# Induction of Metaphase Arrest in Cleaving *Xenopus* Embryos by MAP Kinase

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The natural arrest of vertebrate unfertilized eggs in second meiotic metaphase results from the activity of cytostatic factor (CSF). The product of the c-*mos*$^{xe}$ proto-oncogene is thought to be a component of CSF and can induce metaphase arrest when injected into blastomeres of two-cell embryos. The c-Mos$^{xe}$ protein can directly activate the mitogen-activated protein kinase kinase (MAP kinase kinase) in vitro, leading to activation of MAP kinase. MAP kinase and c-Mos$^{xe}$ are active in unfertilized eggs and are rapidly inactivated after fertilization. Microinjection of thiophosphorylated MAP kinase into one blastomere of a two-cell embryo induced metaphase arrest similar to that induced by c-Mos$^{xe}$. However, only arrest with c-Mos$^{xe}$ was associated with activation of endogenous MAP kinase. These results indicate that active MAP kinase is a component of CSF in *Xenopus* and suggest that the CSF activity of c-Mos$^{xe}$ is mediated by MAP kinase.

Fully grown *Xenopus laevis* oocytes are naturally arrested at the first meiotic prophase. Exposure to progesterone induces the transition between prophase I and second meiotic metaphase (metaphase II), after which meiosis is again arrested until fertilization. During this transition, known as meiotic maturation, a key event is the protein synthesis–dependent activation of meiosis and mitosis (M phase) promoting factor (MPF), which is followed by germinal vesicle breakdown (GVBD), chromosome condensation, and spindle formation (*1*). MPF—a complex of a serine-threonine kinase, p34$^{cdc2}$, and cyclin B (*2*)—is ubiquitous in eukaryotes, and its activity is required for the transition into M phase in both meiosis and mitosis (*3*).

MPF activity rises before GVBD, falls after metaphase I, and then rises again and remains high during metaphase II arrest in unfertilized eggs (*4*, *5*). Fertilization or parthenogenetic activation releases the metaphase II arrest and induces cyclin degradation and MPF inactivation (*3*). A cytostatic factor (CSF), discovered at the same time as MPF (*5*), is responsible for the metaphase arrest and the stabilization of MPF in unfertilized eggs. At present, the only assay for CSF involves the transfer of cytoplasm from unfertilized eggs into one blastomere of a two-cell embryo, leading to metaphase arrest in the injected blastomere when CSF is present (*5*).

The c-*mos* proto-oncogene encodes a serine-threonine protein kinase that is expressed in large amounts in germ-line tissues and is thought to be a component of CSF. *Xenopus mos* mRNA or a fusion protein between *Escherichia coli* maltose-binding protein and *Xenopus* c-Mos (MBP-Mos$^{xe}$) induces metaphase arrest when injected into a two-cell *Xenopus* embryo (*6*, *7*). Mos$^{xe}$ function in CSF is also suggested by synthesis of Mos$^{xe}$ from maternal mRNA during meiotic maturation in response to progesterone, synthesis that is necessary for progesterone-induced meiosis I (*8*, *9*) as well as for meiosis II and CSF arrest (*7*). Either c-Mos mRNA or protein is able to induce maturation when injected into oocytes without any hormonal stimulation (*7*, *8*), but other newly synthesized components, including cyclin-dependent kinase 2 (Cdk2) (*10*), are also required for the CSF function of c-Mos$^{xe}$.

Mitogen-activated protein kinase (MAP kinase) is one of the protein kinases activated during *Xenopus* oocyte maturation after MPF activation (*11*–*14*). Purified MPF can also activate MAP kinase in cell-free oocyte extracts by an unknown mechanism (*15*). MAP kinase activity remains high in unfertilized eggs, decreases to a basal amount at fertilization, and does not increase again until late in development (*14*). MAP kinase kinase, termed MEK, activates MAP kinase by phosphorylation of threonine and tyrosine residues (*16*), and MEK itself is activated by phosphorylation on serine and threonine residues (*17*) by a kinase known as MEK kinase. Several MEK kinases have been described, including c-Raf, a distinct mammalian MEK kinase, and a *Xenopus* MEK kinase (*18*). MBP-Mos$^{xe}$ also directly activates MEK in vitro (*19*), and MAP kinase is activated by MBP-Mos$^{xe}$ without any activation of MPF in *Xenopus* oocyte extracts (*20*).

