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# *RAS* Proteins Can Induce Meiosis in Xenopus Oocytes

**Carmen Birchmeier, Daniel Broek, and Michael Wigler**
Cold Spring Harbor Laboratory
Cold Spring Harbor
New York, New York 11724

## Summary

**Injection of human H-*ras* protein induces maturation of Xenopus oocytes; that is, progression from prophase to metaphase of meiosis. The oncogenic protein encoded by H-*ras*$^{val12}$ is nearly a 100-fold more potent than the protein encoded by the wild-type gene. We do not observe any measurable increase or decrease in cyclic AMP concentration in injected oocytes, and the effects of H-*ras* protein are only partially blocked by cholera toxin. Our results suggest that not all, if any, of the effects of H-*ras*$^{val12}$ protein in this system are mediated by adenylate cyclase.**

## Introduction

The *ras* genes were first isolated as the transforming genes of Harvey and Kirsten sarcoma virus (Ellis et al., 1981). At least three different *ras* genes, H-*ras*, K-*ras*, and N-*ras*, exist in mammals and code for three very similar 21 kd proteins (Shimizu et al., 1983b). The *ras* proteins are localized in the plasma membrane (Willingham et al., 1980), bind guanine nucleotides (Shih et al., 1980; 1982), and have weak GTPase activity (Sweet et al., 1984; McGrath et al., 1984; Gibbs et al., 1984). A large number of tumor cells contain structurally mutated *ras* genes, which are capable of tumorigenic transformation of NIH 3T3 cells upon DNA-mediated gene transfer (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982; Shimizu et al., 1983a; Yuasa et al., 1983; Capon et al., 1983). These oncogenic *ras* genes differ from their normal counterparts by single missense mutations that reduce the GTPase activity of the encoded proteins (Sweet et al., 1984; McGrath et al., 1984; Gibbs et al., 1984). Genes homologous to the mammalian *ras* have been identified in virtually every organism investigated, including yeast, slime molds, and fruit flies (Shilo and Weinberg, 1981; DeFeo-Jones et al., 1983; Powers et al., 1984; Reymond et al., 1984; Neuman-Silberberg et al., 1984). In mice, the *ras* genes appear to be expressed in all cell types, and at all developmental stages (Mueller et al., 1982; 1983). The *ras* proteins are therefore presumed to be involved in a basic and ubiquitous system controlling cell proliferation. Their biochemical function in mammals or vertebrates has, however, not been identified. The *ras* proteins in the yeast Saccharomyces cerevisiae have been shown by genetic and biochemical means to activate adenylate cyclase (Toda et al., 1985; Broek et al., 1985). It was therefore of interest to determine to what extent this function of the *ras* proteins has been conserved during evolution. For this purpose, we have examined the function of *ras* proteins in Xenopus oocytes.

Fully grown Xenopus laevis oocytes are extemely large cells (diameter 1.1–1.4 mm). They can be easily injected, and their size allows biochemical measurements on the injected cells. Moreover, oocytes contain an adenylate cyclase, which, as in mammalian cells, is regulated by the GTP-binding proteins, $G_s$ and $G_i$, which can be ADP-ribosylated in response to exposure to cholera toxin or pertussis toxin, respectively (Sadler et al., 1984; Goodhardt et al., 1984; Olate et al., 1984; Mulner et al., 1985). In addition, oocyte maturation, that is, the induction of meiosis, is modulated by cyclic AMP (cAMP). Oocytes surgically removed from adult Xenopus ovaries are arrested in the prophase of meiosis and can be triggered to undergo meiosis by treatment with progesterone, insulin, IGF-1, and a variety of other agents. Agents, such as cholera toxin or phosphodiesterase inhibitors, which increase cAMP levels, or microinjection of the catalytic subunit of the cAMP-dependent protein kinase (protein kinase A) inhibit maturation (O'Connor and Smith, 1976; Maller and Krebs, 1977; Schorderet-Slatkine et al., 1978). Injection of proteins that inhibit the activity of the protein kinase A, such as the heat stabile kinase inhibitor or the regulatory subunit of protein kinase A, induce meiosis, and progesterone, the physiological inducer of maturation, lowers cAMP levels (Maller and Krebs, 1977; Speaker and Butcher, 1977; Mulner et al., 1979). We therefore decided to determine the effect of injected H-*ras* protein on the maturation of oocytes.

