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The *ras* Oncoprotein and M-Phase Activity

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The endogenous *mos* proto-oncogene product (Mos) is required for meiotic maturation. In *Xenopus* oocytes, the *ras* oncogene product (Ras) can induce meiotic maturation and high levels of M-phase–promoting factor (MPF) independent of endogenous Mos, indicating that a parallel pathway to metaphase exists. In addition, Ras, like Mos and cytostatic factor, can arrest *Xenopus* embryonic cell cleavage in mitosis and maintain high levels of MPF. Thus, in the *Xenopus* oocyte and embryo systems Ras functions in the M phase of the cell cycle. The embryonic cleavage arrest assay is a rapid and sensitive test for Ras function.

IN THE *XENOPUS LAEVIS* SYSTEM, FULLY grown oocytes are arrested in prophase of the first meiotic division. Progesterone releases this arrest, resulting in activation of MPF, germinal vesicle breakdown (GVBD), completion of meiosis I, and production of an unfertilized egg arrested at metaphase II of meiosis (*1*). MPF is composed of the *Xenopus* homolog of the cell cycle regulator $p34^{cdc2}$ and cyclin (*2*) and is present at high levels in unfertilized eggs (*1*). Cytostatic factor (CSF) is also found in unfertilized eggs and is believed to be responsible for the arrest of maturation at metaphase II of meiosis (*1, 3*). Mos has been shown to be an active component of CSF (*4*), and introduction of CSF or Mos into the blastomeres of rapidly cleaving embryos arrests cleavage at metaphase of mitosis (*1, 3, 4*). This arrest by CSF or Mos, at a major cell cycle control point (*5*), results from the stabilization of high levels of MPF (*3, 4, 6,* 7).

The unrestricted proliferation of cells transformed by oncogenes provides a strong argument that proto-oncogenes normally function in the regulation of the cell cycle (*8*). Research emphasis has been directed toward understanding how oncogenes alter the regulation of signal transduction events in the G_0 to G_1 phase of the cell cycle (*9*). The discovery that Mos functions during M phase (*4, 10*) led us to propose that the transforming activity of the Mos in somatic cells is due to the expression of its M-phase activity during interphase (*4, 10, 11*). A similar hypothesis has been presented for the *src*-transforming activity (*12*), and this may be a more general mechanism for how certain oncogenes induce morphological transformation (*4, 10, 11*).

Ras, the transforming guanosine triphosphate (GTP)–binding protein (*13*), and Mos induce progesterone-independent meiotic maturation in *Xenopus* oocytes (*11, 14–17*) (Table 1). We tested Ras in this assay by injecting either Ras^{Lys12} or $H\text{-}Ras^{Val12}$ RNA. Injected oocytes were subsequently examined for GVBD and MPF activity (*18*). Cytosolic extracts prepared from oocytes induced to mature with these products were positive for MPF, indicating that the oocytes were arrested in metaphase (Table 1). In addition, these analyses confirm that Ras (*19*), like Mos, can sustain high levels of MPF after GVBD (Table 1).

In fully grown *Xenopus* oocytes, antisense oligodeoxyribonucleotides destabilize the *mos* maternal mRNA and block progesterone-induced meiotic maturation (*10, 15*). To test whether Ras could induce meiotic maturation in the absence of progesterone and endogenous *mos* mRNA, we injected *mos*-specific antisense or sense oligodeoxyribonucleotides (*10*) into oocytes 3.5 to 4 hours before injecting the test material and subsequently examined them for GVBD and MPF activity (Table 1). GVBD occurred frequently in Mos-negative oocytes injected with Ras (60%), and extracts prepared from oocytes that displayed GVBD were positive for MPF activity (Table 1). Barrett and co-workers have shown that Mos depletion inhibits Ras-induced maturation (*15*). Allende and co-workers reported that Ras can induce GVBD in cycloheximide-treated oocytes (*16*), and Barrett and co-workers also observed this on occasion (*15*). These latter results are more consistent with our data because Mos is not synthesized in oocytes in the presence of cycloheximide (*11, 20*). Moreover, Ras-induced oocyte maturation appears to be Mos-dependent in less mature Dumont stage V (*21*) oocytes, but not in fully grown stage VI oocytes (*22*), presumably because of metabolic changes during oogenesis.

