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H-*ras*^{val12} induces cytoplasmic but not nuclear events of the cell cycle in small *Xenopus* oocytes

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Microinjection of H-*ras*^{val12} protein into fully grown *Xenopus* oocytes has been shown to induce meiotic maturation. In the present study, mRNA encoding the mutant *ras* protein was injected into both fully grown (stage 6) and growing (stage 4) oocytes. The mRNA induced nuclear breakdown in stage 6 oocytes, as expected. However, the mRNA induced neither nuclear breakdown nor maturation promoting factor when injected into stage 4 oocytes. Instead, the response in stage 4 oocytes included an activation pulse of calcium, cortical granule breakdown, elevation of the vitelline envelope, and abortive cleavage furrows, all of which are characteristics of the activation response in mature eggs. In addition, the injected mRNA led to increased rates of endogenous protein synthesis and the migration of subcortical organelles into the oocyte interior. These observations are discussed relative to the suggestion that oncogenic *ras* protein leads to an increase in both diacylglycerol and inositol trisphosphate, which then regulate the various cytoplasmic events described.

Introduction

Full-grown (stage 6) *Xenopus* oocytes are arrested in late G2 of the first meiotic cell cycle and are triggered to enter M phase, i.e., mature, by progesterone and a variety of other agonists acting at the cell surface. This leads in turn to activation of an intracellular inducer of maturation, maturation-promoting factor (MPF), which then is thought to result in the nuclear and cytoplasmic events associated with oocyte maturation (review by Smith, 1989).

The mechanism by which progesterone (and other agonists) induces oocytes to mature remains uncertain. There is general consensus that the initial action of steroids at the oocyte surface results in a decrease in intracellular cyclic adenosine monophosphate (cAMP) levels and that this leads to inactivation of cAMP-dependent protein kinase (protein kinase A, PKA). This then is viewed as resulting in dephosphorylation of a putative maturation-inhibiting phosphoprotein that is a substrate for PKA (review by Maller, 1981; Smith, 1989). However, Birchmeier *et al.* (1985) reported that injection of the oncogenic *ras* protein H-*ras*^{val12} into *Xenopus* oocytes induces germinal vesicle breakdown (GVBD) with no corresponding change in cAMP levels. Deshpande and Kung (1987) and Korn *et al.* (1987) have demonstrated further that antibodies to the *ras* protein inhibit oocyte maturation induced by insulin but not maturation induced by progesterone. These observations have led to the suggestion that oocytes contain two distinct biochemical pathways that can lead to oocyte maturation, one of which does not involve the cAMP pathway.

The members of the *ras* family of oncogenes include the transforming genes of oncogenic viruses and of transformed cell lines (review by Barbacid, 1987). The proteins encoded by the members of this gene family share a number of similarities with a group of GTP-binding proteins (G proteins) that mediate a variety of receptor-induced responses. These include ability to bind GTP (Sweet *et al.*, 1984), shared structural homology with the α subunit of other GTP-binding proteins (Hurley *et al.*, 1984), and function only after attachment to the plasma membrane (Willumsen *et al.*, 1984; Hancock *et al.*, 1989). Moreover, the mutant forms of *ras* protein are defective in GTPase activity, suggesting these proteins are constitutively on relative to the activation of intracellular second-messenger systems. In view of these data, one might anticipate that mutant *ras* protein acts in oocytes at an early step after agonist stimulation, possibly to

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alter endogenous levels of a known second message. However, results obtained to date are confusing.

Sadler and Maller (1989) have reported that injection of mutant *ras* into *Xenopus* oocytes stimulates cAMP phosphodiesterase by 50%. Nevertheless, as indicated above, the protein does not appear to alter endogenous cAMP levels. Lacal *et al.* (1987) and Lacal (1990) have shown that diacylglycerol levels increase by severalfold within minutes after *Xenopus* oocytes are injected with mutant *ras* protein. This suggests an effect of the oncogenic protein via activation of the protein kinase C (PKC) pathway. However, Varnold and Smith (1990) have shown that diacylglycerol levels actually decrease shortly after exposure of oocytes to progesterone and only later increase as oocytes approach GVBD. Perhaps most surprising is the suggestion by Allende *et al.* (1988) that mutant *ras* protein does not even act at an early step in the induction of oocyte maturation. They report that the oncogenic *ras* protein induces GVBD in oocytes treated with cycloheximide, as does MPF, implying that *ras* acts downstream of either progesterone or insulin, possibly to activate pre-MPF.

Xenopus oocytes acquire the capacity to mature in response to progesterone during stage 5 of oogenesis (Reynhout *et al.,* 1975; Taylor and Smith, 1987). Nevertheless, stage 4 oocytes (750–1000 μm in diameter) contain pre-MPF, which can be activated in response to small amounts of active MPF (Taylor and Smith, 1987). Furthermore, these small oocytes can be made responsive to progesterone by injecting stage 6 cytoplasm into them, suggesting that the smaller oocytes lack a cytoplasmic factor that mediates the effect of progesterone (Taylor and Smith, 1987). The current studies were initiated to determine whether *ras* protein might be that factor and/or whether *ras* protein could activate pre-MPF directly. Surprisingly, stage 4 oocytes injected with mutant *ras* mRNA do not undergo GVBD. However, such oocytes do undergo a series of cytoplasmic changes that lead to a response typical of that seen after activation of mature oocytes (unfertilized eggs). In addition, the mutant *ras* mRNA leads to changes at the level of translation and the migration of subcortical organelles. These data are discussed relative to the potential mechanism(s) by which *ras* functions in stage 4 and stage 6 oocytes.

Results

Several studies have shown that microinjection of mutant *ras* protein into stage 6 *Xenopus* oocytes induces GVBD (Birchmeier *et al.,* 1985; Deshpande and Kung, 1987; Korn *et al.,* 1987; Lacal *et al.,* 1987; Allende *et al.,* 1988), although the time of GVBD relative to progesterone-treated controls is slower (Birchmeier *et al.,* 1985). To some extent, this temporal difference could reflect the time required for modification and translocation of injected protein to the oocyte plasma membrane (Willumsen *et al.,* 1984; Hancock *et al.,* 1989). Assuming these processes might be more efficient with proteins translated endogenously, we examined the effects of injecting ras^{val12} mRNA (5 ng/oocyte) into stage 6 oocytes. With the poly A^+ transcript, 24/28 of the injected oocytes exhibited GVBD, compared with 28/30 of the progesterone-treated controls; GVBD was at 6.5 h in both cases. Morphologically, the oocytes injected with mRNA exhibited a typical white spot and looked identical to control progesterone-treated oocytes.