These results suggest that MAP kinase

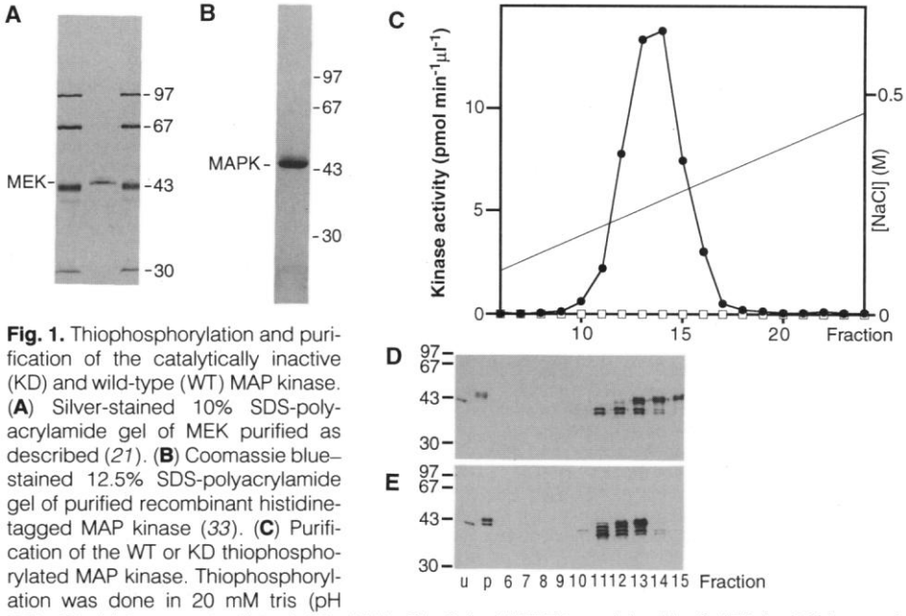


**Fig. 1.** Thiophosphorylation and purification of the catalytically inactive (KD) and wild-type (WT) MAP kinase. (**A**) Silver-stained 10% SDS-polyacrylamide gel of MEK purified as described (*21*). (**B**) Coomassie blue–stained 12.5% SDS-polyacrylamide gel of purified recombinant histidine-tagged MAP kinase (*33*). (**C**) Purification of the WT or KD thiophosphorylated MAP kinase. Thiophosphorylation was done in 20 mM tris (pH 7.5), 15 mM β-mercaptoethanol, 20 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM γ-S-ATP for 3.5 hours at 30°C. Thiophosphorylated WT (●) or KD MAP kinase (□) were concentrated and separated from the γ-S-ATP and MEK by fractionation on a Pharmacia Smart System Mono Q anion-exchange column (PC 1.6/5) with a gradient of 0 to 500 mM NaCl in 20 mM tris (pH 7.5), 15 mM β-mercaptoethanol, 7.5 mM $MgCl_2$, and 0.1 mM EGTA. The kinase activity of the fractions was measured (*24*). (**D** and **E**) Immunoblots of the fractions with an affinity-purified antibody to a COOH-terminal peptide of *Xenopus* MAP kinase (CELIFEETARFQPGY). (D) WT MAP kinase. (E) KD MAP kinase. Abbreviations: u, unphosphorylated MAP kinase; p, thiophosphorylated MAP kinase before chromatographic fractionation. Molecular size markers (in kilodaltons) are indicated on the left. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; E, Glu; F, Phe; G, Gly; I, Ile; L, Leu; P, Pro; Q, Gln; R, Arg; T, Thr; and Y, Tyr.