Because Ras induces meiotic maturation and high levels of MPF in oocytes, we tested whether it influences M-phase events in cleaving embryos, where the cell cycle consists essentially of S and M phases. Ras efficiently arrested embryonic cleavage when one blastomere of each two-cell embryo was injected with either oncogenic Ras protein or RNA (Figs. 1 and 2). This cleavage arrest mimics the arrest caused by CSF or Mos (*4*). Moreover, as little as 1 to 2 ng of Ras could induce the cleavage arrest, which was observable within a few hours (Fig. 2).

Although transforming Ras induced the cessation of embryonic cleavage, both normal and nontransforming mutant forms of Ras had no observable effect on cleavage, even when introduced at concentrations approximately ten times the minimum effective dose for the transforming Ras. Thus, 15 ng of either normal Ras or $Ras^{Lys12Ser186}$, a protein that cannot associate with the plas-

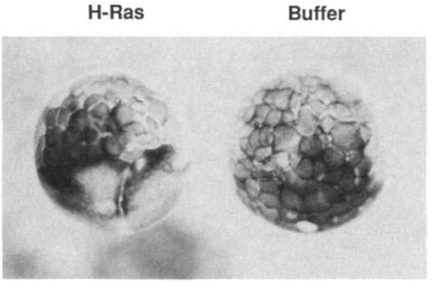

Fig. 1. Morphology of embryos injected with $H\text{-}Ras^{Val12}$ RNA. Animal-pole view of embryos injected with either capped $H\text{-}Ras^{Val12}$ RNA (*18*) or buffer. The RNA or buffer was microinjected into one blastomere (bottom half) of a two-cell embryo and examined several hours later.

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Table 1. Influence of Ras on oocyte maturation in the presence or absence of Mos. *Xenopus laevis* females were obtained from *Xenopus* I (Ann Arbor, Michigan). Oocytes were removed from the surrounding follicle tissue by the addition of MBS containing collagenase A (2 mg/ml; Boehringer Mannheim) (*38*) and incubated for 2 hours. The oocytes were washed extensively with MBS, and stage VI (*21*) oocytes were removed and allowed to recover overnight. We microinjected groups of 10 to 30 oocytes using an Attocyte injector (ATTO Instruments, Rockville, Maryland) with 40 nl of the appropriate reagent diluted to the desired concentration in 88 mM NaCl and 15 mM tris (pH 7.5). In the cases where *mos* sense or antisense oligodeoxyribonucleotides [described as A to D by Sagata and co-workers (*10*)] were used in injections, oocytes were cultured for 3.5 to 4 hours before the second indicated treatment or injection. GVBD was determined 14 to 18 hours later by the appearance of a white spot at the animal pole. In addition, all oocytes were soaked in 10% trichloroacetic acid for 10 min, then dissected and examined under a binocular microscope for the presence or absence of the germinal vesicle. Oocytes were scored for GVBD 14 to 18 hours later. Where indicated, MPF activity was tested (*18*). (+) denotes where activity was found, (−) where none was observed, and (n.d.) where activity was not determined. Amounts are given in nanograms per oocyte.