Translation of ras^{val12} mRNA in stage 4 oocytes

The primary translation product of *ras* mRNA is a protein with apparent molecular mass of 23 kDa. This undergoes posttranslational modification in a two-step process in which the precursor protein is converted to a cytosolic protein of 21 kDa, which, after further modification, becomes a membrane-associated protein of 21 kDa (Gutierrez *et al.,* 1989). Figure 1A shows that stage 4 oocytes injected with the mutant *ras* mRNA synthesize a prominent protein that has an apparent molecular mass of 21 kDa and is absent in control (uninjected) oocytes. In this particular experiment, oocytes were microinjected with [^{3}H]leucine and radioactive proteins were analyzed 60 min later. Under these conditions, the bulk of the radioactive p21 is located in the cytosolic fraction, which also contains a protein with apparent molecular mass of 23 kDa just above the 21-kDa protein; we assume this is the unmodified precursor form of *ras* (see Figure 1B). Nevertheless, radioactive p21 also is readily observed in the membrane fraction. This suggests a more rapid posttranslational modification and translocation of p21 to the membrane when protein is translated in the oocyte compared with protein injected into the oocyte.

Figure 1B shows the results of an additional experiment in which stage 4 oocytes injected with mutant *ras* mRNA were incubated continuously (8 h) in radioactive amino acids (tran^{35}S label). In this case, it is obvious that a much

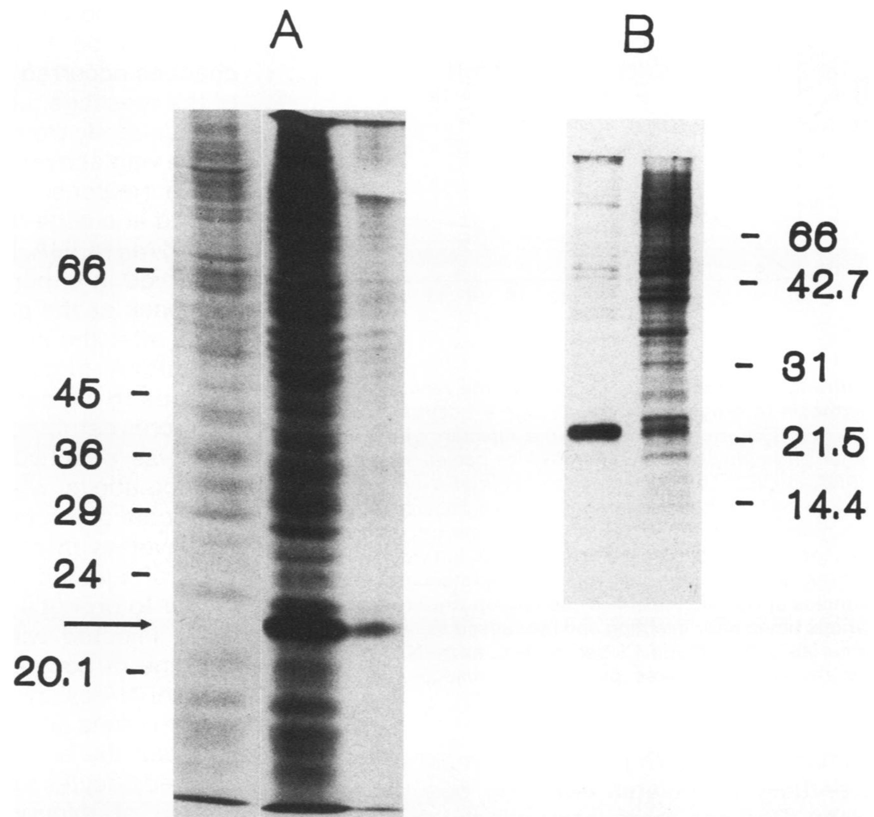


***Figure 1.* Translation of synthetic *ras* mRNA in stage 4 oocytes.** Stage 4 oocytes were injected with 5 ng of *ras* mRNA and incubated in OR2 for 5–6 h. They were then injected with 1 μCi [^{3}H]leucine and incubated for 1 h (A) or incubated an additional 8 h in OR2 containing tran^{35}S label (ICN, 10 mCi/ml) at 100 μCi/ml (B). In both cases, protein was isolated from soluble and insoluble fractions as described in Materials and methods, and electrophoresed on 12.5% SDS-PAGE. The gels were fixed and treated with Enhance (Dupont) for fluorography (A) or exposed to film directly for autoradiography (B). The three lanes in A represent, from left to right, total protein from 10 control oocytes, cytosolic protein from 10 *ras* mRNA–injected oocytes, and membrane-associated protein from 10 *ras* mRNA–injected oocytes. The two lanes in B are membrane-associated and cytosolic protein, respectively, from the equivalent of 2.5 *ras* mRNA–injected oocytes. Relative mobilities of molecular weight markers are indicated by the numbers in the margins (kDa × 10^{-3}). Arrows point to the position of *ras* protein.

greater proportion of the radioactive p21 is associated with the membrane fraction. Actual quantitation of the labeled *ras* protein was estimated by cutting the *ras* bands from the gel and counting them directly. The membrane-associated p21 represented 74% of the total *ras* protein (membrane-associated plus the cytosolic proteins of 23 and 21 kDa), whereas total *ras* protein represented ~6% of the total radioactive protein.

The rate of protein synthesis in stage 4 oocytes averages ~8 ng/h per oocyte (Taylor and Smith, 1985). If we assume that synthesis of *ras* protein in stage 4 oocytes injected with mutant *ras* mRNA is ~6% of total protein synthesis, then the approximate rate of *ras* protein synthesis would be 0.5 ng/h per oocyte. However, because Figure 1A represents a comparison of labeled proteins from equal numbers of control and mRNA-injected oocytes, it appears that the level of incorporation of radioactive leucine into endogenous proteins is increased considerably in oocytes containing p21 protein. To explore this observation further, rates of protein synthesis were measured in oocytes injected with mutant *ras* mRNA as well as in oocytes injected with globin mRNA. Taylor *et al.* (1985) have reported that stage 4 oocytes contain "spare translational capacity"; total protein synthesis can be increased by the amount of translation product from injected message. The injection of globin mRNA into stage 4 oocytes increases total protein synthesis with no effect on endogenous synthesis (Taylor *et al.,* 1985). Thus, globin mRNA–injected oocytes serve as a control for potential effects of the *ras* mRNA on endogenous protein synthesis.