## Results

### Induction of Oocyte Maturation by Injected *ras* Proteins

Mutant and wild-type human H-*ras* proteins were purified from E. coli carrying an expression plasmid (Gross et al., 1985). Upon injection of H-*ras* protein into Xenopus oocytes, maturation was induced, as judged by the appearance of a white spot in the pigmented half of the oocyte, by the breakdown of the germinal vesicle, and by the appearance of a meiotic spindle (Figure 1). To confirm that the activity responsible for the induction of maturation was not due to a buffer component like GTP, heat denatured H-*ras*$^{val12}$ protein was injected, and no maturation was observed (Figure 2a).

Differences in the biological activity of the mutant and wild-type proteins were observed. H-*ras*$^{val12}$ protein, the product of the oncogenic gene with a missense mutation that specifies valine instead of glycine in position 12, induced maturation in 94% of the oocytes when 10 ng of the protein was injected (Figure 2a). The time course of induction of maturation for H-*ras*$^{val12}$ injected cells was slower than for progesterone-treated cells. Progesterone treatment of H-*ras*$^{val12}$-injected oocytes resulted in a time course of maturation faster than that observed when only one of these effectors was applied (Figure 2a). The in-

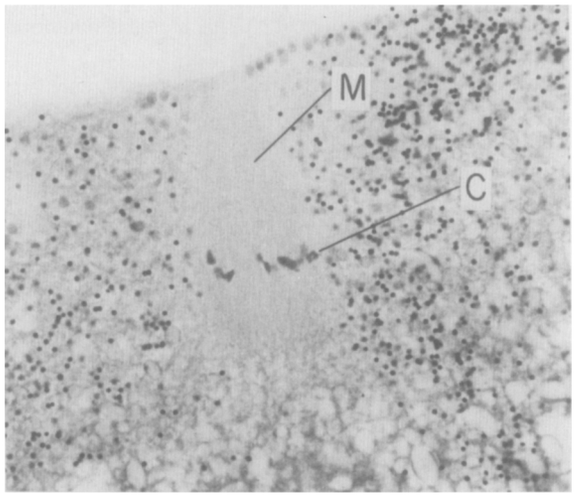


Figure 1. Metaphase Spindle of First Meiotic Division in Xenopus Oocyte Injected with H-*ras*$^{val12}$ Protein

Collagenase-treated Xenopus oocytes were injected with 10 ng of H-*ras*$^{val12}$ protein and were incubated for 10 hr. Oocytes that showed a clear white spot were processed as described in Experimental Procedures. Arrows indicate chromosomes (C) and metaphase spindle (M). Photomicrograph magnification 1500×.

jected wild-type H-*ras* protein was almost 100 times less efficient in the induction of maturation. At the highest concentration tested, 400 ng of this protein induced maturation in only 55% of the injected oocytes (Figure 2b). In addition, an opaque ring especially prominent after fixation in trichloroacetic acid was observed around the white spot in the pigmented half of oocytes injected with H-*ras*$^{val12}$. Finally, if more than 20 ng of the H-*ras*$^{val12}$ protein was injected, oocytes visibly degenerated, displaying a characteristic dispersal of pigment. No toxicity was observed even when 400 ng of the wild-type protein was injected. These experiments indicate that injected H-*ras* proteins induce meiosis in Xenopus oocytes, and that the Xenopus oocytes are much more sensitive to oncogenic H-*ras* protein than to wild-type protein.

