Regulation In Expression Of The mRNA-Binding Protein pp56 During Early Development Of Xenopus

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CONTENTS

ABSTRACT

Most of the mRNA synthesized during oogenesis in Xenopus is not translated in oocytes but is stored to be used later during early embryogenesis. This stored maternal mRNA is complexed with proteins, the two most abundant being phosphoproteins of 56 and 60 kD (pp56 and pp60). Both pp56 and pp60 are shown by UV cross-linking to be bound directly to the RNA but not to the poly(A) sequence which apparently binds a protein of 72 kD. Immunostaining of sectioned ovary with monospecific anti-pp56 reveals its location to be perinuclear in early oocytes with a more even cytoplasmic distribution later. Immunoblotting of protein from different developmental stages shows that pp56 is expressed throughout embryogenesis although it appears to be subjected to specific proteolysis during early stages. However the most dramatic changes relate to the levels of phosphorylation of pp56 and pp60 which are suppressed at stages during which maternal mRNA is being translated. Assays of mRNA-bound protein kinase activity and phosphatase activity support the interpretation that reversible phosphorylation is involved in the translational control of maternal mRNA. Finally, initial studies were conducted to identify possible, specific sequences, here the cyclin B1 message, masked by these phosphoproteins and contained within the stored mRNP fractions.

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INTRODUCTION

Xenopus laevis oocytes synthesize and accumulate vast quantities of messenger ribonucleic acid (mRNA), or maternal message, which is stored, bound with proteins, as messenger ribonucleoprotein (mRNP) particles, until utilization in embryogenesis (reviewed, Davidson, 1986). Production of these maternal gene products is necessary for the twelve rapid and synchronous cell divisions which occur from cleavage to mid-blastula (Laskey et al., 1979; Woodland, 1982); for it is at mid-blastula that the embryo cells begin to produce noticeable quantities of zygotic mRNA, thereby becoming less dependent on maternal translational material (Bachvarova & Davidson, 1966; Newport & Kirschner, 1982; Kimelman et al., 1987). In Xenopus oocytes, these maternal mRNA molecules appear to be stable over extended periods (Ford et al., 1977) and are prevented from being translated even in the presence of spare translational machinery (Taylor et al., 1985). Therefore special features are expected to attend the structural organization of the RNA molecules.

The non-polysomal mRNP which accumulates in Xenopus oocytes sediments at 40-80S and has a protein:mass ratio of 4:1. Most of the protein mass is comprised of a few polypeptides in the size range of 50-60kD which appear to be associated with all size classes of mRNP particle (Darnbrough and Ford, 1981; Cummings and Sommerville, 1988). On in vivo phospholabelling of oocytes and in vitro phospholabelling of isolated mRNP particles, two mRNP proteins, of 56 and 60kD are revealed to be the cell components most heavily phosphorylated during early oogenesis (Dearsly et al., 1985). Phosphorylation of pp60 and pp56 in the mRNP particles is correlated with tight binding of the phosphoproteins to oocyte mRNA and non-translation of mRNA both in oocytes and in in vitro translation systems (Kick et al., 1987). Since mRNA extracted from stored mRNP particles can be translated efficiently in vitro, the block to translation and stabilization of mRNA molecules would seem to depend more upon the presence of special blocking proteins than some deficiency in RNA structure (Richter and Smith, 1984). Since phosphorylaton of mRNP proteins is a consistent feature of non-polysomal particles, it is tempting to speculate that the phosphorylating enzyme, protein kinase, plays a role in regulating the availability of mRNA for translation. Indeed, in reconstitution experiments using the 60kD phosphoprotein and rabbit globin mRNA, tight interaction and non-translation in cell-free systems are achieved only as long as phosphorylation is maintained (Kick et al., 1987). Nevertheless, the proposition of reversible phosphorylation as a key mechanism in the regulation of translation of maternal mRNA requires a more detailed analysis of protein kinase activity not only during oogenesis but also through the early stages of embryogenesis when specific mRNA species are released for translation.

That the translational program during early embryogenesis may relate to the presence, and state of modification, of RNA-binding proteins is an intriguing possibility. Also important is the question of whether the mRNA proteins can be identified as known translation factors (Audet et al., 1987; Patrick et al, 1989) or whether the key regulatory proteins are peculiar to early development (Richter & Smith, 1983). Here the expression of the mRNP protein kinase and the mRNP phosphoproteins through oogenesis and early embryogenesis is examined and how changes in levels of expression might relate to the mobilization of maternal mRNA into polysomes is discussed. The relevance of the protein kinase and phosphoproteins to the masking of maternal mRNA in oocytes and release of mRNA for translation during early embryogenesis is examined in this study.

The objectives of this study were: to confirm that the phosphoproteins interact directly with mRNA sequences, to identify the sequences bound to the phosphoproteins, to assay the relative activities of the protein kinase throughout early development, when major changes in translational activity occur, and finally, to identify specific, blocked maternal mRNAs, namely the possibility of cyclin as a masked maternal message.

EXPERIMENTAL PLAN

In the initial experiments of this study, UV cross-linking was utilized to covalently bond mRNA to its contacting proteins in mRNP particles from previtellogenic (PV) ovary and maturing ovary (oocyte stages I-IV) and also in living XTC cells (representing the final stages of embryogenesis). After affinity chromatography on oligo-(dT)-cellulose to isolate cross-linked mRNP proteins, sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to identify these proteins through gel staining. Also in vitro phospholabelling prior to SDS-PAGE could reveal which of these proteins were phosphorylated. After identifying cross-linked phosphoproteins of 56 and 60 kD (pp56 and pp60, repectively) a more sensitive and discriminating assay of pp56 and pp60 was utilized by immunoblotting with polyclonal antibodies raised against mRNP phosphoproteins, followed by labelling of protein-antibody complexes with protein A (I^{125}).

The enzyme which phosphorylates the mRNA-bound proteins, pp60 and pp56, has previously been identified as a casein kinase II (LaRovere et al., 1989). An assay was developed to track the prevalence of casein kinase II through oogenesis and embryogenesis, for it is known that not the presence of these proteins, but rather their actual phosphorylation, inhibits translation (Cummings et al., 1989). mRNA particles isolated on oligo-(dT)-cellulose were destabilized in EDTA and then placed on a heparin-Sepharose affinity column to dissociate the protein kinase from the particles. Isolated kinase from the different stages of oogenesis and embryogenesis were then assayed for the capacity to phosphorylate B-casein using ^{32}P-γ-ATP as substrate.