Figure 2 shows an example of one experiment in which the rate of incorporation of [^{3}H]leucine into total protein in *ras* mRNA–injected oocytes was ~4-fold greater than in globin mRNA–injected controls. In two additional experiments, the increase was 2- and 2.3-fold, respectively, or an overall average of 2.8-fold. Based on this value, the estimated rate of *ras* protein synthesis in these oocytes becomes ~1.4 ng/h per oocyte. These numbers obviously are approximations. Thus, a reasonable conclusion from the data would be that stage 4 oocytes injected with 5 ng of mutant *ras* mRNA synthesize *ras*

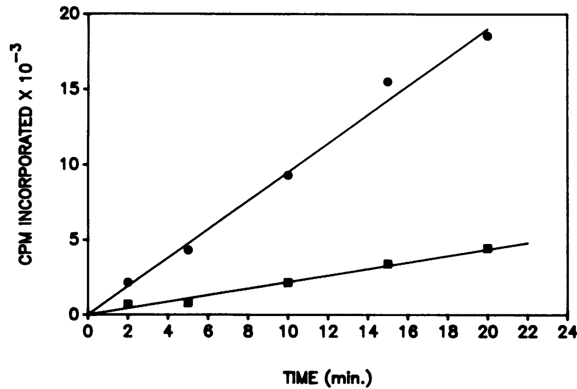


Figure 2. **ras induces an increase in the rate of protein synthesis in stage 4 oocytes.** Stage 4 oocytes between 750 and 800 μm in diameter were injected with 1 ng of either polyadenylated globin mRNA or polyadenylated *ras* mRNA in OR2. The oocytes were cultured overnight. The *ras*-injected oocytes had formed abortive cleavage furrows, and globin-injected oocytes resembled control uninjected oocytes. These oocytes were injected individually with 200 000 cpm/50 pmoles of [^{3}H]leucine and incubated in OR2. Samples of two oocytes from each group were removed at various times after injection and processed as described in Materials and methods. Closed circles, *ras* mRNA-injected oocytes. Closed squares, globin mRNA-injected oocytes.

protein at ~1 ng/h per oocyte, most of which eventually associates with the oocyte membrane. Perhaps more interestingly, the results show clearly that the *ras* protein that is translated from injected mRNA leads to a stimulation of endogenous protein synthesis.

Morphologic effects of injected ras transcripts in stage 4 oocytes

The injection of *ras* mRNA (1-5 ng/oocyte) into stage 4 oocytes did not result in GVBD. Rather, in several experiments involving >250 individual oocytes, 100% of the recipients underwent a series of changes leading to a response normally associated with parthenogenetic activation or fertilization of mature eggs. Although populations of stage 4 oocytes from different females varied with respect to the timing of events, the first obvious change (usually 5-6 h after injection of message) was a dispersal of pigment in the animal hemisphere, producing a mottled appearance (see Smith, 1989). This was followed 1-2 h later by obvious elevation of the vitelline envelope, and abortive cleavage furrows were evident after an additional overnight incubation (Figure 3A). All of these changes except pigment changes also were induced by injection of the *ras* mRNA into albino oocytes. Because these oocytes contain no melanosomes, intact germinal vesicles (GVs) can readily be visualized in activated oocytes undergoing abortive cleavage divisions (Figure 3B). It should be emphasized that all of these changes occurred spontaneously after injection of the message, i.e., injected oocytes were not subsequently treated with any stimuli known to cause egg activation.

The responses described above were observed at a wide range of concentrations of injected *ras* mRNA. No significant difference was observed in either the time of initiation of the response or the percentage of responding oocytes after the injection of from 0.5 to 10 ng of *ras* mRNA. In contrast, the responsiveness of oocytes to injection of the mutant protein was far more restricted. Injection of 5 ng of *ras* protein was effective in inducing the events described above, whereas 10 ng was fatal, and 1 ng produced no recognizable effects (Table 1). However, even at the most effective concentrations of injected protein, the oocytes required longer to produce the same effects seen in oocytes injected with mRNA, and a lower percentage of oocytes injected with protein than with mRNA exhibited the morphologic changes. These results are somewhat puzzling, especially because the amount of *ras* protein in mRNA-injected oocytes that would accumulate over the course of experiments eventually would be greater than the amount of injected protein which is lethal. Presumably, the oocyte can more readily accommodate a relatively slow accumulation of p21 protein than a single bolus of larger amounts of protein.

Birchmeier *et al.* (1985) reported that progesterone treatment of H-*ras*^{val12}-injected stage 6 oocytes resulted in GVBD at an earlier time than in steroid-treated controls. In contrast, treatment of *ras* mRNA-injected stage 4 oocytes with progesterone prevented any of the morphologic changes just described. In two experiments, a total of 20 oocytes were injected with the message and immediately thereafter were incubated in progesterone (10 μg/ml). None of the oocytes exhibited the pigment changes, vitelline envelope elevation, or pseudocleavage furrows. Similar results were obtained if oocytes were exposed to progesterone first and then injected with the mutant *ras* mRNA. Thus, *ras* protein does not appear to represent the putative cytoplasmic factor (Taylor and Smith, 1987) that mediates the initial action of progesterone.

ras **protein does not activate MPF in stage 4 oocytes.** Deshpande and Kung (1987) have reported that MPF activity appears in the cyto-