Treatment or injection	Amount	Pre-treatment*	Assays	Injected oocytes	GVBD ± SD (%)	MPF activity
Progesterone		S	12	125	83 ± 14	+
		AS	12	145	11 ± 7	−
H-*ras*^{Val12} RNA	1	—	2	20	60	n.d.
	5	—	2	23	96	n.d.
	10	B	8	80	93	+
	10	S	8	130	86 ± 15	+
	10	AS	8	130	60 ± 20	+†
H-Ras^{Lys12}	15	B	8	80	90	+
	15	S	8	194	88 ± 8	+
	15	AS	8	202	53 ± 22	+†
c-*mos*^{xe} RNA	1	—	1	20	5	n.d.
	50	—	1	10	100	+

*B (buffer); S (sense) or AS (antisense) oligodeoxyribonucleotides; 120 ng of oligodeoxyribonucleotides were injected per oocyte; — (no pretreatment). †Only oocytes displaying GVBD were used in the MPF assay.

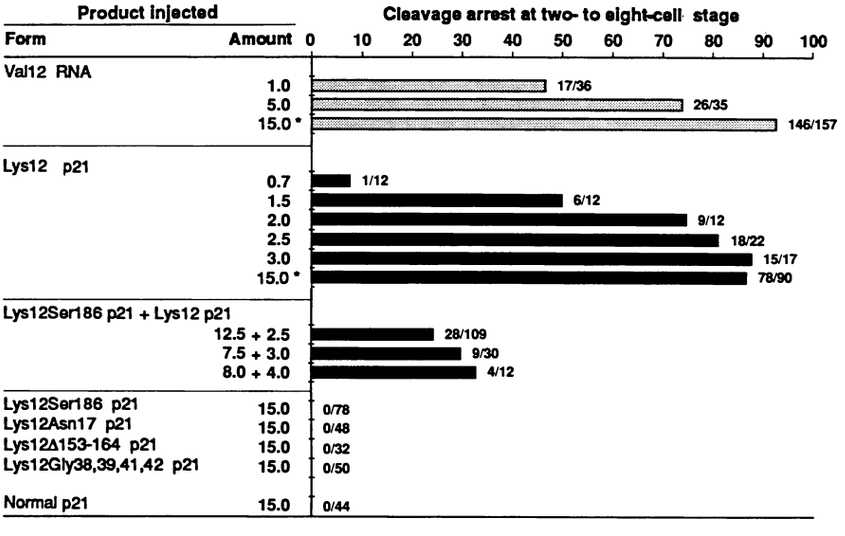

Fig. 2. Induction of cleavage arrest by injected RNA or Ras protein (indicated as p21). Ovulated eggs were obtained and fertilized in vitro (*15*). The fertilized eggs were dejellied on 0.3× modified Ringer's solution (MR) containing 2% cysteine (pH 7.9) (*3*) then washed and placed in 0.3× MR for 1.5 hours at 21°C. The two-cell embryos were microinjected with 30 nl of solution containing the appropriate RNA or protein and incubated several hours longer in 0.3× MR containing 5% Ficoll 400 (Pharmacia). The few injected blastomeres that ceased cleavage with irregular pigment patterns were omitted from the tabulated data. The fractions at the end of each histogram bar represent the number of embryos arrested in cleavage over the number of embryos injected. Crude MPF extracts were prepared (*18*) from groups of ten embryos 5 to 6 hours after they had been injected with the indicated solutions, as described in Table 1. These extracts were tested for MPF activity (*18*). Amounts are given in nanograms of Ras product injected per cell. Asterisks indicate that 100% of more than 60 cycloheximide-treated recipient oocytes displayed GVBD after microinjection of embryonic extract.