Restriction of translation may also be governed by the location of mRNA in the cytoplasm of the cell. Immunostaining was utilized as a means to identify the mRNP particles regulated by pp56. XTC cells and ovary sections were probed with anti-pp56 followed by fluorescein isothiocyanite (FITC) to identify the specific location of the mRNP particle through fluorescence.

In order to conduct more specific and restricted studies, immunoprecipitation experiments were conducted to isolate mRNP particles containing the specific protein pp56. Antibodies, like anti-pp56, were used in conjunction with protein A on either S. aureus envelopes of Sepharose for isolation and later release of mRNA sequences. In this way a specific mRNA, cyclin message, could be probed for in various types of cell fractions.

Cyclin mRNA is an interesting message to examine in initial studies to identify mRNA

blocked in these mRNP particles. Much is known about oocyte Maturation Promoting Factor (MPF), cdc^2, cyclin expression and cyclin mRNA, but little is known about the mechanisms which mask and initiate cyclin mRNA translation (Newport and Kirschner, 1982; Murray and Kirschner, 1989; Murray et al., 1989; Minishull et al., 1989). These studies indicate the possible means to answer questions relating to the translational control of specific mRNA sequences.

MATERIALS AND METHODS

XENOPUS LAEVIS BREEDING

A mature female Xenopus laevis was injected with human chorionic gonadotropin (500 I.U.) to stimulate ovulation and placed in complete darkness for several days with her male counterpart to breed. Embryos were removed from the tank periodically and jelly coats detached with a 30sec. wash in 0.1M dithiothreitol(DTT) Embryos were then manually separated and selected through the various stages of embryogenesis according to Niewkoop and Faber, 1967. These stages -- cleavage (stage 3), blastula (stage 8), gastrula (stage 12), neurula(N2) (stage 20), tailbud (stage 27), and tadpole (stage 40 or 42) -- were frozen in HB (50mM NaCl, 10mM Tris, 2mM $MgCl_2$, 5mM 2-mercaptoethanol(2-ME), 8.5% glycerol) in aliquots of 25 embryos/0.25ml HB. The early development of X. laevis is shown in Figure 1.

TISSUE ISOLATION AND RADIOLABELLING

Ovaries were excised from both previtellogenic and mature female Xenopus laevis and washed in modified Barth's solution -- 88mM NaCl, 1mM KCl, 2.4mM $NaHCO_3$, 0.82mM $MgSO_4$:7 H_20, 0.33mM $Ca(NO_3)_2$:4 H_20, 0.41mM $CaCl_2$:6 H_20, 7.5mM HEPES-NaOH pH7.6, plus 5 units of penicillin, streptomycin and kanamycin per ml and phenol red as a pH indicator (Gurdon, 1974). Individual oocytes were released from this tissue through stirring at room temperature for 2hrs. in 0.2% collagenase in OR-2 minus Ca^{2+} -- 82.5mM NaCl, 2.5mM KCl, 1mM $MgCl_2$, 1mM Na_2HPO_4, 5mM HEPES, 0.5% polyvinylpyrrolidone, 3.8mM NaOH, pH7.8 -- followed by three washes in OR-2 minus Ca^{2+}, then three washes in modified Barths (Rungger and Turler, 1978). Specific oocyte developmental stages were then selected manually according to Dumont(1974): stage I, clear (0.1-0.2 mm); stage II, white (0.3-0.4 mm); stage III, fawn (0.5-0.6 mm); stage IV, black/green (0.7-0.8 mm); stage V, brown/green (1.0-1.2 mm). Oocyte stages are represented also in Figure 1.

Both individual oocytes and whole ovary tissue were often in vivo radiolabelled with ^{32}P or ^{3}H. Some were incubated in Barths for 6hrs. at 18^0C in the presence of (^{32}P)phosphate (500μCi/ml, Amersham International). Other tissue was incubated with ^{3}H-adenosine, ^{3}H-cytosine, or ^{3}H-uridine (0.5mCi/ml) in Barths for 40-72hrs. at 18^0C.

CELL CULTURE

XTC-2 cells (Pudney et al, 1973) were grown at 25^0C in GMEM media(6.4g/l NaCl, 0.4g/l KCl, 2.75g/l Na_2CO_3) supplemented with 10% foetal calf serum, 2% penicillin, 2%