### Effects of Injected H-*ras*$^{val12}$ on Oocyte Adenylate Cyclase

A very early response of Xenopus oocytes to progesterone, the physiological inducer of meiosis, is a drop in cAMP levels, which is possibly the signal for induction of maturation (for a review, see Maller, 1983; Sadler and Maller, 1985). We determined therefore, whether we could observe a similar drop in Xenopus oocytes injected with H-*ras*$^{val12}$ protein. Preliminary experiments indicated that we could not accurately measure cAMP levels using the protein binding assay, a method employed to observe changes of cAMP in Rana pipiens oocytes (Speaker and Butcher, 1977). This problem, previously encountered by others, might arise from the presence of substances interfering with this assay. We therefore used an alternate assay (Mulner et al., 1979), involving the injection of [$\alpha$-$^{32}$P]ATP into oocytes followed by a determination of the amount of radioactivity converted into [$^{32}$P]cAMP. Details of this method are given in Experimental Procedures. Using this method, others have detected decreases in cAMP levels upon progesterone treatment and increases upon cholera toxin treatment (Mulner et al., 1979). In our experiments, we detect a 70% drop in radioactive cAMP upon progesterone treatment and a 4- to 6-fold increase upon cholera toxin treatment. The progesterone-induced drop in the amount of cAMP is observed whether the progesterone treatment is initiated 15 min or 4 hr after radioisotope injection (data not shown). Therefore, measurement with this method reflects the equilibrium level of cAMP in the oocyte. Using this technique, we failed to detect any significant difference between oocytes injected with H-*ras*$^{val12}$ protein or injected with bovine serum albumin (Table 1). We conclude from this that the injected H-*ras* protein does not produce a sustained depression or elevation of adenylate cyclase activity in Xenopus oocytes. Very small changes or changes of a transient nature would not be detected in our assay and can therefore not be completely excluded.

### Fate of Microinjected *ras* Proteins

A transient biochemical effect of *ras* protein on Xenopus oocytes could be expected if the H-*ras* protein were stable for only short times after injection. We therefore determined the fate of the protein injected into the Xenopus oocytes. H-*ras*$^{val12}$ protein was partially purified from a metabolically labeled E. coli culture carrying the expression plasmid. At various times after injection of the $^{35}$S-labeled H-*ras*$^{val12}$ protein, total oocyte protein soluble in 1% Triton X-100 was isolated and analyzed by sodium dodecylsulfate gel electrophoresis (SDS-PAGE) and fluorography (Figure 3, lanes a–e). A protein of 23 kd, present initially, was seen to disappear gradually, while a new species of apparent molecular weight 21 kd accumulated. The time course suggested a precursor–product relationship between these two species and a precursor half-life on the order of 2–3 hr. The 21 kd species can be observed within half an hour after injection (data not shown). This protein species comigrated with H-*ras*$^{val12}$ protein immunoprecipitated from a 3T3 cell line transfected with the activated human H-*ras* gene (Figure 3, lane j). As has been previously shown, the primary translation product of the H-*ras* protein is modified after transcription, a process which is associated and probably responsible for the insertion of the soluble precursor protein into the plasma membrane (Sefton et al., 1982). The primary translation product and the membrane bound form migrate on SDS-PAGE with apparent molecular weights of 23 kd and 21 kd, respectively. We observed that in Xenopus oocytes, too, the modified 21 kd species is preferentially soluble in buffers that contain detergents (Figure 3, lanes f–i). Therefore, we can conclude that the injected H-*ras* protein is stable in Xenopus oocytes and that it is modified and associated with a membrane fraction.

### Partial Cholera Toxin Inhibition of H-*ras* Action

Cholera toxin, which raises cAMP production by activating adenylate cyclase, and 3-isobutyl-1-methylxanthine, which