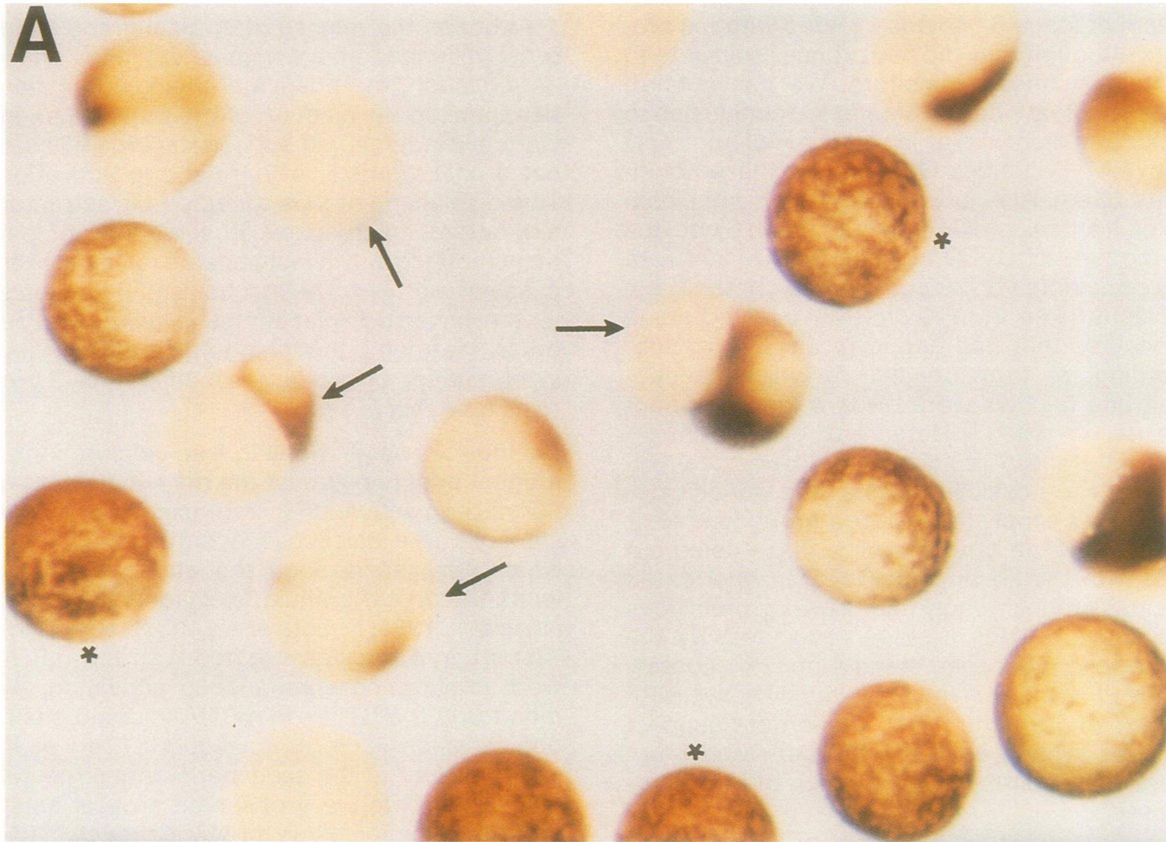
plasm of stage 6 oocytes injected with *ras* protein, although they did not expose recipients to protein synthesis inhibitors. We can confirm their observations and extend the conclusion to include cycloheximide-treated recipients; only active MPF induces GVBD under these conditions (Gerhart *et al.,* 1984). Thus, cytoplasm taken from *ras* mRNA–injected stage 6 oocytes at the time of GVBD induced GVBD in 74% of the recipients (17/23 oocytes). In contrast, cytoplasm taken from *ras* mRNA–injected stage 4 oocytes that had begun to display pigment mottling, or from oocytes in which pseudocleavage furrows were evident, had no effect on the GV when injected into cycloheximide-treated stage 6 recipients (0/30 oocytes). Although the possibility exists that donor cytoplasm was simply taken at the wrong time after injection of the *ras* transcript, these data coupled with the absence of GVBD in *ras*-treated oocytes argue strongly that the *ras* protein does not activate MPF in stage 4 oocytes. By the same token, because stage 4 oocytes contain pre-MPF, which can be activated by active MPF (Taylor and Smith, 1987), these data suggest that *ras* protein activates MPF in stage 6 oocytes only indirectly via induction of other processes.

***ras* induces cortical granule breakdown in stage 4 oocytes.** As indicated above, elevation of the vitelline envelope and the subsequent appearance of pseudocleavage furrows both are indicative of egg activation. At the ultrastructural level, egg activation results initially in breakdown of cortical granules, which discharge their contents into the perivitelline space, resulting in vitelline envelope elevation. Figure 4 shows electron micrographs of the cortical region from stage 4 control oocytes and oocytes fixed at several times after injection of *ras* mRNA. In *ras*-injected oocytes examined before elevation of the vitelline envelope (Figure 4A), both intact cortical granules and numerous pigment granules are observed at the oocyte periphery. Figure 4B shows a portion of the cortex from an oocyte that exhibited an elevated vitelline envelope at the time of fixation; cortical granules clearly are absent. Evidence of cortical granule release also is apparent in an oocyte exhibiting pseudocleavage furrows (Figure 4C). Control stage 4 oocytes injected with synthetic actin mRNA 17 h earlier still exhibited a normal complement of cortical granules and subcortical organelles, i.e., pigment granules, mitochondria, and yolk platelets (Figure 4D). Hence, injection of a non-*ras* mRNA did not induce the cortical activation response.

Although the release of cortical granule material described above mimics the initial phase of activation, the other morphologic modification induced by injection of *ras* mRNA, i.e., inward displacement of subcortical organelles, is not a characteristic of normal egg activation. This displacement was detected ultrastructurally before macroscopic observations of pigment mottling and in fact appears to be the likely cause of mottling. The mechanism(s) that leads to the inward displacement is unknown, but other treatments that lead to pigment mottling involve action on processes mediated by G proteins (see Discussion).

***ras* induces oscillations in free calcium in the stage 4 oocytes.** One of the main indicators of normal egg activation is a transient increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$), which spreads through the egg cytosol and leads to cortical granule exocytosis (Busa and Nuccitelli, 1985; Kubota *et al.,* 1987). To confirm that stage 4 oocytes injected with *ras* mRNA were undergoing spontaneous activation, we monitored the $[Ca^{2+}]_i$ in mRNA-injected albino oocytes also injected with aequorin (Cork *et al.,* 1987). In four of the six experiments large calcium transients were observed several hours after the injection of *ras* mRNA, although in two of the four cases the oocytes could not be retrieved intact from the cell chamber; morphologic observations were not possible. In the other two experiments, when the oocytes were removed from the chamber after observing the large calcium transient, they appeared activated as indicated by elevated vitelline envelopes.