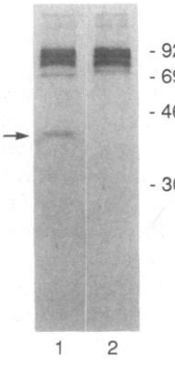

Fig. 3. Lack of Mos in H-Ras^{Val12}–arrested embryos. Forty embryos at the two-cell stage, prepared as described in Fig. 2, were injected in both blastomeres with a mixture of either 0.25 μCi of ^{35}S-labeled cysteine plus 0.3 ng of capped c-Mos^{xe} transcripts (lane 1) (*18*) or 0.25 μCi of [^{35}S]-labeled cysteine plus 10 ng of capped H-Ras^{Val12} transcripts (lane 2) (*18*). Embryos were cultured for 3 hours, then extracts were prepared and analyzed by immunoprecipitation with a monoclonal antibody to *Xenopus* c-Mos product expressed in *Escherichia coli* as described (*10*). Molecular size markers (in kilodaltons) are represented on the right. Arrow indicates the position of pp39^{Mos}.

ma membrane (*23*), had no effect on the division of embryonic cells (Fig. 2). Likewise, the injection of a dominant negative mutant with a preferential affinity for guanosine diphosphate, Ras^{Lys12Asn17} (*24*), was ineffective at ceasing cell division, as was Ras$^{Lys12\Delta153\text{-}164}$ (Fig. 2), which is defective in GTP binding (*25*). In addition, Ras that harbors glycine substitutions at positions 37, 38, 41, and 42 in the guanosine triphosphatase activating protein (GAP)–binding and effector domain (*26*) did not exert influence on the embryonic cell cycle (Fig. 2). To eliminate the possibility that arrest of embryonic cell division was due to some toxic effect of the *ras* oncoprotein, we co-injected two- to fivefold excess of the cytosollocalized Ras mutant, Ras^{Lys12Ser186}, along with Ras^{Lys12}. In these experiments, Ras^{Lys12Ser186} acted as a competitive inhibitor similar to the cytosolic mutant form of yeast RAS1^{Leu68} (*27*) and suppressed the Ras-induced cleavage arrest (Fig. 2). Thus, only Ras that displays oncogenic activity can cause embryonic cleavage arrest.

Extracts from both Mos and Ras-arrested embryos exhibited high levels of MPF, as assayed in cycloheximide-treated oocytes (Fig. 2). Moreover, extracts from embryos arrested by either Ras or authentic CSF had equally high levels of MPF-associated histone H1 kinase activity when compared to the amount detected in extracts from control-activated eggs (*28*). Thus, Ras can arrest cleaving embryos in mitosis, as evidenced by the presence of high levels of MPF and the associated histone H1 kinase activity. This biological activity of Ras provides an assay for oncogenic potential that can be performed in a few hours and

does not rely on the further morphological alteration of an already immortalized cell.

The above results raise the question of whether Mos is required for the CSF-like activity. Even though Mos is not always required for Ras-induced meiotic maturation (Table 1), it is routinely synthesized (*29*). Because endogenous *mos* RNA is present through the late blastula stage (*10*) and could be translated during mitosis, we examined embryos arrested in cleavage by Ras for Mos expression. H-Ras^{Val12} RNA transcripts were co-injected with ^{35}S-labeled cysteine into both blastomeres of two-cell embryos and compared to blastomeres injected with 0.3 ng of *mos* RNA, an amount too low to display CSF activity (*4*). After 3 hours, when cleavage arrest was visible in Ras-injected blastomeres, extracts were subjected to immunoprecipitation analyses with a *Xenopus* Mos-specific monoclonal antibody (*11*). Radioactively labeled pp39 Mos was detected only in the *mos* RNA-injected embryos, not in embryos arrested by Ras (Fig. 3). This suggests that Mos does not participate in the Ras-induced arrest.

Our studies identify an activity for Ras that links its function to the M phase of the cell cycle. Moreover, cleavage arrest is a rapid assay for Ras oncogenic potential. The rise in MPF activity at the end of interphase is responsible for entry into mitosis, whereas its decline allows entry into the next interphase (*5*). Ras can induce meiosis or arrest embryonic cells in mitosis and therefore must directly or indirectly influence M-phase events. It is possible that the pathological effect of Ras is manifested through its M-phase activity. Although it is known that insulin-induced meiotic maturation occurs through a pathway requiring endogenous Ras as well as Mos function (*10, 19, 30*), oncogenic Ras, in fully grown stage VI oocytes, can induce maturation through a Mos-independent pathway (Table 1). The high levels of MPF observed in the mature oocytes or in the Ras-arrested blastomeres are consistent with an arrest in metaphase.