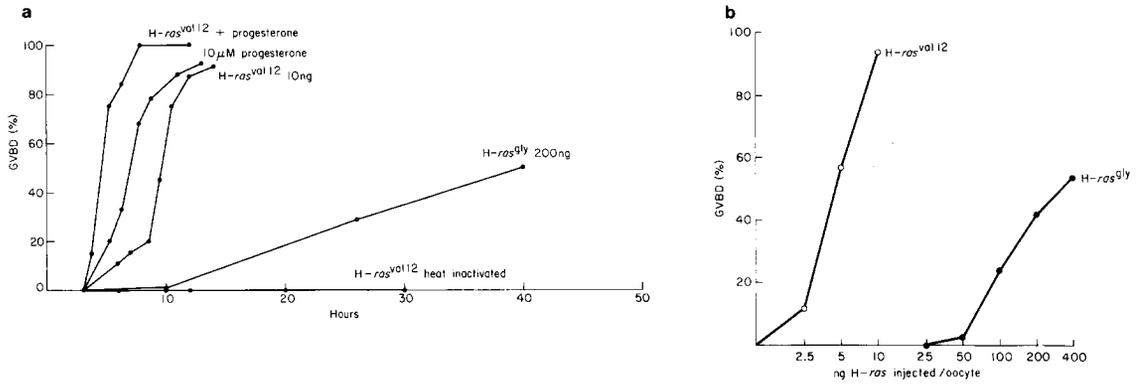


Figure 2. Time Course and Dose Response Curve of Induction of Meiosis by Injected H-*ras* Protein

(a) Time course of germinal vesicle breakdown (GVBD) is shown for Xenopus oocytes incubated in 10 μM progesterone or injected with 10 ng of H-*ras*$^{val12}$ or 200 ng of H-*ras*$^{gly}$ as indicated. After the injection, oocytes were transferred to fresh Barth medium with or without 10 μM progesterone, as indicated. Heat-inactivated H-*ras*$^{val12}$ protein had been boiled for 3 min. Oocytes were fixed in 6% trichloroacetic acid after the indicated incubation times and were examined for the presence of the germinal vesicle. (b) Germinal vesicle breakdown (GVBD) in Xenopus oocytes is shown after injection of various amounts of H-*ras*$^{val12}$ or H-*ras*$^{gly}$ protein. After injection, the oocytes were incubated for either 12 hr (oocytes injected with H-*ras*$^{val12}$) or 20 hr (oocytes injected with H-*ras*$^{gly}$), fixed, and examined for the presence of the germinal vesicle. For each experimental point, a minimum of 24 oocytes was analyzed.

Table 1. cAMP Levels in Oocytes Injected with H-*ras*$^{val12}$ Protein or Treated with Progesterone or Cholera Toxin

| Treatment | Time | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 | Experiment 5 | Experiment 6 | Experiment 7 |
|---|---|---|---|---|---|---|---|---|
| BSA Injection | 15 min | | | 122 | 106 | 119 | | |
| | 30 min | | | 101 | 98 | | | |
| | 45 min | | | 113 | | | | |
| | 1 hr | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | 2 hr | | | 103 | | | | |
| | 3 hr | 82 | 104 | | | | | |
| | 4 hr | | | | | | | |
| BSA Injection and Progesterone Treatment | 1 hr | 21 | 32 | | 38 | | | |
| H-*ras*$^{val12}$ Protein Injection | 15 min | | | 111 | 86 | 118 | | |
| | 30 min | | | 112 | 125 | | | |
| | 45 min | | | 103 | 98 | | | |
| | 1 hr | 95 | 124 | 90 | 122 | 100 | | |
| | 2 hr | | | 97 | | | | |
| | 3 hr | 100 | 105 | | | | | |
| | 4 hr | | | | | 87 | | |
| BSA Injection and Cholera Toxin Treatment | 1 hr | | | | | | 413 | 623 |