Although the aequorin measurements were not calibrated in terms of calcium concentrations, the activation pulse observed (Figure 5) was very similar in magnitude and duration to the activation pulses seen in mature eggs (Cork *et al.,* 1987). Based on our previous aequorin measurements, the peak $[Ca^{2+}]$ reached ~700 nM in the oocyte represented in Figure 5, and ~250 nM in a second oocyte. For about 1 h before the final pulse, smaller bursts of increased $[Ca^{2+}]_i$ were seen as shown in Figure 5. The oocyte shown in Figure 5 also exhibited additional smaller oscillations in $[Ca^{2+}]_i$ beginning soon after the oocyte was placed in the measuring chamber (Figure 5, inset). We followed these oscillations for ~30 min before changing the chart recorder scale to allow measurements of the activation peak. During that 30 min, the oscillations were very regular, with a frequency of ~0.5 min^{-1} and an amplitude of ~3% of the basal $[Ca^{2+}]$. The possibility exists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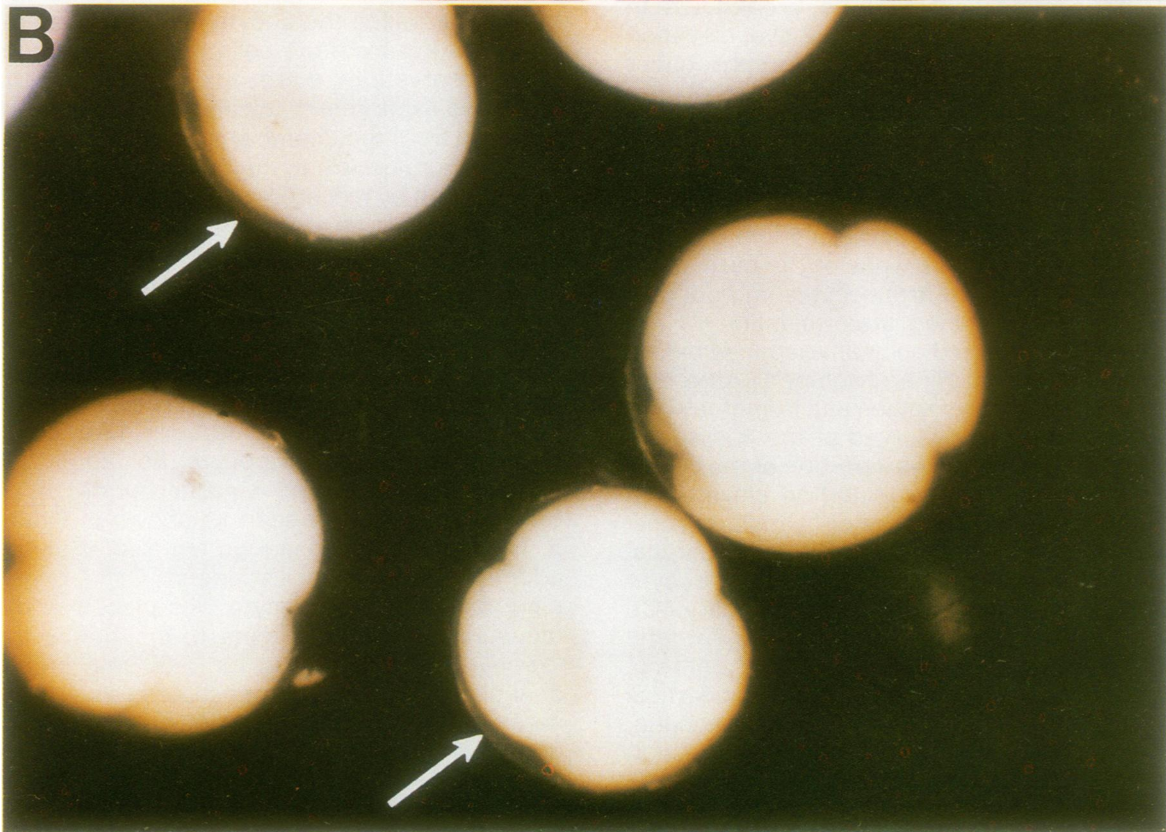
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that these oscillations continued and increased in amplitude throughout the entire 7 h before activation.

Discussion

ras induces an activation response in stage 4 oocytes

The most striking observation in the present study is that mutant *ras* protein induces in stage 4 oocytes a response resembling that normally associated with activation of eggs at the time of fertilization. This includes an activation pulse of calcium, cortical granule breakdown, elevation of the vitelline envelope, and abortive cleavage divisions. It should be emphasized that this response occurs spontaneously, i.e., in the absence of any external activation stimulus. The mechanism by which *ras* protein induces these responses is not yet clear. However, based on several studies on the process of activation in mature unfertilized eggs, several possibilities can be considered.

It now appears that the hydrolysis of phosphatidyl-4,5-bisphosphate (PIP_2) by phospholipase C plays a major role in the activation of mature *Xenopus* eggs. Both products of this hydrolysis, inositol-1,4,5-trisphosphate (IP_3) and diacyglycerol (DAG), appear to be involved in the activation response (Bement and Capco, 1989a, 1990; Nuccitelli *et al.,* 1989). Several studies have linked increased phosphoinositide turnover to the product of *ras* oncogenes in transformed cells in culture, and injection of oncogenic *ras* protein into stage 6 *Xenopus* oocytes induces a rapid increase in DAG levels (Lacal *et al.,* 1987; Lacal, 1990). The injection of p21 at a dose (10 ng) that induced GVBD resulted in no change in inositol phosphates, although high doses (100 ng) induced an increase (Lacal, 1990). Lacal (1990) suggested that the p21-induced DAG increase resulted from activation of phosphatidylcholine metabolism, as opposed to hydrolysis of PIP_2, and that the increase in inositol phosphates at high doses of p21 results indirectly from PKC regulation of phosphatidylinositol (PI) kinase, increasing PIP_2 levels.

We have not yet measured effects of *ras* on DAG and IP_3 levels in stage 4 oocytes. However, based on the above discussion, we suggest that mutant *ras* protein translated from injected mRNA induces the activation response in stage 4 oocytes by leading to increased levels of both second messages. The oscillating calcium peaks seen in Figure 5 (inset) soon after injection of *ras* mRNA are identical to the oscillations seen after injection of IP_3 into stage 6 oocytes (Parker and Miledi, 1986; Berridge, 1988). Berridge (1988) suggests that such oscillations are caused by a calcium-induced calcium release, which may function as a signaling mechanism in a variety of systems (review by Berridge and Galione, 1988). These oscillations could eventually trigger the activation pulse of calcium from a calcium-sensitive store.