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may mediate some of the actions of c-Mos$^{xe}$, and therefore we investigated the role of MAP kinase in the induction of CSF arrest. Purified MAP kinase from unfertilized eggs (*13*) did not induce metaphase arrest when injected into two-cell embryos. However, MAP kinase is dephosphorylated and inactivated by specific phosphatases that are active after fertilization (*14, 21*). Because thiophosphorylated proteins are generally resistant to dephosphorylation by protein phosphatases (*22*), we thiophosphorylated bacterially expressed, histidine-tagged *Xenopus* MAP kinase (Fig. 1B) with MEK purified from unfertilized eggs (Fig. 1A). The thiophosphorylated MAP kinase was then separated from unreacted adenosine-5′-O-(3-thiotriphosphate) (γ-S-ATP) and MEK and concentrated (Fig. 1C). This purification and concentration proved to be essential for achieving consistent results and the viability of injected embryos. As a control, a catalytically inactive (kinase-dead) Lys$^{57}$ to Met mutant of the histidine-tagged MAP kinase was also prepared and concentrated by the same protocol.

Thiophosphorylation of either the wild-type (WT) or the kinase-dead (KD) MAP kinase decreased MAP kinase electrophoretic mobility by about 2 kD (Fig. 1, D and E), as shown for orthophosphorylated MAP kinase (*23*). The thiophosphorylated form of MAP kinase was partially separated from the unphosphorylated form of MAP kinase during chromatography on a Mono Q column (Fig. 1, D and E). The activity of both WT and KD MAP kinase (Fig. 1C) was monitored with epidermal growth factor (EGF) receptor peptide as a substrate (*24*). The thiophosphorylation of WT MAP kinase by MEK resulted in the activation of MAP kinase activity, whereas no MAP kinase activity was detected after thiophosphorylation of the KD MAP kinase (Fig. 1C).

Injection of the thiophosphorylated WT MAP kinase into one blastomere of a two-cell embryo induced cleavage arrest of the injected blastomere (Fig. 2, A and D), whereas the thiophosphorylated KD MAP kinase did not induce arrest (Fig. 2, C and F). We also injected the unphosphorylated forms of the bacterially expressed WT and KD MAP kinase, which were both catalytically inactive; neither induced cleavage arrest even when the amount of protein injected was ten or twenty times higher than the effective amount of thiophosphorylated MAP kinase. Thus, the embryonic-cleavage arrest induced by MAP kinase is dependent on its protein kinase activity and not on some other property. In these experiments we injected into one blastomere 50 nl of thiophosphorylated MAP kinase that expressed a total activity of 0.66 pmol/min. This amount of enzyme (~5 ng) expressed an activity equivalent to one-tenth or less of that measured in one unfertilized egg, in which all the endogenous MAP kinase is fully active. As a positive control, we injected the MBP-Mos$^{xe}$ protein (*25*), which also induced arrest (Table 1 and Fig. 2, B and E) (*7*). Cytological analysis of serial sections of arrested blastomeres injected with thiophosphorylated WT MAP kinase revealed a prominent spindle arrested at metaphase (Fig. 2G).

To determine whether MPF activity was increased in arrested blastomeres after injection of thiophosphorylated WT MAP kinase, we transferred into resting oocytes cytoplasm taken from the arrested and nonarrested sides of the embryos. In several experiments, a mean of 61 ± 5.7% of the oocytes underwent GVBD within 3 hours after the transfer of cytoplasm from the arrested blastomere; GVBD was not induced by the transfer of cytoplasm taken from the nonarrested blastomere. Control experiments demonstrated that injection of thiophosphorylated WT MAP kinase into oocytes at a concentration sufficient to induce CSF arrest did not induce GVBD. Therefore, the arrested blastomere contained high MPF activity, indicating that cleavage arrest induced by thiophosphorylated WT MAP kinase occurs in M phase. These data show that MAP kinase, like the c-Mos$^{xe}$ or Ras oncoproteins (*26*), has CSF activity. Ras also stimulates MAP kinase and MEK in oocytes and eggs (*27*), and its actions are at least partially dependent on c-Mos$^{xe}$ (*28*). Thus, MAP kinase appears to function downstream of proteins that exhibit CSF activity.