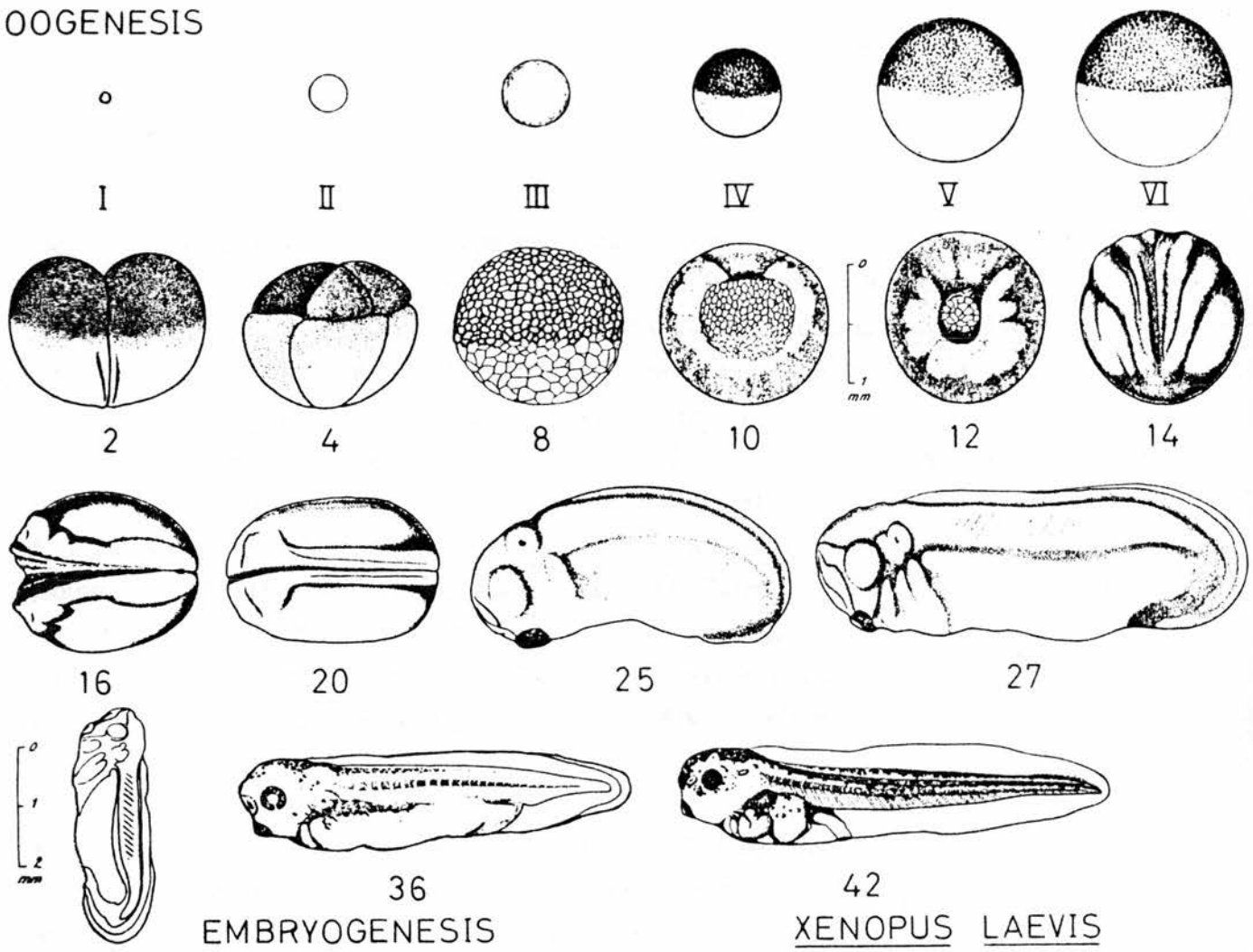

FIGURE 1. Xenopus laevis developmental stages of oogenesis (Dumont, 1974) and embryogenesis (Niewkoop and Faber, 1967).

streptomycin and 1% L-glutamine in an atmosphere of 5% CO_2 in air.

ANTIBODIES

Antibodies were raised in rabbits against the 56kD and 60kD polypeptides of mRNP particles of Xenopus laevis as described previously (Dearsly et al., 1985).

IMMUNOSTAINING

Both XTC cells grown up on coverslips and sectioned ovary were immunostained with anti-pp56 and anti-pp60. XTC cells were fixed in methanol for 15min. followed by acetone for 5min., all at -20^{0}C. Ovary was quick frozen with CO_2 under pressure, sectioned in 10micron increments on a Reichert-Jung (West Germany) cryostat machine at -18^{0}C, placed on subbed coverslips and fixed for 10min. in formaldehyde vapor. All coverslips were then washed at room temp. in phosphate-buffered saline solution(PBS) 3 times, 10min. each, before incubating in antibody dilutions of 1/20 to 1/100 for 45min. at room temp. Coverslips were rewashed as before in PBS and then incubated in fluorescein isothiocyanate(FITC) conjugated goat anti-rabbit IgG diluted to 1/50 in PBS for 45min. at room temp. in complete darkness. Slips were washed once again as before and finally mounted on slides with 90% glycerol in TBS. All samples were examined under a Zeiss fluorescence microscope and photographed using TMAX 400 film.

RNP ISOLATION FROM OVARY, OOCYTES AND EMBRYOS

Ovary, staged oocytes and embryos were either sonicated in HB or homogenized manually on ice with 20 strokes of a teflon/glass homogenizer. The homogenates were clarified by centrifugation at 10K for 10min. at 0^{0}C and the supernatants(SN^{10}) were then either layered on glycerol gradients or bound to oligo-d(T).

32ml. gradients of 15-40% glycerol in HB were prepared, supernatants layered on and tubes balanced. Gradients were generally spun at 18,000rpm for 16hrs. at 2^{0}C in a Beckman 6 X 40ml swing-out rotor and then fractionated into 1 to 1.5ml aliquots while being monitored by a U.V. analyser at 254nm.

Unlabelled gradient fractions were often used for in vitro phospholabelling. Fractions were made up to 2mM $MnCl_2$, followed by the addition of 4μCi (2pmole/ml) of [γ-^{32}P]ATP (3,000 Ci/mmole, Amersham International). Tubes were incubated at room temperature for 1hr. before centrifuging at 37,000rpm for 4hrs. at 0^{0}C in an MSE 10 X 10 ml angle rotor to pellet mRNP particles.

Oligo-(dT)-cellulose was swollen in dH_2O and then equilibrated in RNP Buffer 1(100mM

NaCl, 10mM Tris, 2mM $MgCl_2$, 5mM 2-ME) or RNP Buffer 2(200mM NaCl, 10mM Tris, 2mM $MgCl_2$, 5mM 2-ME, 8.5% Glycerol). Samples of mRNP particles, generally 50µg RNP in 1ml buffer, were loaded into Eppendorf tubes with 0.5ml oligo-(dT)-cellulose and shaken for 15min. Reactions either continued in these tubes or were transferred onto a column packed with glass wool and oligo-d(T)-cellulose Several washes were conducted in the respective equilibrating buffers to collect unbound material. Some reactions conducted in RNP Buffer 1 were then incubated for 30min. with 10λ of RNase A(1mg/ml) to cleave the RNP from the poly(A) tail; and then eluted in Elution Buffer 1 (EB) (10mM Tris, 5mM 2-ME, 2mM EDTA), followed by 20% deionized formamide in EB to elute the poly(A) tail. Formamide samples were dialyzed for 1-2hrs. against EB. Reactions conducted in RNP Buffer 2 were eluted with 60% deionized formamide in either dH_20 or RNP Buffer 2. These elutes were dialyzed against ImmunoHB (200mM NaCl, 10mM Tris, 2mM $MgCl_2$, 5mM 2-ME, 8.5% Glycerol, 0.5% NP-40).

RNP ISOLATION FROM XTC CELLS

GMEM media was drained from the tissue culture flask and replaced with 10ml of Cell Wash(80mM NaCl, 10mM KCl, 2mM $MgCl_2$, 1mM $CaCl_2$, 10mM PBS) or Barths. Some flasks were then incubated with 0.25mCi of ^3H at 25^0C for 90min., washed in GMEM media and raised in Cell Wash or Barths. All flasks were then scraped with a rubber policeman and drained into centrifuge tubes. Cells were centrifuged at 2,000rpm for 10min., drained and raised in HB with the addition of 500 units/ml Human Placental Ribonuclease Inhibitor(HPRI) and 0.5% Deoxycholate(DOC) before sonicating. Samples were again centrifuged at 10,000rpm for 10-60min. Supernatants were run on glycerol gradients and oligo-d(T) cellulose as above.