Xenopus oocytes were injected with 10 ng protein and 2 μCi [α-$^{32}$P]ATP in experiments 1–4, 6, and 7 and 4 μCi [α-$^{32}$P]ATP in experiment 5; after injection, the oocytes were transferred to fresh Barth medium containing 10 μM progesterone or 50 ng/ml cholera toxin when indicated, and incubated for various times. The amount of [$^{32}$P]cAMP present after the indicated incubation times was determined as described in Experimental Procedures. Oocytes from a single female were used for each experiment. The value of [$^{32}$P] cAMP obtained for oocytes injected with bovine serum albumin (BSA) and incubated for 1 hr was taken as 100, and other values obtained in the same experiment were adjusted accordingly. The amount of the injected [α-$^{32}$P]ATP converted into [$^{32}$P]cAMP differed for oocytes from different females. For oocytes injected with BSA and incubated for 1 hr in the presence or absence of 1 μM 3-isobutyl-1-methylxanthine, values from 0.018% to 0.045% and from 0.015% to 0.011%, respectively, were obtained. One micromolar 3-isobutyl-1-methylxanthine was present during the injection and incubation in experiments 1, 2, 3, 4, and 7 and was absent in experiments 5 and 6. [α-$^{32}$P]ATP and protein were coinjected in experiments 1, 2, 4, 6, and 7; [α-$^{32}$P]ATP was preinjected and incubated for 1 hr before protein injection in experiments 3 and 5. In experiments 6 and 7, oocytes were treated with collagenase to remove follicle cells.

raises cAMP levels by inhibiting phosphodiesterase, both block oocyte maturation induced by progesterone (O'Connor and Smith, 1976; Schorderet-Slatkine et al., 1978). We therefore sought to determine whether all the effects observed upon H-*ras*$^{val12}$ injections could be blocked by treatment of oocytes with cholera toxin or 3-isobutyl-1-methylxanthine. Cholera toxin inhibited the maturation induced by injected H-*ras*$^{val12}$ protein, as judged by the absence of germinal vesicle breakdown. However, neither the opaque ring in the pigmented half of the oocyte, nor the toxic effect of high doses of H-*ras*$^{val12}$ could be totally inhibited by this agent. In addition, in most of the treated oocytes we observed that the germinal vesicle had migrated to a position located directly under the oocyte

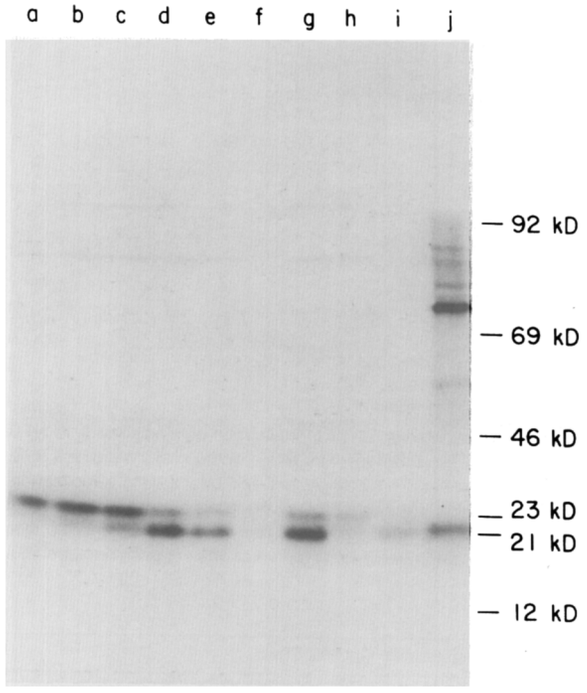


Figure 3. Fate of Microinjected H-*ras* Protein

SDS-PAGE analysis of oocytes injected with $^{35}$S-labeled H-*ras*$^{val12}$ protein. In lanes a–e, after various times of incubation, oocytes were homogenized in phosphate-buffered saline (PBS) containing 1% Triton X-100, and a 10,000 ×g supernatant of the homogenate was analyzed. The oocytes were extracted (a) 5 min; (b) 1 hr; (c) 2 hr; (d) 4 hr; (e) 6 hr after injection. In lanes f–i, oocytes were injected and incubated for 4 hr, homogenized in PBS with or without 1% Triton X-100, and centrifuged at 30,000 ×g for 2 hr. f and g are pellet and supernatant, respectively, of homogenate with detergent. h and i are supernatant and pellet, respectively, of homogenate without detergent. (Lane j) H-*ras*$^{val12}$ protein immunoprecipitated from 3T3 cells containing the human T24 *ras* gene (see Experimental Procedures).