To examine the state of phosphorylation of endogenous MAP kinase in the arrested embryos, we dissected blastomeres from the arrested and nonarrested sides of the embryo. The dissection was performed

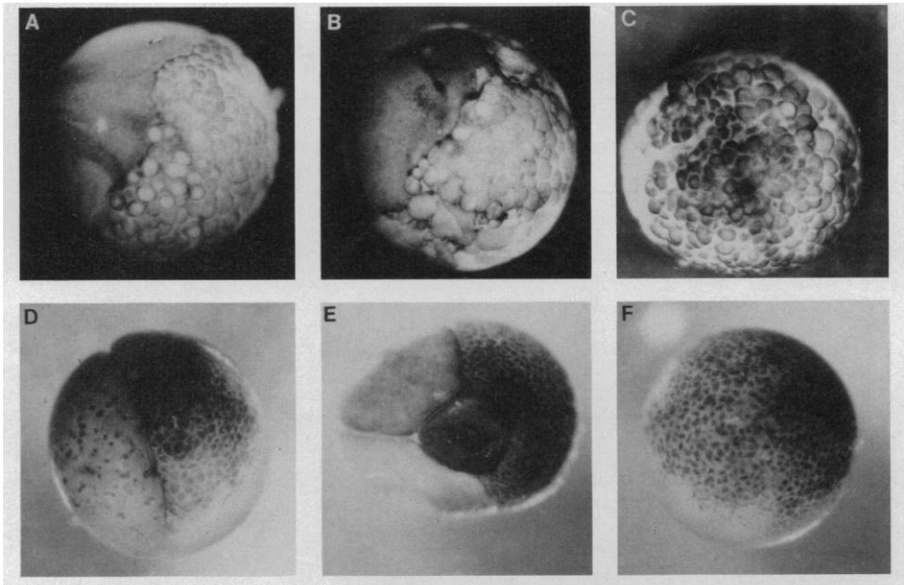


**Fig. 2.** CSF arrest in *Xenopus* embryos injected with thiophosphorylated WT MAP kinase or MBP-Mos$^{xe}$. (**A** and **D**) Embryos with arrested and nonarrested blastomeres after injection of 50 nl of thiophosphorylated WT MAP kinase into one of the blastomeres at the two-cell stage. (**B** and **E**) Embryos with arrested and nonarrested blastomeres at two-cell and four-cell stages, respectively, after injection of 50 nl of MBP-Mos$^{xe}$ into one of the blastomeres at the two-cell stage. (**C** and **F**) Control embryos after injection of 50 nl of thiophosphorylated KD MAP kinase into one of the blastomeres at the two-cell stage. Stage 7 (4 hours) (A to C) and mid–stage 8 (7 hours) (D to F) embryos were injected at the two-cell stage in two different experiments and cultured for 4 to 6 hours at 20°C in MMR [100 mM NaCl, 8 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 5 mM Hepes (pH 7.8)] containing 2% Ficoll before being fixed in Perenyi's fluid (6% $HNO_3$, 0.15% $Cr_2O_7$, and 29% ethanol). (**G**) Embryos injected with thiophosphorylated WT MAP kinase were fixed in Perenyi's fluid, embedded in paraffin, sectioned at 7 μm, stained with Mayer's hemalum, and counterstained with light green. The bar represents 3 μm.

after removal of the vitelline membrane in a buffer containing protease inhibitors and phosphatase inhibitors. The arrested blastomere and the nonarrested side of the embryo were then homogenized, and MAP kinase in each sample was analyzed by protein immunoblotting. This technique distinguishes activated and nonactivated MAP kinase by revealing electrophoretic shifts that accompany phosphorylation and activation (*13, 23*). In blastomeres arrested after the injection of MBP-Mos$^{xe}$, 100% of the MAP kinase appeared in the shifted upper form (Fig. 3), which migrated with the same mobility as active MAP kinase from unfertilized eggs (Fig. 3). In the other half of the embryo, composed of nonarrested cells, endogenous MAP kinase was found in its lower, inactive form (Fig. 3). These results show that the injection of MBP-Mos$^{xe}$ induced the in vivo phosphorylation of endogenous MAP kinase in the arrested blastomeres (*19*).