UV CROSS-LINKING OF mRNA TO ASSOCIATED PROTEINS

Oocytes sonicated directly in UV buffer (50mM NaCl, 2mM $MgCl_2$, 10mM Tris, 5mM 2-ME), as well as gradient fractions pelleted and raised in UV buffer, were UV irradiated to cross-link mRNA to associated proteins. Samples, generally of 400λ, were placed on ice in 3cm. diameter watch glasses, stirring continuously, and 0.1mM of protease inhibitors PMSF and PCMB was added. Ultraviolet light with a wavelength of 260nm and producing a measured output of 250ergs/mm^2/sec was generated from two lamps contained in a silver foil-lined box. Irradiation times lasted for 1sec. to 45min with an optimum of 30min. Cross-linked material, equilibrated to 0.5% SDS and 0.5M NaCl, was often run through oligo-(dT)-cellulose to further purify the mRNP. The affinity chromatography procedure was identical to that described above, however a different RNP Loading Buffer was utilized (0.5M NaCl, 10mM Tris, 1M EDTA, 0.5% SDS) and samples were denatured at 60^0C for 3min before mixing with oligo-(dT)-cellulose. Once the particles are cross-linked, SDS can be present to denature all proteins and prevent any fortuitous protein binding to RNA.

IMMUNOPRECIPITATION OF RNP PARTICLES

Either anti-pp56, anti-p48, or anti-goat serum was added at a dilution of 1:50 to RNP samples and incubated for 1-2hrs. on ice. Samples were precipitated with either protein A - Sepharose (50µl/ml) or Staphalococcus-aureus envelopes (50µl/ml). Protein A - Sepharose was swollen in dH_20, washed and equilibrated in ImmunoHB. S. aureus was centrifuged, drained, and also rinsed and equilibrated in ImmunoHB. Either form of protein A was added. Samples were incubated at 4^0C for 1-2hrs. with constant rotation, beads collected through centrifugation, and supernatants collected. Beads were washed twice with ImmunoHB and supernatants were collected. Bound RNP particles were released by the addition of SDS to 1% in ImmunoHB.

PROTEIN KINASE ASSAY

A protein kinase assay was developed to examine oocytes at stages 1, 3 and 5, and embryos at cleavage, blastula, gastrula, neurula, stage 27 and stage 42. RNP particles were isolated similar to as before. Briefly, 25 oocytes or embryos were sonicated in 250λ HB, spun at 10,000rpm for 10min., added to 500λ oligo-(dT) in RNP Buffer 3 (100mM NaCl, 10mM Tris, 5mM 2-ME, 2mM $MgCl_2$) and shaken for 10-30min. on ice. Samples were centrifuged at 2,000rpm for 2min. and supernatants(SN^2) collected. RNP Buffer 3 was added again, shaken for 10min. on ice, SN^2 and collected. RNP particles were then isolated with 60% deionized formamide in Elution Buffer(10mM Tris, 5mM 2-ME, 2mM EDTA), which was added, shaken on ice for 10min., SN^2 and collected. This elution was repeated twice.

Protein kinase was then isolated from these particles through affinity chromatography on Heparin/Sepharose. Oligo-(dT) fractions were dialyzed against Kinase Loading Buffer(KLB) (25mM Tris, 5mM 2-ME, 2mM EDTA). Each was then added to 500λ of Heparin/Sepharose in KLB, shaken for 10-30min., SN^{10} and collected. Kinase Wash Buffer(KLB plus 0.3M NaCl) was added next, shaken for 10min., SN^{10} and collected. This wash was repeated. Finally, protein kinase was eluted by the addition of Kinase Elution Buffer(KLB plus 1M NaCl), which was shaken for 10min., SN^{10} and collected. This also was repeated.

Eluted fractions were assayed for the capacity to phosphorylate B-casein using ^{32}P-γ-ATP as substrate. Each fraction was split into two. One half was made up to 0.5µg/ml of heparin; the other was left as is. Reaction mix was then added to each tube and left incubating at room temp. for 5-100min., with an optimum incubation of 60min. Reactions were run in either Reaction Mix 1 (2λ 1mM ATP, 4λ [γ-^{32}P]-ATP, 11λ 1M $MgCl_2$, 360λ B-Casein(0.5mg/ml), 540λ dH_20) or Reaction Mix 2 (60λ 2mM ATP, 5λ [γ-^{32}P]-ATP, 72λ 1M $MgCl_2$, 600λ B-Casein(2mg/ml), 7.26ml dH_20). For one study, additional cold ATP,

see Fig 16 , was added. After incubation, reactions were stopped by freezing after the addition of 10λ 2mM ATP andd 50λ BSA(1mg/ml).

PROTEIN PHOSPHATASE ASSAY

Assays for phosphatase activity and the presence of inhibitors of protein kinase activity were carried out in conjunction with Alison Cummings. Experimental methods are detailed in Cummings et al., 1989.

TRICHLOROACETIC ACID (TCA) PRECIPITATION

The radiolabel of samples from several experiments was quantitated through scintillation counting. Samples were either pipetted or filtered with suction onto Whatman paper disks, glass fibre filters, or nitrocellulose. Filters were precipitated in 10% tri-chloroacetic acid (TCA) on ice twice for 15min. each and then rinsed 5 times for 2min. each in 5% TCA on ice, followed by 2 rinses in chilled 96% ethanol. After drying, filters were placed in scintillation vials with 5ml of dH_20 for ^{32}P or 5ml of toluene for ^{3}H to be scintillation counted.

PROTEIN SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

After ethanol precipitation of mRNP samples, 10λ of ribonuclease mixture -- RNase A (100u/ml), RNase T_1 (200u/ml) and RNase T_2 (200u/ml) -- was added to each dried pellet and left to digest for 1hr. at room temperature. 30λ of SDS sample buffer -- 1% SDS, 5% 2-Mercaptoethanol(2-ME), 5% glycerol, 10mM Tris, pH6.9 -- along with 5λ of saturated bromophenol blue in urea solution were aliquoted to each Eppendorf and samples were then denatured at 95^0C for 5min. before loading.

