

In this work, we have found that all but one of the most highly conserved serine residues within the various *mos* proteins were dispensible for the maturation-inducing and CSF activities of *mos*^{xe}. However, the *mos*$^{xe(A105)}$ mutant was completely inactive in these biological assays. The *mos*$^{xe(A105)}$ mutant protein was stably expressed and phosphorylated in microinjected oocytes. However, we did not detect any differences in the phosphopeptide maps of the *mos*$^{xe(A105)}$ protein compared with the wild-type *mos*^{xe} protein. It is likely that serine-105 is a structurally important residue and not a site of phosphorylation given its close proximity to a glutamate residue (glutamate-109) that is highly conserved among known protein kinases (Hanks et al., 1988). Work from another laboratory (Okazaki et al., 1991) has demonstrated that deletion of the NH_2-terminal 50 amino acids of *mos*^{xe} has no effect on either its maturation-inducing or CSF activities. This is consistent with our results that mutagenesis of the NH_2-terminal serine residues S3, S16, S18, S25, and S26, has no effect on the biological activity of *mos*^{xe}.

This work identifies serine-3 as a major in vivo site of phosphorylation. Serine→alanine mutagenesis at this residue removes both chymotryptic peptides No. 1 and No. 2. That both of these peptides contain serine-3 was shown by secondary digestions with proline-specific peptidase which yielded identical phosphopeptide products. We have not yet identified the phosphorylated serine residue(s) responsible for chymotryptic peptide No. 3 and No. 4. However, it is clear that these phosphopeptides do not arise from phosphorylation at any of the 13 conserved serine residues examined in this work. We would suggest that this additional phosphorylation(s) is not likely to be of biological significance. In this work, we have examined all serine residues conserved in at least three out of the four species of *mos* protein: *Xenopus*, chicken, human, and mouse. Other investigators have demonstrated that *mos* proteins from diverse organisms are biologically active in *Xenopus* oocytes (Yew et al., 1991). Thus the phosphorylation(s) giving rise to chymotryptic peptides No. 3 and No. 4 must be species-specific. If this additional phosphorylation is of biological significance, one would have to argue that a key regulatory site used in *Xenopus mos*^{xe} is not conserved in the other *mos* species even though they are also biologically active when expressed in *Xenopus* oocytes.

On occasional peptide maps, a number of minor chymotryptic phosphopeptides were observed, including the labeled peptide No. 5. The occurrence of minor phosphopeptides is best illustrated in the right hand map shown in Fig. 5. The yield of these minor peptides was variable and also dependent upon excellent recovery of radio-labeled protein throughout the peptide mapping procedure. Because of the failure of proteases, especially chymotrypsin, to cleave with absolute specificity, most of these minor phosphopeptides probably represent partial or secondary digestion products. For instance, several of the minor phosphopeptides are absent in the map of *mos*$^{xe(A3)}$ (Fig. 5, *left panel*) and presumably represent peptides containing serine-3. However, unlikely though it seems, we cannot rule out the possibility that one or more minor phosphopeptides may be biologically significant.

One potential criticism of our experimental approach concerns the fact that endogenous *mos*^{xe} protein would have been expressed and recovered together with any of the 12 biologically active serine→alanine mutant *mos*^{xe} proteins. This could have been avoided by first microinjecting oocytes with an antisense *mos*^{xe} oligonucleotide to inhibit endogenous *mos*^{xe} expression (Sagata et al., 1988; Freeman et al., 1990). For a number of technical reasons, including the large numbers of oocytes required to recover sufficient radiolabeled *mos*^{xe} protein for peptide mapping, we chose not to attempt to eliminate endogenous *mos*^{xe} expression in these experiments. As discussed previously, microinjection of RNA leads to expression of mutant *mos*^{xe} proteins at levels 10–20-fold compared with endogenous *mos*^{xe}. This means that in peptide maps of the biologically active serine→alanine *mos*^{xe} mutants a maximum of 5–10% of the recovered protein may represent endogenous *mos*^{xe} protein. This level of contamination would not affect any of the results reported here.

The phosphorylation of the *mos*^{xe} protein might regulate its ability to induce CSF arrest. Previous studies demonstrated that the half-life of the *mos*^{xe} protein from matured eggs is significantly greater than its half-life in pre-GVBD oocytes (Watanabe et al., 1989). In addition, there is evidence that *mos*^{xe} phosphorylation may be necessary to enhance or preserve CSF activity. During normal maturation, CSF activity is detected only after GVBD, rising in conjunction with the transition into the second meiotic metaphase (Meyerhof and Masui, 1979). In metaphase egg extracts, CSF activity can be enhanced as well as sustained by the addition of phosphate inhibitors and ATP (Shibuya and Masui, 1988; Moses and Masui, 1990). Interestingly, the *mos*^{xe}

protein in CSF extracts is also directly modified by these agents; the abundance of mos^{xe} protein does not change but the apparent molecular mass of the protein increases, presumably because of hyperphosphorylation (Sagata et al., 1989*b*). In the work reported here, we observed a strict correlation between the ability of different serine→alanine mutants to induce oocyte maturation and to function in CSF assays. Taken together, these arguments strongly suggest that phosphorylation is not involved in converting a pre-GVBD CSF-inactive form of mos^{xe} into a post-GVBD CSF-active form. In this regard, it was recently shown that the overexpressed mos^{xe} proteins from pre-GVBD oocytes and from post-GVBD oocytes possess similar CSF activities (Kanki and Donoghue, 1991), although such assays do not address whether the mos^{xe} protein from pre-GVBD oocytes may undergo additional phosphorylation in the injected blastomeres.

In addition to its other activities, it should be noted that *mos* translation is required for progression from meiosis I to meiosis II, both in *Xenopus* oocytes (Kanki and Donoghue, 1991) and murine oocytes (O'Keefe et al., 1989). For technical reasons, the work presented in this study has not addressed whether phosphorylation of *mos* protein (possibly even at serine-3) may be required for its activity to promote progression from meiosis I to meiosis II.

There are five serine-proline motifs present in mos^{xe} that could act as potential sites of $p34^{cdc2}$ phosphorylation, although none of these fit the more rigorous Ser-Pro-X-Lys/Arg consensus sequence (Moreno and Nurse, 1990). Three of these serines (S3, S16, and S26) were mutated in this study and found not to be required for the maturation inducing or CSF activities of mos^{xe}. We have demonstrated that serine-3 is a major phosphorylation site in mos^{xe}. However, it seems unlikely that this residue is phosphorylated in vivo by $p34^{cdc2}$ since phosphorylation of this site occurs in both the $mos^{xe(R90)}$ and $mos^{xe(A105)}$ mutants that fail to induce oocyte maturation or activate MPF.

It has long been a matter of speculation whether phosphorylation may regulate the activity of mos^{xe} with respect to the initiation of meiotic maturation or its ability to function as CSF. Our results indicate that significant phosphorylation at unique sites in the mos^{xe} protein does not occur during maturation and are not likely to be catalyzed by $p34^{cdc2}$. In addition, the major phosphorylations of mos^{xe} can occur in the absence of autophosphorylation. Finally, phosphorylation of conserved serine residues does not appear to regulate the ability of mos^{xe} to induce oocyte maturation or function as CSF.

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