Presumably, an increase in DAG would activate PKC, and this could lead to increased IP_3 as suggested by Lacal (1990). In addition, activated PKC could play a role in formation of the calcium-sensitive calcium store released at the activation pulse. In this regard, a cortical endoplasmic reticulum (CER) forms after GVBD in stage 6 oocytes (Charbonneau and Grey, 1984); this CER is thought to sequester the calcium released at activation (Busa *et al.,* 1985; Nuccitelli *et al.,* 1989). Bement and Capco (1989b) have reported that agents that activate PKC induce formation of a CER in stage 6 oocytes independent of any action on the nuclear events associated with maturation. We have not detected an extensive CER in initial ultrastructural studies on stage 4 oocytes injected with *ras* mRNA, although such oocytes were examined only at two times (2 and 5 h) before cortical granule release. Nevertheless, one possible role of PKC in the activation response in stage 4 oocytes would be to cause the formation of a CER that sequesters calcium into a calcium-sensitive pool. Once established, oscillating Ca^{2+} resulting from IP_3 action would trigger the activation pulse via action on the CER.

ras effects in stage 6 oocytes

In contrast to the effect on stage 4 oocytes, *ras* protein in stage 6 oocytes does not lead to an activation-like response. Rather, oocytes are induced to undergo GVBD and meiotic arrest at

Figure 3. **Morphologic effects of *ras* on stage 4 oocytes.** Stage 4 pigmented oocytes 650–700 μm in diameter (A) or albino oocytes 700–750 μm in diameter (B) were injected with 5 ng *ras* mRNA and incubated overnight in OR2. (A) The injected oocytes all exhibit elevated vitelline envelopes and abortive cleavage furrows (arrows). Note that in most of the *ras*-injected oocytes, pigment has almost disappeared from the surface. Control oocytes (asterisks) at early stage 4 have just accumulated animal hemisphere pigment and remain unchanged during overnight incubation in OR2. (B) Albino oocytes injected with *ras* mRNA. The arrows denote the location of intact GVs, which are readily visualized in one of the "blastomeres" resulting from abortive cleavages.

Table 1. Dose response of stage 4 oocytes to injected *ras* protein

Treatment of oocytes	Number of responding oocytes		
	15 h	24 h	36 h
Stage 4 + 3.75 ng *ras* RNA	10/10, 10/10		
Stage 4 + 10 ng *ras* protein	0/10*		
Stage 4 + 5 ng *ras* protein	0/10	6/10	
Stage 4 + 2.5 ng *ras* protein	0/10	0/10	1/10, 3/10
Stage 4 + 0.5–1 ng *ras* protein	0/10	0/10	0/10

Stage 4 oocytes, 750–800 μm diameter, were microinjected with either 3.75 ng *ras* mRNA or with the indicated amounts of bacterially expressed *ras* protein. The protein samples were diluted so that a constant injection volume of 10 nl was used to deliver the protein. Oocytes were maintained in OR2 and monitored periodically under a dissecting microscope for the first sign of activation, i.e., lifting of the vitelline envelope, which was then scored as a positive response.

* Fatal.

the second meiotic metaphase. At this time, we can only speculate as to the nature of these differences in oocyte responsiveness. However, one explanation suggests that stage 6 oocytes develop a factor during maturation that somehow interacts with *ras* protein and/or *ras* substrates to regulate the *ras*-induced pathway. One possibility for such a factor is a protein to which GTPase activating protein (GAP) binds, as discussed in the recent study by Gibbs *et al.* (1989). On the other hand, maturing stage 6 oocytes in which GVBD has occurred also develop a factor, cytostatic factor (CSF), that maintains arrest at second meiotic metaphase (review by Masui and Shibuya, 1987) and that also maintains MPF in active form (Sagata *et al.*, 1989). CSF is inactivated by the calcium pulse that occurs when mature eggs are activated. Conceivably, CSF also prevents a premature activation response during normal oocyte maturation. In support of this view, Bement and Capco (1989a) have reported that brief treatment of stage 6 oocytes (no CSF) with phorbol 12-myristate 13-acetate (PMA) rapidly induces cortical granule exocytosis, followed later by the appearance of abortive furrows, but the oocytes retain an intact GV—the same response observed in stage 4 oocytes injected with *ras* mRNA. Because stage 4 oocytes presumably lack CSF, the sequence of events activated by the *ras* protein could proceed to completion, leading to spontaneous activation. This hypothesis appears to be testable. Sagata *et al.* (1989) recently have reported that the protooncogenic c-*mos* protein exhibits CSF activity, suggesting the possibility that in stage 4 oocytes injected with *ras* mRNA and then c-*mos*, spontaneous activation would not occur.

ras induces multiple effects in Xenopus *oocytes*

In addition to the activation response discussed above, injection of oncogenic *ras* mRNA into stage 4 oocytes leads to two other clear-cut responses. On the one hand, injected oocytes exhibit an intracellular movement of subcortical organelles, including pigment granules, which produces initially a mottled appearance of the animal hemisphere surface. Eventually, such oocytes appear externally to be almost without pigmentation and resemble albino oocytes (Figure 3A). Birchmeier *et al.* (1985) observed that injection of mutant *ras* protein into stage 6 oocytes also induced dispersal of pigment in the animal hemisphere, but only if relatively high amounts of the protein (>20 ng) were injected. They suggested that the abnormal pigmentation was indicative of oocyte degeneration. This does not appear to be the case because injection of *ras* mRNA into albino oocytes that contain no melanosomes still results in elevation of the vitelline envelope and pseudocleavage furrows with no obvious indication of degenerative changes (Figure 3B). In addition, pigmentation changes of the type described here also have been observed by injection of agents that affect cytoskeletal proteins as well as treatment of oocytes with activators of PKC (see Smith, 1989). Further, Lucas *et al.* (1987) have linked activation of PKC to melanosome dispersion in pigment cells of the lizard. We suggest that the mottled pigmentation resulting from injection of *ras* transcript results from activation of PKC, which then mediates the internal movement of organelles from the subcortical cytoplasm (Figure 4).