Proteins were separated on 12% polyacrylamide gels. The separating gel consisted of 12ml 60% acrylamide, 22.5ml 1M Tris-SO_4, 8.5ml glycerol, and 17ml dH_20, which was degased. 0.9ml of 10% SDS was added, followed by 150λ 25% ammonium persulphate(AMPS) and 120λ tetramethylethylene diamine(TMED) to catalyze polymerization. For the stacking gel, 1ml 60% acrylamide, 1.25ml 0.5M Tris-SO_4 and 7.5ml dH_20 were degased with the later addition of 0.15ml 10% SDS, 50λ25% AMPS and 60λ TMED. Gels were run at 80V for 18hrs. in a running buffer of 15g Tris, 72g glycine and 7.5g SDS made up to 5L with dH_20. After separation, gels were placed in coomassie blue stain solution -- 1g coomassie blue, 100ml acetic acid, 400ml methanol, 500ml dH_20 -- for several hours until gel was sufficiently stained. It was then washed twice in destain solution -- 100ml acetic acid, 250ml methanol, 650ml dH_20 -- for a total of 18hrs. Gels were then dried under vacuum and heat, and subjected to autoradiography.

PROTEIN TRANSFER AND IMMUNOBLOT

After SDS-PAGE, some gels were transferred onto nitrocellulose by a 30V charge for 18hrs. in a running buffer of 15g Tris, 43g glycine, 600ml methanol and 1.6l dH_20. Filters were incubated in 0.9% NaCl, 10mM Tris-HCl, pH7.4, 5% bovine serum albumin(BSA) for 45min. at room temp. with agitation. They were then incubated with either anti-pp56 or anti-pp60 in 1:25 or 1:10 dilutions, respectively, in the same buffer for 2hrs. at room temp. Blots were given a series of washes of 10min. each -- one in Tris-Saline, two in Tris-Saline with 0.5% Nonidet P-40(NP-40), one in Tris-Saline -- before labelling with Protein A (I^{125}) (1μCi) in BSA Tris-Saline for 45min. at room temp. They were washed as above, air dried and autoradiographed.

RNA ISOLATION AND SLOT BLOT

RNA was isolated and purified from protein and cell debris through phenol/isoamyl alcohol/chloroform (PIC) extraction, followed by ethanol precipitation. Samples were first made up to 0.1mg/ml Proteinase K in 0.1% SDS and incubated for 30min. at room temp. Cloroform and isoamyl alcohol were mixed in a ratio 24:1 and then mixed 1:1 with phenol. Equal volumes of sample and PIC were mixed and centrifuged at 2,000rpm for 5min., with the lower phenol phase discarded. This procedure was repeated a second time to further purify the RNA, with the upper aqueous phase transferred to a clean tube. Samples were then precipitated at -20^0C for several hours in 2.5 volumes of ethanol; centrifuged at 10,000rpm for 20min.; drained; dried and raised in 100λ depc dH_20. Quantitation and purity of RNA was estimated through O.D.(260/280) readings of 1/200 dilutions on a UV spectrophotometer. RNA samples were then mixed with equal volumes of Denaturation Solution (170λ deionized glyoxal, 20λ 0.5M NaH_2PO_4, pH6.5, 310λ dH_20), incubated at 50^0C for 1hr. and chilled on ice ready to load.

Nitrocellulose was prewet in dH_20, followed by 20X SSC, before placing in Manifold Apparatus. Wells were rinsed with 2X SSC, samples loaded, and wells rinsed again. Filters were air dried before baking in the vacuum oven at 80^0C for two hours. They were then rewet with 20mM Tris-HCl, pH 8 at 100^0C to deglyoxylate and allowed to cool to room temperature.

NORTHERN GEL ELECTROPHORESIS

Isolated RNA, used in slot blot analysis, was also separated through gel electrophoresis on 1.4% agarose gels. 2.1gms. of agarose was heated in 15ml. of 10X MOPS(Morpholinopropanesulfonic acid) and 132ml. of dH_20 until dissolved and then cooled to 60^0C before adding 2.7ml. of 37% formaldehyde solution and 12μg of ethidium bromide. Gels were poured and allowed to set while samples were prepared. 25μg of each sample were mixed with 24ug of Blue Juice Mix (80λ 10X MOPS, 80λ saturated bromophenol blue, 130λ 37% formaldehyde, 360λ deionized formamide) and denatured at

65^0C for 5 min. Samples were centrifuged briefly, quick chilled on ice and then loaded. Gels were run at 20Volts and 12mAmps for 18hrs. in a continuous running buffer consisting of 20mM MOPS, 5mM NaOAc, and 1mM EDTA at pH 7.0. Gels were photographed under UV transilluminator with FP4 ASA 100 film. They were then rinsed in 20X SSC, diffusion blotted onto nitrocellulose for 24hrs, and baked in the vacuum oven at 80^0C for two hours.

RIBOPROBES

Dr. Tim Hunt, Cambridge University, provided cyclin B1 antisense templates, JS23(5mg/ml) and R3(11mg/ml), in pGEM with a T7 promotor, for radioactive RNA synthesis in vitro for use in probing Northern and slot blots. Either 0.8λ JS23 or 0.4λ R3 was mixed with 1λ EcoR1 buffer and made up to 10λ with dH_20. 0.5λ EcoR1(6U) was added to each reaction to cut the vector and incubated at 37^0C for 15 min., then placed on ice. T7 mix was assembled -- 10λ linearized DNA, 20λ 5X salts, 20λ 5X NTPs(20mM stocks -- 20λ ATP, 20λ GTP, 20λ CTP, 5λ UTP, 35λ dH_20), 2λ ^{32}P-UTP(20μCI), 5λ BSA(0.1mg/ml), 4λ HPRI, 5λ dithiothreitol(DTT) (0.1M), 33λ dH_20, 1λ T7 RNA polymerase -- and placed at 37^0C for 45 min. 2.5λ 0.5M EDTA (pH7.5) were added to the reaction before phenol extracting twice. The aqueous phase was transferred to a clean tube with the addition of 5λ 5M NaCl and 250λ 96% EthOH to be precipitated for several hours at -20^0C. Precipitates were then centrifuged at 10K for 20 min., supernatants discarded, RNA pellets dried and raised in 100λ dH^20. 2λ were counted.