Table 2. Partial Inhibition of H-*ras* Effects by Cholera Toxin

| Treatment | Absence of Germinal Vesicle | | Migration of Germinal Vesicle |
|---|---|---|---|
| | −CT | +CT | +CT |
| H-*ras*$^{val12}$ 10 ng/oocyte | 17/26 | 0/27 | 18/27 |
| H-*ras*$^{val12}$ 20 ng/oocyte | 23/25 | 0/28 | 25/26 |
| Progesterone 10 μM | 17/20 | 0/22 | 1/25 |

Oocytes were treated with collagenase to remove follicle cells and were allowed to recover overnight. Oocytes exposed to cholera toxin were pretreated with the toxin for 1 hr before injection with H-*ras*$^{val12}$ protein or exposure to progesterone. After a 12 hr incubation, during which cholera toxin and progesterone were continuously present when indicated, oocytes were fixed in 6% trichloroacetic acid and scored for migration or absence of the germinal vesicle.

membrane and, occasionally, had become flattened (Table 2). The same concentration of cholera toxin inhibited germinal vesicle breakdown and migration of the nucleus in progesterone-treated oocytes (Table 2). Similar results were obtained when oocytes were treated with 3-isobutyl-1-methylxanthine (data not presented).

## Discussion

We have examined the effects of injecting the oncogenic H-*ras*$^{val12}$ protein and its normal counterpart on Xenopus oocytes. These proteins induce meiosis in the oocyte, although the oocyte is much more sensitive to the oncogenic H-*ras* protein than to the wild-type protein. The amount of injected H-*ras*$^{val12}$ protein needed to induce maturation efficiently corresponds to 0.0033% of the total protein in oocytes. In many cultured mammalian cells, we estimate that H-*ras* protein constitutes approximately 0.005% of total protein. Thus the Xenopus oocyte is a sensitive indicator of oncogenic *ras* protein function.

It has been observed that the protein product from v-*src* accelerates the time course of progesterone-induced maturation upon injection into oocytes (Spivack et al., 1984). The same phenomenon was observed when H-*ras*$^{val12}$ protein was injected prior to the exposure of oocytes to progesterone. This suggests that the biochemical pathways recruited by v-*src* overlap with the pathways recruited by the H-*ras*$^{val12}$ protein. However, it has not been shown that the v-*src* product is alone sufficient to induce oocyte maturation, since large quantities of the v-*src* product have not been injected into oocytes. We therefore do not know whether there are essential biochemical pathways recruited by *ras* proteins that are not recruited by the v-*src* oncoprotein.

We do not understand how injected H-*ras* proteins induce maturation of oocytes. A drop in cAMP levels has previously been proposed as one signal for induction of meiosis; indeed, such a drop can be observed upon administration of progesterone. However, no significant alterations of cAMP levels can be observed in oocytes injected with H-*ras*$^{val12}$ protein. We cannot of course completely rule out a short-lived change in cAMP concentration, or a change smaller than we can detect. However, in contrast to progesterone treatment, not all the morphological changes observed after H-*ras* protein injections could be inhibited by either cholera toxin or phosphodiesterase inhibitor. This seems to imply that not all, if any, of the effects of H-*ras*$^{val12}$ proteins in this system are mediated by adenylate cyclase.

These findings were unexpected in view of our results obtained in the yeast, Saccharomyces cerevisiae. In that organism both yeast and mammalian *ras* proteins strongly activate adenylate cyclase (Toda et al., 1985; Broek et al., 1985). It is still not clear whether the *ras* proteins interact directly with the S. cerevisiae adenylate cyclase, or whether they act through other as yet unidentified proteins. Indeed, preliminary biochemical and genetic data support the latter hypothesis (D. Broek, S. Powers, and T. Kataoka, unpublished). If such intermediate proteins exist, the interaction of *ras* with these proteins might be con-

served in evolution, thereby explaining the observed structural and functional homology of the yeast and mammalian *ras* proteins (Kataoka et al., 1985; DeFeo-Jones et al., 1985). The interaction between these putative proteins and adenylate cyclase might have changed during evolution, and H-*ras* proteins in vertebrates could therefore regulate other, still unidentified biochemical pathways.