In blastomeres arrested after injection of thiophosphorylated WT MAP kinase, the endogenous MAP kinase did not become phosphorylated (Fig. 3), and migrated with the same mobility as in resting oocytes or in the nonarrested halves of embryos injected with thiophosphorylated KD MAP kinase (Fig. 3). This implies that the exogenous MAP kinase is sufficient to induce CSF arrest and does not act through activation of the endogenous enzyme, suggesting that MEK (or MEKs) and c-Mos$^{xe}$ are not active. Because active endogenous MAP kinase is present during meiotic metaphase but not during mitotic metaphase (*13, 14*), we propose that the CSF arrest induced by c-Mos$^{xe}$ is mediated by the activation of MAP kinase. These results also provide a rationale for the deactivation of MAP kinase and c-Mos$^{xe}$ that invariably occurs after fertilization in *Xenopus*. The CSF activity of Ras (*26*) may also be explained at least in part by activation of Mos$^{xe}$ and MAP kinase (*28*). However, Mos$^{xe}$ may also lead to activation of Raf-1 (*29*), which can function as a MEK kinase as well (*18*). Although our data indicate that MAP kinase is a component of CSF (Fig. 4), the enzyme is also active during meiosis I (*13, 14*). MAP kinase may not be sufficient for metaphase arrest at meiosis I because of a need for additional components to form active CSF. For example, Cdk2 is active at metaphase II and at mitosis but not at metaphase I (*10*), and ablation of Cdk2 prevents CSF arrest in meiosis II even in the presence of active c-Mos$^{xe}$ (*10*). This may explain why metaphase arrest does not occur at meiosis I despite the presence of high c-Mos$^{xe}$ and MAP kinase activity. Thiophosphorylated WT MAP kinase was unable to cause GVBD when injected into oocytes at a concentration sufficient to induce CSF arrest, even though injection of MBP-Mos$^{xe}$ could (*7*). Similarly, addition of c-Mos$^{xe}$ to oocyte extracts activates MAP kinase but not Cdc2 (*20*). This suggests that the ability of c-Mos$^{xe}$ to cause entry into meiosis I is dependent on additional components. The target or targets of MAP kinase that produce CSF arrest are unknown, but MAP kinase affects spindle dynamics in egg extracts (*11, 30*) and also activates other protein kinases such as pp90$^{rsk}$ and MAPKAP kinase-2 (*13, 31*). MAP kinase is involved in a variety of signaling pathways during interphase in many systems; participation in CSF activity is an important physiolog-

**Table 1.** Injection of thiophosphorylated MAP kinase and MBP-Mos$^{xe}$ into two-cell stage blastomeres. In each experiment, injected embryos were derived from eggs laid by one frog. One blastomere of a two-cell stage embryo was injected with 50 nl of either thiophosphorylated KD MAP kinase, thiophosphorylated WT MAP kinase, or MBP-Mos$^{xe}$. The average percentage of cleavage arrest (mean ± SEM) for cells injected with thiophosphorylated KD MAP kinase, thiophosphorylated WT MAP kinase, or MBP-Mos$^{xe}$ were 5.5 ± 2.5%, 63 ± 7%, and 65 ± 12%, respectively.

| Experiment | KD MAP kinase | | WT MAP kinase | | MBP-Mos$^{xe}$ | |
|---|---|---|---|---|---|---|
| | Cleavage arrest (%) | No. of injected cells | Cleavage arrest (%) | No. of injected cells | Cleavage arrest (%) | No. of injected cells |
| 1 | 0 | 17 | 55 | 40 | 26 | 15 |
| 2 | 0 | 14 | 87 | 23 | 72 | 22 |
| 3 | — | — | 79 | 39 | — | — |
| 4 | 13 | 15 | 36 | 22 | — | — |
| 5 | 10 | 49 | 51 | 47 | — | — |
| 6 | 0 | 25 | 52 | 25 | 57 | 14 |
| 7 | 10 | 39 | 59 | 54 | 71 | 62 |
| 8 | — | — | 88 | 9 | — | — |
| 9 | — | — | — | — | 100 | 9 |