HYBRIDIZATION AND AUTORADIOGRAPHY

Hybridization Buffer - 50% deionized formamide
5X SSC (standard sodium citrate)
50mM sodium phosphate, pH6.5
100μg/ml herring DNA
5X Denhardt's (0.1% Ficoll, 0.1% BSA,
and 0.1%polyvinylpyrrolidone)
Mix 4X the above solution with
1X 50% dextran sulphate

Nitrocellulose blots were prehybidized in Hybridization Buffer(1ml. per $10cm^2$) at 60^0C for one hour, and then hybridized with the riboprobe(1,000,000 counts/ml) in the same buffer at 60^0C for 16hrs. Blots were washed in 2X SSC/0.1% SDS at 60^0C and autoradiographs were established using Kodak X-omat RP film with intensifier screens at -70^0C. Following exposure, films were developed for 2-4min. in Kodak D19 developer, washed in water, and fixed for 5min. in Kodak Unifix.

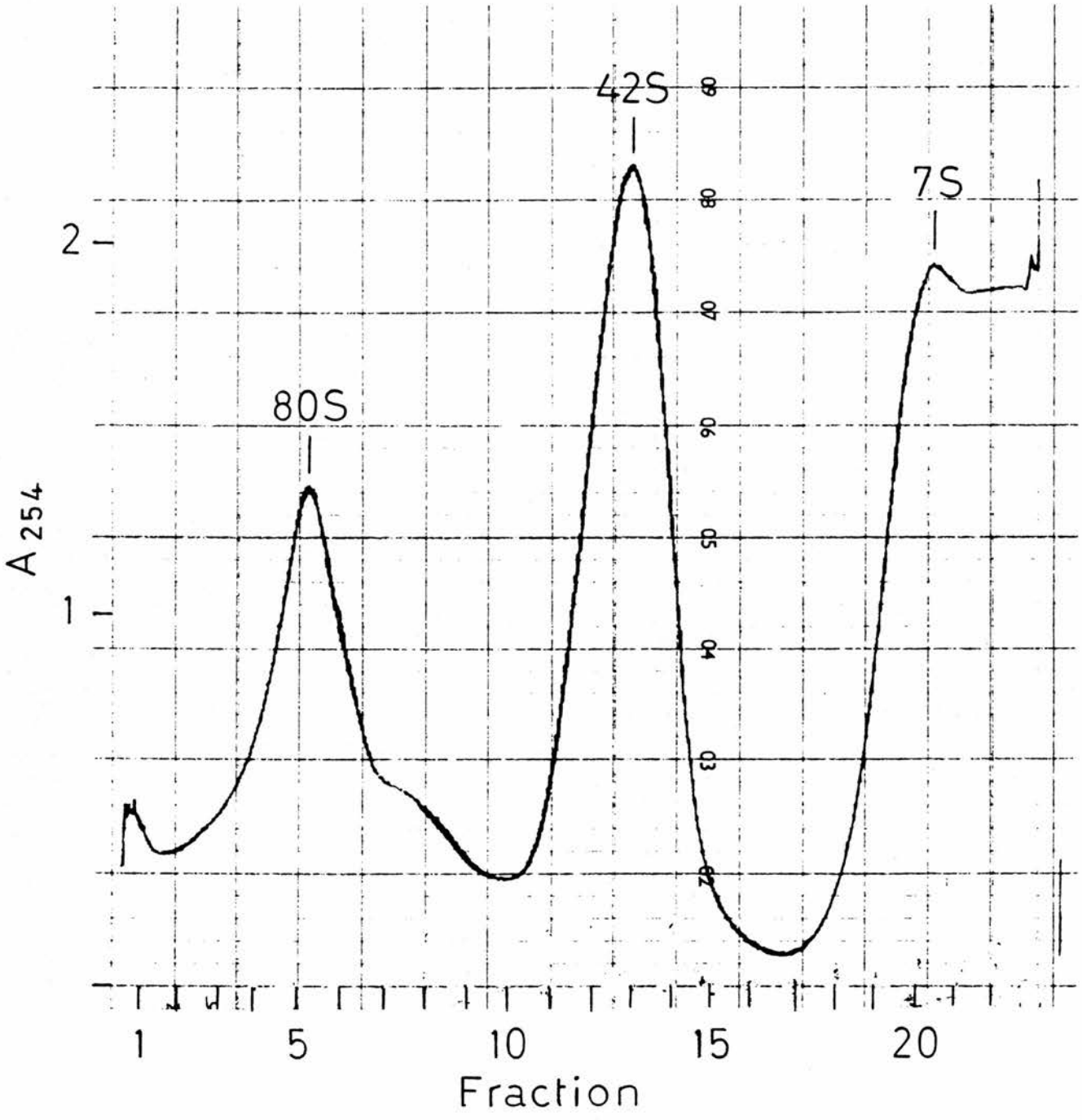

FIGURE 2. Separation of RNP particles derived from stage I (previtellogenic) oocytes on a glycerol gradient containing 50mM NaCl. Monoribosomes sediment at 80S, the storage particle containing 5S RNA and tRNA at 42S and the storage particle containing 5S RNA alone at 7S. Free mRNP particles sediment between the 42S and 80S peaks and mRNP-ribosome complexes sediment at 80-100S (Cummings and Sommerville, 1988). Polyribosomes sediment at greater than 100S.

RESULTS

FRACTIONATION AND DESCRIPTION OF GLYCEROL GRADIENT PARTICLE PEAKS Figure 2.

On sedimenting homogenates of oocytes (normally at stage I or previtellogenic, PV) through linear gradients of 15-40% glycerol, individual peaks of absorbance at 254nm can be discerned. The peak at 80S represents monoribosomes. Whereas this peak is relatively small in PV oocytes, it grows substantially at stage II through to full grown (stage VI) oocytes. This increase reflects the production of ribosomes due to increased nucleolar activity (see also Fig.26). A second peak at 42S represents storage particles for 5S RNA and tRNA which are present maximally at stage I and have been degraded by stage III, as 5S RNA (and tRNA) is incorporated into ribosomes. The peak at 7S represents stored 5S RNA plus its bound protein. Messenger ribonucleoprotein (mRNP) particles occur in free form over the sedimentation range of 40-80S (Darnbrough and Ford, 1981), whereas ribosome-bound mRNP occurs in the range of 80-100S (Cummings and Sommerville, 1988). Polyribosomes are present in relatively small numbers in oocytes (see also Fig.26) and would sediment at greater than 100S.