It is not known if inducers of maturation other than progesterone decrease cAMP levels in Xenopus oocytes. $Ca^{2+}$ ionophores have been observed to trigger meiosis (Wasserman and Masui, 1975), and an increase in intracellular $Ca^{2+}$ concentration has been observed as an early response to progesterone and a variety of other inducers (Wasserman et al., 1980; Moreau et al., 1980). In addition, insulin, another inducer of maturation, affects intracellular pH and phosphorylation of the ribosomal protein S6 even in the presence of cholera toxin (Smith and Maller, 1984). Our studies are consistent with the idea that there is indeed an alternate pathway triggering meiosis in Xenopus oocytes that bypasses changes in intracellular cAMP levels.

The Xenopus oocyte has proven to be a useful system for testing a specific hypothesis concerning the function of the H-*ras* protein in vertebrates, and it may be valuable in elucidating the still enigmatic pathway on which this protein acts in higher eukaryotes. Since *ras* proteins bind guanine nucleotides and have weak structural similarity to the mammalian G proteins (Hurley et al., 1984; Lochrie et al., 1985; Medynski et al., 1985; Yatsunami and Khorana, 1985; Tanabe et al., 1985), which mediate signals from receptors to effectors, a role for *ras* proteins in a signal transduction pathway is an attractive hypothesis. The availability of antibodies that inhibit the action of *ras* (Mulcay et al., 1985) make it feasible to test whether *ras* communicates the signals induced by any of the hormones known to activate oocytes.

## Experimental Procedures

### Animals and Oocytes

Xenopus laevis were obtained from Xenopus I, Ann Arbor, Michigan. Ovarian fragments were surgically removed from animals that were anesthetized by hypothermia. Fully grown stage VI oocytes (Dumont, 1972) were manually dissected and kept in Barth medium (Maniatis et al., 1982). To remove follicle cells, the ovarian fragment was incubated for 1.5 hr with 1 mg collagenase/dispase (Boehringer Mannheim) per ml of Barth medium; the incubation was stopped by washing the fragment five times in 50 ml of Barth medium. The enzyme-treated oocytes were allowed to recover overnight before injection.

### Purification and Injection of H-*ras* Proteins

H-*ras* protein was purified as described from E. coli carrying the expression plasmids pSKT24 or pSKTcH-*ras* (Gross et al., 1985; Feramisco et al., 1984). The protein was stored in 20 mM Hepes (pH 7.4), 20 mM NaCl, 4 mM $MgCl_2$, 0.5 mM $\beta$MSH, and 5 $\mu$M GTP at high protein concentrations (3–10 mg/ml) at −70°C. The protein was diluted with 20 mM Hepes (pH 7.4), 50 mM NaCl, and 2 mM $MgCl_2$ to the desired concentration, and 50 nl of this solution was injected into the cytoplasm of Xenopus laevis oocytes as previously described (Kressmann and Birnstiel, 1980).

### Microscopy

Oocytes were fixed for 3 hr in the following solution: 25 ml saturated $HgCl_2$, 5 ml 5% trichloroacetic acid, and 15 ml formaldehyde. They were embedded in Technovit (Kulzer, Germany) as recommended by the producer. Serial sectioning and staining were performed as previously described (Hausen et al., 1985).