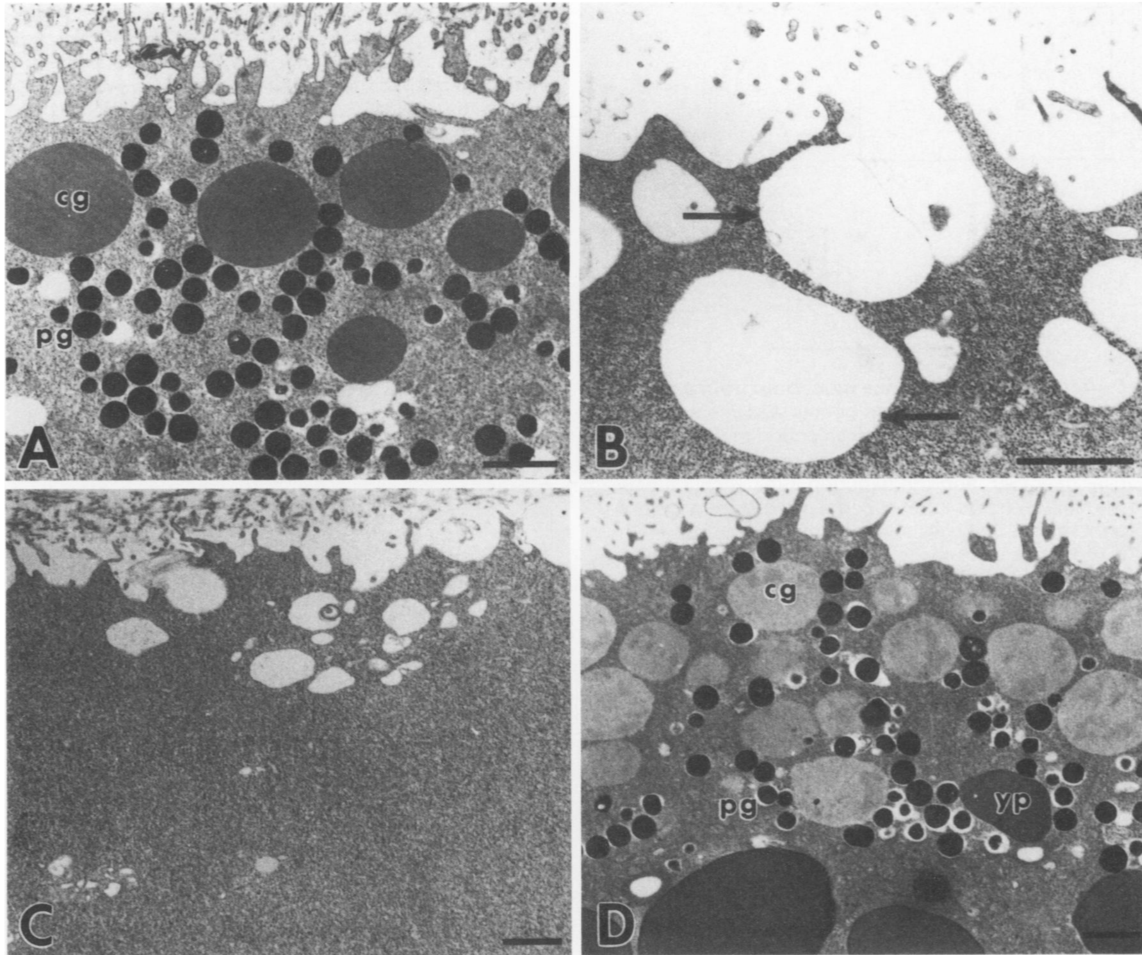

Figure 4. **Electron micrographs of the cortical region of stage 4 oocytes injected with *ras* mRNA.** Stage 4 oocytes were fixed and prepared for electron microscopy at various times after injection with 5 ng *ras* mRNA (A–C) or with synthetic actin mRNA transcripts (D). (A) 3.5 h after injection. No structural alterations are evident; cortical granules (cg) and pigment granules (pg) occupy the subcortical cytoplasm. (B) 10.5 h after injection. Oocytes exhibited an elevated vitelline envelope and cortical granule contents have been released. Note the absence of subcortical pigment granules. (C) 17 h after injection. Oocytes exhibited abortive cleavage furrows. Cortical granules are absent and the subcortical cytoplasm is devoid of organelles. (D) 17 h after injection. These control oocytes exhibit normal morphology and contain normal cortical granules, pigment granules, and scattered yolk platelets. Bars equal 1 μm.

The current data show clearly that injection of oncogenic *ras* transcript leads to a marked stimulation in the rate of endogenous protein synthesis. This effect is both surprising and striking. The rate of protein synthesis in control stage 4 oocytes is about one-third that in stage 6 oocytes and about one-sixth that in mature oocytes (Wasserman *et al.,* 1982; Taylor and Smith, 1985). Thus, the effect of *ras* transcript in stage 4 oocytes is to elevate endogenous protein synthetic rate to a level at least as high as in stage 6 oocytes and, in some cases, as high as seen only in mature oocytes. Injection of *ras* protein also leads to increased protein synthetic rate in stage 6 oocytes (unpublished data). However, in this case, because *ras* protein also induces maturation, active MPF appears (Barrett *et al.,* 1990). MPF itself stimulates protein synthetic rate, both in stage 6 oocytes (Wasserman *et al.,* 1982) and in stage 4 oocytes (Taylor and Smith, 1987), making it difficulty to discriminate between direct and indirect effects of the *ras* protein. This is not a problem in stage 4 oocytes because *ras* does not activate MPF. Thus, either the appearance of *ras* protein increases translational efficiency in the stage 4 oocytes or, as discussed by Taylor *et al.* (1985), increased protein synthesis would result from recruitment of additional mRNA from the maternal stockpile.

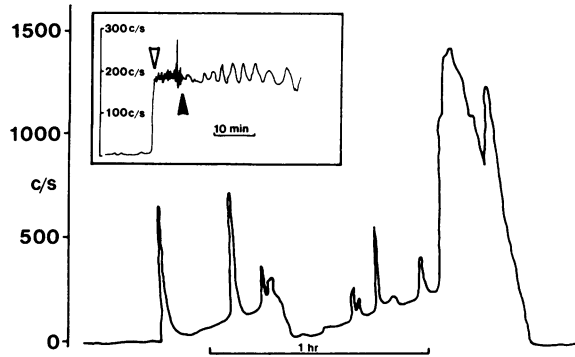

Figure 5. **Aequorin luminescence monitored from a single stage 4 oocyte.** The albino stage 4 oocyte was injected with 10 nl of 1 mg/ml aequorin 1 h after injection of 5 ng of *ras* mRNA. Approximately 1 h later, it was placed in the photomultiplier chamber, and the aequorin luminescence was monitored. (Inset) Initial luminescence record from before opening the photomultiplier shutter (open arrow) to ~1 h later. At the point marked by the closed arrow the chart speed was increased 10X relative to that indicated on the time scale bar. (Main figure) The luminescence trace starts ~6 h after the initial record ends. Two series of calcium transients were observed, each lasting ~30 min. The second series ended with a large calcium transient, the peak of which represents a calcium concentration of ~700 nM. After removal from the chamber, the oocyte showed all signs of activation, including abortive cleavage furrows.