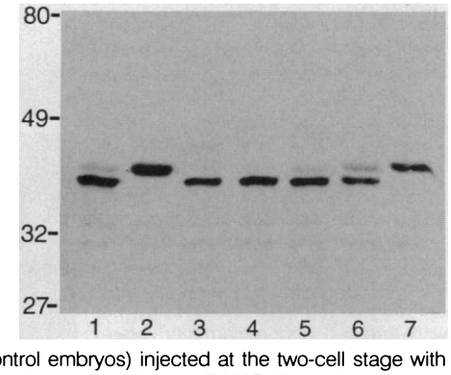


**Fig. 3.** Immunoblot of MAP kinase in *Xenopus* embryos injected with thiophosphorylated WT or KD MAP kinase, or MBP-Mos$^{xe}$. Fully grown oocytes (lane 1) or eggs (lane 2) were homogenized in 5 volumes of EB [80 mM β-glycerophosphate (pH 7.4), 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 100 μM sodium orthovanadate, 10 mM NaF, pepstatin A (10 μg/ml), leupeptin (10 μg/ml), and chymostatin (10 μg/ml)] and centrifuged for 5 min in Eppendorf tubes. From the supernatant the equivalent of 3/10 of an oocyte was subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel and immunoblotted with a polyclonal antibody to the COOH-terminus of MAP kinase. Stage 5 embryos (16 cells in the control embryos) injected at the two-cell stage with either thiophosphorylated KD MAP kinase (lane 3), thiophosphorylated WT MAP kinase (lanes 4 and 5), or MBP-Mos$^{xe}$ (lanes 6 and 7) were dissected into two parts in EB buffer after removal of the vitelline membrane. The dissected embryo halves were then homogenized in 5 μl of EB buffer per half as described for the oocytes and eggs. An equivalent amount of cytosolic extract was electrophoresed on each lane of a 12.5% SDS-polyacrylamide gel. Lane 3, the injected half of embryos injected at the two-cell stage with thiophosphorylated KD MAP kinase; lane 4, non-injected, nonarrested halves of embryos injected at the two-cell stage with thiophosphorylated WT MAP kinase; lane 5, arrested halves of embryos injected at the two-cell stage with thiophosphorylated WT MAP kinase; lane 6, noninjected halves of embryos injected at the two-cell stage with MBP-Mos$^{xe}$; lane 7, arrested halves of embryos injected at the two-cell stage with MBP-Mos$^{xe}$. In lanes 3 and 5 the amount of exogenous MAP kinase (5 ng) in the samples was too low to be detected at this exposure of the immunoblot. Prestained molecular size markers (in kilodaltons) are indicated on the left.

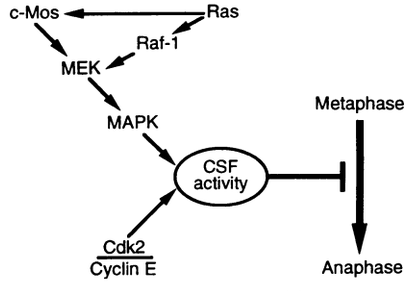


**Fig. 4.** Model for mechanism of CSF arrest. The model proposes that CSF activity of both Ras and c-Mos$^{xe}$ is mediated by the activation of MAP kinase, which together with active Cdk2 forms CSF and blocks the metaphase to anaphase transition in unfertilized eggs.

ical function of this enzyme during M phase.

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24. MAP kinase activity was assayed for 15 min at 30°C in 20 mM Hepes (pH 7.5), 2 mM EGTA, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), bovine serum albumin (0.1 mg/ml), 100 μM [$\gamma$-$^{32}$P]adenosine triphosphate (ATP) (1 to 5 cpm/fmol), with 200 μM EGF receptor peptide as substrate.
25. The pTZXA$^+$ plasmid (*7*) contained c-*mos*$^{xe}$ cDNA in pTZ18R vector (Pharmacia). A 1.4-kilobase Eco RI–Hind III fragment containing the full-length c-*mos*$^{xe}$ was subcloned into the Eco RI and Hind III sites of pMAL-cRI (New England Biolabs) and used to transform *E. coli* NM522 to produce the 77-kD maltose-binding protein fused to c-Mos$^{xe}$ (MBP-Mos$^{xe}$).
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33. *Xenopus laevis* MAP kinase cDNA (*32*) was inserted into the pRSET vector (by L. E. Heasley and G. L. Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) and expressed in BL21(DE3) as described (*21*). The eluate was dialyzed against 20 mM Hepes (pH 7), 1 mM EGTA, 1 mM EDTA, 15 mM β-mercaptoethanol, 1 mM benzamidine, and 500 mM NaCl, then loaded onto a FPLC phenyl Superose HR 5/5 and eluted with a 24-ml gradient of 0 to 60% ethylene glycol and 500 to 0 mM NaCl.