### Metabolic Labeling and Immunoprecipitation of H-*ras*$^{val12}$ Protein

E. coli carrying the expression plasmid pSKT24 were grown in M9 medium containing 0.5% glucose, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, 100 $\mu$g/ml Thiamine, 5 $\mu$g/ml Biotin, and 0.2 mM for all amino acids except methionine. At a culture density equivalent to an absorbance of 1.0 at 600 nm, the cells were centrifuged to form a pellet and resuspended in fresh medium, heat induced at 42°C, and incubated for 1 hr with 100 $\mu$Ci/ml $^{35}$S-met. The $^{35}$S-labeled protein was then purified as described above. The specific activity of the isolated H-*ras*$^{val12}$ protein was approximately 100,000 cpm/$\mu$g. A1-1 cells, a 3T3 cell line containing the activated human H-*ras* gene, were metabolically labeled with $^{35}$S-methionine overnight as described (Shimizu et al., 1983b). The cells were then grown for an additional 12 hr in media without radioisotope. A cleared lysate obtained from a nonionic detergent extract was used for immunoprecipitation with rat anti-*ras* monoclonal antibody Y13-259 (Furth et al., 1982). Immunocomplexes were collected with goat anti-rat antibody coupled to Sepharose 4B (Pharmacia). Details of this method were published previously (Powers et al., 1984). Sodium dodecylsulfate-polyacrylamide gel electrophoresis was carried out using the method of Laemmli (1970). After treatment with Enlightning (New England Nuclear), the gel was processed for fluorography.

### Determination of cAMP Levels

Oocytes were injected with 2 or 4 $\mu$Ci [$\alpha$-$^{32}$P]ATP and 10 ng protein each and incubated for the indicated times. Injection of [$\alpha$-$^{32}$P]ATP did not interfere with meiosis. The incubation was stopped by transfer of batches of 25 oocytes to ice cold 10% trichloroacetic acid, and 100 nmol of cAMP (unlabeled cAMP marker) and 100,000 cpm of $^3$H-cAMP were added. The oocytes were homogenized by sonication and centrifuged for 5 min at 10,000 ×g to remove insoluble material. An aliquot of the supernatant was removed to determine the total amount of soluble $^{32}$P-labeled material. The supernatant was neutralized by addition of 1 M Trisma base, applied to a Dowex 50W-4X column and eluted with $H_2O$. The eluate from the Dowex column was lyophilized, redissolved in $H_2O$, and subjected to two-dimensional thin-layer chromatography on polyethyleneimine cellulose plates, which were developed twice in the first dimension in 60 mM sodium acetate (pH 5.6), and once in the second dimension in 1.5 M $(NH_4)_2SO_4$. The unlabeled cAMP marker was located under UV light. The cAMP was eluted from the plates in 0.5 M $NH_4HCO_3$, and the salt was removed by repeated lyophilization in a speed-vac centrifuge. The eluted material was resuspended in 25 mM Tris (pH 7.4), 25 mM imidazole, 3 mM magnesium acetate, and 10 $\mu$M $CaCl_2$ and was divided into two equal aliquots. One aliquot was treated with phosphodiesterase from bovine brain in the presence of calmodulin (obtained from Sigma) to determine the amount of hydrolizable material, and the reaction was stopped by boiling. Imidazole was added to hydrolyzed and nonhydrolyzed samples to a final concentration of 0.1 M, and the samples were applied to a neutral alumina column. The amount of $^{32}$P and $^3$H in the eluate was determined by scintillation counting. Dowex and neutral alumina column chromatography were used according to Solomon et al. (1973), and thin-layer chromatography on polyethyleneimine cellulose plates according to Boehme and Schulz (1974).

In contrast to the $^3$H-labeled material, which could be hydrolyzed completely, 10%–30% of the $^{32}$P-labeled material could not be hydrolyzed by phosphodiesterase, the exact amount varying with each experiment. We attributed this to contamination still present after the extensive purification. We subtracted the amount of nonhydrolizable $^{32}$P-labeled material in the sample that was hydrolyzed from the value of $^{32}$P-labeled material obtained in the duplicate sample that was not hydrolyzed. The difference was assumed to represent [$^{32}$P]cAMP. The value thus obtained was adjusted for overall recovery, as judged from the amount of [$^3$H]cAMP recovered. Overall recovery was typically 30%. The percentage of [$^{32}$P]cAMP present in the total amount of soluble, $^{32}$P-labeled material was calculated.

In a control experiment, [$\alpha$-$^{32}$P]ATP was injected and incubated for either 15 min or 4 hr. Half of the injected oocytes were then treated with progesterone for 1 hr. A 68% and 71% drop of [$^{32}$P]cAMP was observed in response to progesterone for the oocytes preincubated for 15 min or 4 hr, respectively.

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