CSF activity induced by Mos or Ras raises the question of how embryonic cleavage arrest relates to transformation of somatic cells. Cells acutely infected with Moloney murine sarcoma virus express high levels of Mos (*31*), subsequently round up, and then detach from the monolayer (*32*). This morphological alteration is reminiscent of the mitotic phenotype and could be an effect of CSF or Mos activity (*4*). We have proposed that the selection for the Mos-transformed phenotype is a selection for cells expressing levels of Mos that are ample for transformation but insufficient for CSF arrest (*4*). Ras has been reported to induce growth arrest at G_2 (*33*) or G_2/M (*34*) when overexpressed in either REF52 (rat embryo fibroblast) or primary Schwann cells, respectively. Durkin and Whitfield (*35*) have shown that in NRK cells, Ki-*ras* p21 promotes G_2/M transition in serum-free medium. High levels of Ras expression increase the rate of abnormal mitosis in NIH 3T3 cells (*36*).

Our data show that activated Ras can induce oocyte maturation with either Mos-dependent or -independent pathways. Masui and co-workers have described a secondary CSF activity (*37*) that develops after the primary CSF or Mos activity has been inactivated (*4, 20*), indicating that parallel pathways exist. Transforming Ras exhibits CSF-like activity in embryos without the assistance of Mos. CSF may mediate cell cycle arrest through a feedback mechanism that stabilizes high levels of MPF (*5*). Presently, we do not know whether oncogenic Ras functions in M phase by inducing MPF activity or whether it stabilizes MPF activity by functioning through a feedback control mechanism that prevents MPF degradation.

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18. Cloned *Xenopus mos* was inserted into the Sac I restriction site of a modified pTZ18 vector that has a polyA tail. The H-*ras*^{Val12} cDNA was ligated into the Sal I and Bam HI restriction sites of the SP64 vector (Promega). All RNAs were capped and transcribed by the method recommended by the supplier (Stratagene) with either T7 or SP6 RNA polymerase. The Ras^{Lys12} p21 proteins were purified as described (*38*). Crude MPF extracts were prepared as described (*11*). Briefly, groups of 10 to 20 oocytes were homogenized in 20 to 40 μl of MPF extract buffer [80 mM sodium β-glycerophosphate (Sigma), 20 mM EGTA, 15 mM $MgCl_2$, 20 mM Hepes (pH 7.2), 1 mM adenosine triphosphate (ATP) (Boehringer Mannheim), and 5 mM NaF]. The homogenate was centrifuged at 16,000*g* for 5 min at 4°C, and the supernatant was used for microinjections. Groups of 10 to 20 oocytes were incubated in modified Barth solution (MBS) (*39*) containing cycloheximide (10 μg/ml; Sigma) for 1 hour and then injected with 40 nl of the supernatant from each appropriate donor group. After 2 to 3 hours of culturing the oocytes in the presence of cycloheximide, we examined recipient oocytes for GVBD.
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28. We performed H1 kinase assays by transferring 2 μl of extracts into 50 μl of stabilization buffer [80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM phenylmethyl-sulfonyl fluoride, leupeptin (10 μg/ml), aprotinin (10 μg/ml), 5 μM protein kinase inhibitor (Sigma)]. We performed kinase assays by adding 10 μl of this sample to 5 μl containing 1 μg of histone H1, 1 mM ATP, and 1.5 μCi γ-^{32}P-labeled ATP. The reaction was incubated at room temperature for 15 min, then stopped by the addition of an equal volume of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. Samples were analyzed by SDS-PAGE on a 12% gel that was then fixed and dried; x-ray film was exposed to the gel for 2 hours.
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