IDENTIFICATION AND DESCRIPTION OF MESSENGER RIBONUCLEOPROTEINS

Most of the mRNA synthesized during oogenesis in Xenopus is not translated in oocytes, but is stored to be used later during early embryogenesis. This stored maternal mRNA is complexed with a simple set of abundant proteins (Fig.3a), two of which, at 56 and 60kD, remain the proteins most tightly bound to poly(A)$^+$ RNA isolated on oligo-(dT)-cellulose (Fig.3b). However, on incubating oocyte mRNP particles with poly(A)-Sepharose, the protein bound most effectively to the resin is one of 72 kD (Fig.3c). It would appear that the 56 and 60 kD proteins are bound generally to mRNA, whereas the 72kD protein specifically interacts with the poly(A)-tail. In addition to these major proteins, the 100kD protein is probably translation Elongation Factor-2 (EF-2), whereas the 50kD component is probably EF-1 . The other proteins at 75kD and 40kD are so far unidentified.

To ascertain the role played by these proteins in the stabilization, localization and translation of maternal mRNA, we have examined their expression through oogenesis and early embryogenesis, as recorded below. We have been helped in this analysis by the fact that the 56 and 60 kD components are heavily phosphorylated in oogenesis and so can be traced easily by labelling either in vivo (with ^{32}P-phosphate) or in vitro (with ^{32}P-γ-ATP).

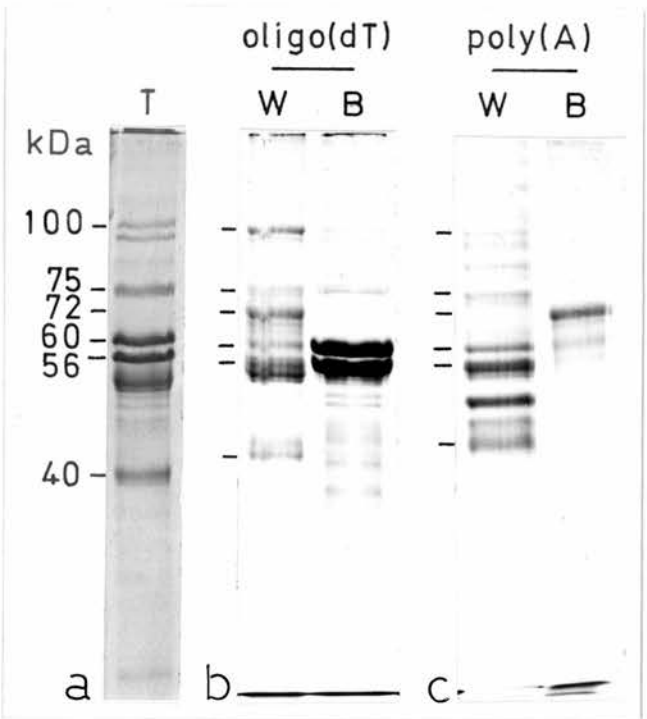

FIGURE 3. Protein components of free mRNP particles derived from the 60-70S region of a glycerol gradient similar to that shown in Figure 2. Proteins were separated by SDS-PAGE. Track T shows total protein on a stained gel. On binding the mRNP particles to an oligo(dT) affinity column, loosely bound protein can be washed off with 1M NaCl (track W).. Proteins tightly bound to polyadenylated mRNA are eluted with 60% formamide (track B). On applying the mRNP particles to a poly(A)-Sepharose affinity column polyadenylated mRNA will not bind, but proteins with an affinity for the poly(A) sequence will bind. Fractions eluting with 1M NaCl (track W) and 2M guanidinium hydrochloride (track B) are shown. A 72kD protein appears to be the most tightly bound component (Baer and Kornberg, 1983; Sachs et al, 1986).

UV CROSSLINKING

Experimental Strategy

In sufficient dose, 254nm UV light provides enough energy to induce the photoactivation of RNA. This RNA, in its short-lived reactive state, will now covalently bind all proteins which directly contact it; thus crosslinking the RNA to the protein and stabilizing the RNP particle. Given the short radius of this UV light source, we are virtually guarenteed that only contacting proteins are bound. This procedure has been used to identify proteins bound to hnRNA (Dreyfuss et al., 1988): here it is used to identify proteins bound to oocyte mRNA.

Experimental details of the UV crosslinking procedure are listed in the Materials and Methods section. However, a general description of this technique, as well as the labelling and further isolation of the mRNP particles, is presented in Figure 4. Previtellogenic(PV) ovary, mature oocytes and XTC cells were either sonicated or homogenized, centrifuged through glycerol gradients and fractionated. Specific gradient fractions, as described above, which contain the mRNP particles, were then pooled together and incubated with ^{32}P-γ-ATP. This radiolabel will only be incorporated into phosphorylated proteins and thus allows these proteins to be traced and later identified.

^{32}P labelled mRNP proteins were covalently cross-linked to their corresponding mRNAs through irradiation with 254nm UV light. Proteins were then heat denatured in the presence of SDS and NaCl, and affinity selected on oligo-(dT)-cellulose. This affinity chromatography purifies the mRNP particles from other kinds of RNP material and any unbound proteins. Oligo-(dT) -- which binds the poly(A)-tail of the particle-associated mRNA -- with cellulose holds the cross-linked mRNP complex while all unbound proteins are eluted in high salt and 1% SDS. RNase A, which cleaves at pyrimidine (uridine and cytosine) residues, then releases the mRNP fragments from its associated poly(A)-tail which remains bound to the matrix. The poly(A)-tails with any UV-linked proteins were then eluted with 20% deionized formamide, which denatures the poly(A):oligo-(dT) base pairing. Cross-linked proteins were then released from their complexes through the digestion of the associated mRNA with RNases. RNase T_1, which cleaves A residues, was added particularly to dissociate the poly(A)-tail. Released and purified mRNP proteins could then be separated and identified through SDS-PAGE followed by autoradiography to detect phospholabelled proteins. Thus this technique allows a definitive isolation of those proteins which are bound to the mRNA in the cytoplasm of the cell.