In recent years, studies on a variety of cell types have sought to identify the intracellular signaling system(s) in which *ras* is involved. To date, *ras* has been implicated in all of the major intracellular second-messenger pathways (see Price *et al.*, 1989), and frequently results have been different depending on the cell type used. Part of the problem, as pointed out by Price *et al.* (1989), is that transfected cell lines have been used, making it difficult to distinguish between short-term and long-term effects. In addition, multiple *ras*-like GTP-binding proteins have been discovered in several cell types (see Wolfman *et al.*, 1989), implying the possibility that *ras*-type proteins could regulate multiple functions. Although the current study does not suggest an easy resolution of these problems, it does suggest that stage 4 oocytes can serve as a model system to dissect some functions of the *ras* protein under conditions in which many of the events associated with progression through the cell cycle do not simultaneously occur.

Materials and methods

Subcloning and plasmids

Plasmid pEMS9 carrying a mutant copy of the T24 Ha-*ras*-1 cDNA was a gift of Dr. E.J. Taparowsky, Purdue University. The coding region of EMS9 (Fasano *et al.*, 1983) was removed by digestion with *Sal*I and *Mst*II and isolated by standard methods (Maniatis *et al.*, 1982). The fragment was initially ligated into the *Bgl*II site of pSP64T with linkers (Krieg and Melton, 1984), and subsequently transferred to the *Bam*H1 site of pSP65A (Galili *et al.*, 1988). This new plasmid Ha-*ras*/65A could be linearized with *Hin*dIII or *Xba*I to produce poly(A+) or poly(A−) transcripts, respectively, and was used for all experiments reported here. Initial experiments showed that the poly(A−) transcript was translated with considerably less efficiency than the poly(A+) message, as also observed with other mRNAs (Galili *et al.*, 1988). All experiments reported in this study utilized the poly(A+) *ras* mRNA.

Globin transcripts described in the legend to Figure 2 were generated from *Sal* 1 linearized pBXlm which was provided by Dr. D. Melton of Harvard University. pCyIIIA was a gift from Dr. C. Flytzanis, Baylor College of Medicine. The insert from this clone was subcloned into pSP64A, which was provided by Dr. B. Larkins, University of Arizona. Transcripts from this clone were used to provide actin mRNA referred to in the legend to Figure 4.

Handling of animals and cells

Frogs were anesthetized by hypothermia, and pieces of ovary were surgically removed and placed into OR2 (Wallace *et al.*, 1973) at room temperature. In all experiments, oocytes were manually removed from their follicles with watchmakers forceps and maintained in OR2 until use. The oocytes were sized with an ocular micrometer and staged according to Dumont (1972).

Maturation was induced by progesterone treatment (10 μg/ml) in OR2. For MPF experiments, cytoplasm was removed with a micropipette and 5–10 nl MPF was injected into recipient stage 6 oocytes. The small volume was used because of the difficulty in removing cytoplasm from stage 4 oocytes. For cycloheximide experiments, oocytes were preincubated (10 μg/ml) for 0.5 h before transfer to healing medium (Ford and Gurdon, 1977), also containing 10 μg/ml cycloheximide. After injection in this medium, oocytes were allowed to heal for 30 min and then were transferred back to OR2 with cycloheximide for the duration of experiments.

Transcription

In vitro transcription was as described by Melton *et al.* (1984) with the following modifications. 7mGpppG (Pharmacia, Piscataway, NJ) was included in transcription at 1 mM. Five microcuries of 3H-GTP was included for quantitation, and the final GTP concentration was 100 μM. After removal of template with RQ1 DNase (Promega, Madison, WI), the reaction was extracted with phenol-chloroform and precipitated with ethanol. The transcripts were resuspended in H_2O and passed over a NICK Column (Pharmacia) that was equilibrated in 0.3 M NaOAc, pH 5.2, and reprecipitated with ethanol. RNA was resuspended in H_2O at 0.5–1 mg/ml.

Analysis of translation products

For translation experiments oocytes were injected with 5–10 nl of RNA solution, which was either 0.5 or 1 mg/ml in H_2O. Oocytes were incubated for ~5 h to allow loading onto polysomes and then injected with 1 μCi of [^{3}H]leucine. Isotope was incorporated for 1 h, then the oocytes were homogenized in 50 mM NaCl containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged for 5 min, and the supernatant was removed and precipitated with 4 vol acetone. The pellet was resuspended in

the same cocktail as above, but containing 1% Triton essentially as described by Birchmeier *et al.* (1985). This suspension was recentrifuged, and the supernatant was precipitated with acetone. The first and second supernatants were considered the soluble and membrane-bound fractions, respectively. The acetone precipitates from both fractions were boiled for 5 min in Laemmli (1970) sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were treated with Enhance (Dupont, Wilmington, DE) before using for fluorography.

Rates of protein synthesis

Oocytes were treated as described in the text and the rates of incorporation of [^{3}H]leucine into protein were determined exactly as described by Wasserman *et al.* (1982).

Electron microscopy

Oocytes were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, in 100 mM $NaPO_4$, pH 7.4, overnight. They were then postfixed in 2% osmium tetroxide, 5% sucrose for 2 h. If the oocytes were to be processed for the formation of the CER, they were fixed overnight in 2.5% glutaraldehyde, 0.1% tannic acid in 100 mM phosphate buffer at pH 7.4 as described by Charbonneau and Grey (1984). Oocytes were then dehydrated in a graded series of ethanol and propylene oxide and embedded in a mixture of epon and araldite. Ultrathin sections were sequentially stained with uranyl acetate and lead citrate. Micrographs were taken in a Phillips 300 electron microscope.

Free calcium measurements

Albino *Xenopus* oocytes were injected with synthetic *ras* pA + mRNA and allowed to equilibrate for 1 h in OR2. Busa and Nuccitelli (1985) have shown that prolonged incubation in Ca^{++}-free medium can alter the properties of the oocyte plasma membrane. For this reason the oocytes were transferred to calcium-free OR2, quickly injected with 10–15 nl of 1 mg/ml aequorin solution, and returned to OR2 as quickly as possible. Individual oocytes were then placed in a flow chamber attached to a photomultiplier tube, and aequorin luminescence was measured as described in Cork *et al.* (1987).

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