34. We thank B. Lattes for technical assistance in the preparation of MBP-Mos$^{xe}$, G. Vande Woude (NCI) for the pTZXA$^+$ plasmid, and J. Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA) for *X. laevis* MAP kinase cDNA. Supported by a grant from the NIH (GM26743) and by a grant to the Molecular Biology Program from the Lucille P. Markey Charitable Trust. L.M.R. is a recipient of a National Research Service Award (F32CA0981) and J.L.M. is an Investigator of the Howard Hughes Medical Institute.

2 August 1993; accepted 14 September 1993


# Requirement of Tyrosine Phosphorylation for Rapid Activation of a DNA Binding Factor by IL-4


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**Interleukin-4 (IL-4) is an immunoregulatory cytokine produced by activated T lymphocytes to promote the growth and differentiation of cells that participate in immune defense. This study demonstrates the rapid activation of a specific DNA binding factor by IL-4. The IL-4 nuclear-activated factor (IL-4 NAF) appeared within minutes of IL-4 stimulation and recognized a specific DNA sequence found in the promoters of IL-4–responsive genes. Activation of this putative transcription factor required tyrosine phosphorylation, and antibodies specific for phosphotyrosine recognize the IL-4 NAF–DNA complex. Thus, IL-4 appears to transduce a signal to the nucleus through tyrosine phosphorylation of a latent DNA binding factor.**


Interleukin-4 (IL-4) is a T cell lymphokine produced in response to mitogen or antigen stimulation (*1*). Initially discovered by its proliferative effect on B lymphocytes, it is now recognized to function in the survival, growth, and differentiation of T lymphocytes and other cells of the hematopoietic lineage and induces the expression of genes that function in immune recognition. In B lymphocytes, IL-4 activates transcription of the constant region of the immunoglobulin heavy chain genes (murine $C_\gamma1$ and $C_\epsilon$ and human $C_\gamma4$ and $C_\epsilon$), promoting isotype class switching (*2*, *3*). IL-4 also induces transcription of the major histocompatibility class II (MHCII) genes (*4*) and a gene encoding a receptor for immunoglobulin E (IgE), FcεRII (*5*, *6*).

Interleukin-4 initiates these biological responses by binding to a specific cell surface receptor. The gene encoding the human IL-4 receptor has been isolated but does not appear to encode a receptor with intrinsic enzymatic activity (*7*). Although little is known of the IL-4 signal transduction pathway, studies have described the activation of phosphatidylinositol-3 kinase after IL-4 stimulation and provided evidence for the activation of both a tyrosine kinase and phosphatase (*8*, *9*). Here, we describe the tyrosine kinase–dependent activation of a putative transcription factor by IL-4.

Several of the biological effects of IL-4 are antagonized by another T cell lymphokine, interferon-γ (IFN-γ). Interferon-γ inhibits the immunoglobulin class switch to IgG1 and IgE induced by IL-4 (*10*) and also suppresses IL-4 induction of MHCII genes and the FcεRII gene in B cells (*11*). However, in macrophages both IFN-γ and IL-4 can induce the expression of MHCII genes (*12*). Therefore, IFN-γ and IL-4 can have either antagonistic or analogous effects on transcription. For this reason, we examined the ability of IL-4 to activate a DNA binding factor that could recognize an IFN-γ–stimulated response element.

To determine if IL-4 activates a transcription factor with DNA binding specificity for an IFN-γ response element, we analyzed electrophoretic mobility-shift assays (EMSAs) (Fig. 1). Nuclear extracts were prepared from human monocytic cells treated with IL-4, IFN-γ, or IL-4 and IFN-γ and were incubated with DNA corresponding to the IFN-γ response region of the FcγRI gene


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