Time Dose Response Curve

Cross-linking of mRNA to protein was assayed through increasing levels of UV irradiation

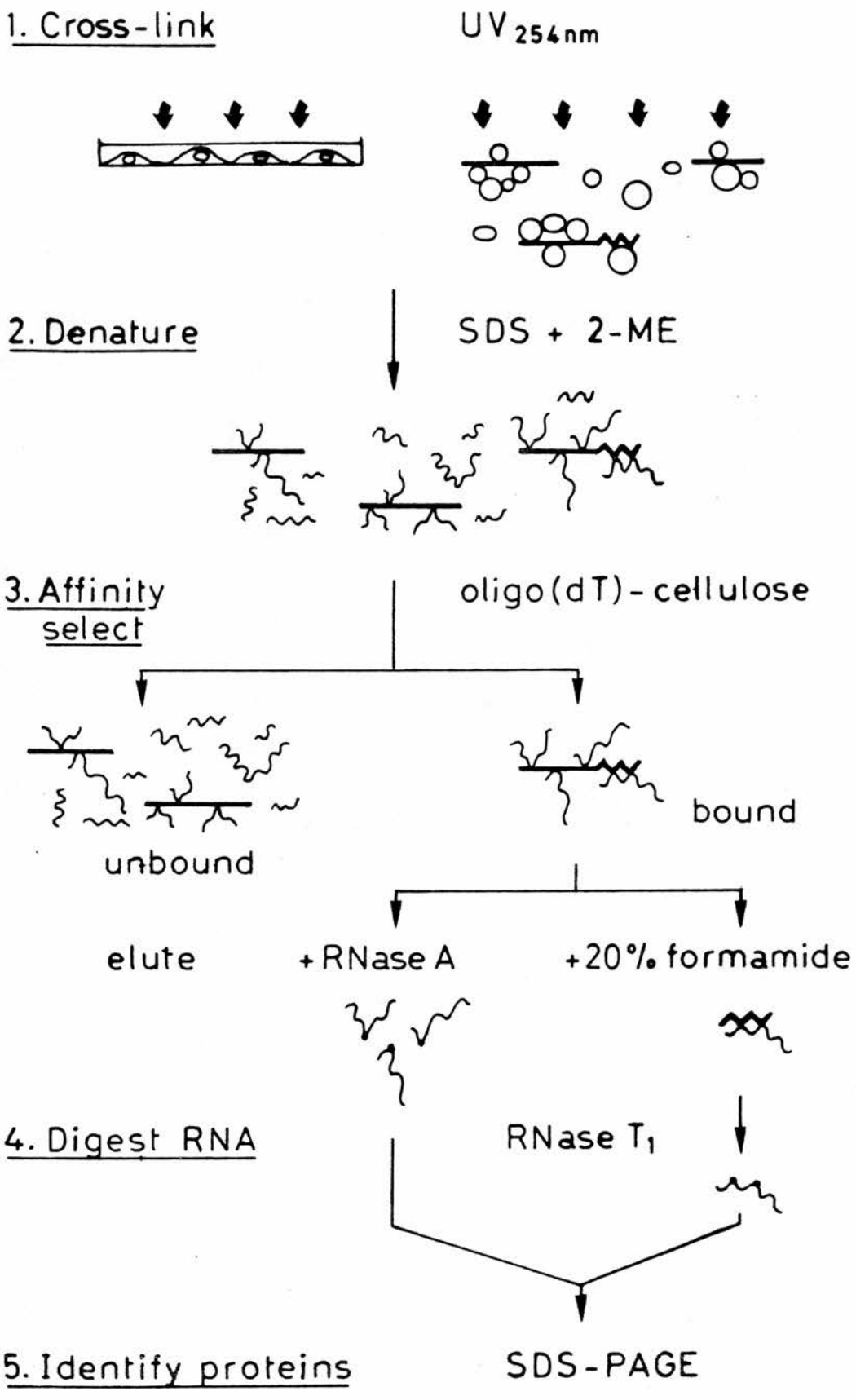

FIGURE 4. Experimental strategy for UV-crosslinking and identification of proteins crosslinked to polyadenylated RNA.

in order to establish the optimum dosage. Both XTC cells and PV ovary were pulse-labelled in vivo with ^{3}H uridine. PV ovary was sonicated, while XTC cells were left intact; thus, irradiation of oocyte components was in vitro, while irradiation of XTC cells was in vivo. All material was subjected to 254nm UV light generating an output of 250ergs/mm^2/sec through increasing periods of time, with XTC samples taken at 1, 4, 100, 400, 1,000 and 4,000 secs, and oocyte samples removed after 0, 100, 300, 600, 1,200 and 2,400 secs irradiation. Cross-linking was quantitated by comparing total ^{3}H RNA at each time point with ^{3}H RNA loss from the aqueous phase after PIC extraction in the presence of SDS and NaCl. All cross-linked mRNA would be partitioned to the phenol phase with its accompanying proteins, thus presenting a loss of radioactive counts in the aqueous phase. The percentage of RNA cross-linked is measured by the equation $(x-y)/x$, where x is the trichloroacetic acid-precipitated [^{3}H]uridine radioactivity of the total irradiated sample and y is the precipitated radioactivity from the aqueous phase of the corresponding extracted, irradiated sample. This percentage is graphed against its accompanying dose in Figure 5 and indicates that there is a dose-dependent shift of mRNA to the phenol phase both in XTC cells and oocyte mRNP particles, which increases with progressive UV irradiation, despite the presence of SDS, which under normal circumstances would strip proteins from RNA. The percentage of cross-linked RNA plateaus by 10^6 ergs/mm^2. Continued irradiation risks sample evaporation.

Run 1

The initial cross-linking study was conducted on the 60-70S sedimenting fraction (free mRNP particles) selected from a glycerol gradient of PV ovary. This fraction is indicated in the UV trace in Fig.2. After in vitro labelling with ^{32}P-γ-ATP and subsequent cross-linking for 30min as described in Materials and Methods, the irradiated sample was purified through affinity chromatography on oligo-(dT)-cellulose. Each eluted fraction was Cerenkov counted, with the calculated ^{32}P radioactivity plotted in the accompanying graph (Fig.6a). As is evident, there are three peaks, each representing a release of material from the oligo-(dT)-cellulose column. The first peak represents all unbound RNA, as well as any free nucleotides, which can be seen from the height of this peak. The column was washed down to background through six fractions, followed by the addition of 20% formamide to elute fractions 7-9 with a peak at fraction 8. To ensure complete elution of the bound mRNP particles, 60% formamide was also applied and collected in fractions 10-12. This was followed by elution in 0.1M NaOH for fractions 13-15 to hydrolyze any remaining RNA. This peak is in fraction 14.

After RNase digestion, fractions 2, 3, 8, 9 and 14 were examined by SDS-PAGE, with the resulting autoradiograph shown in Fig.6b. The only phosphorylated proteins are present in lane 8 at 56 and 60 kD. These represent the proteins cross-linked to mRNA in the mRNP

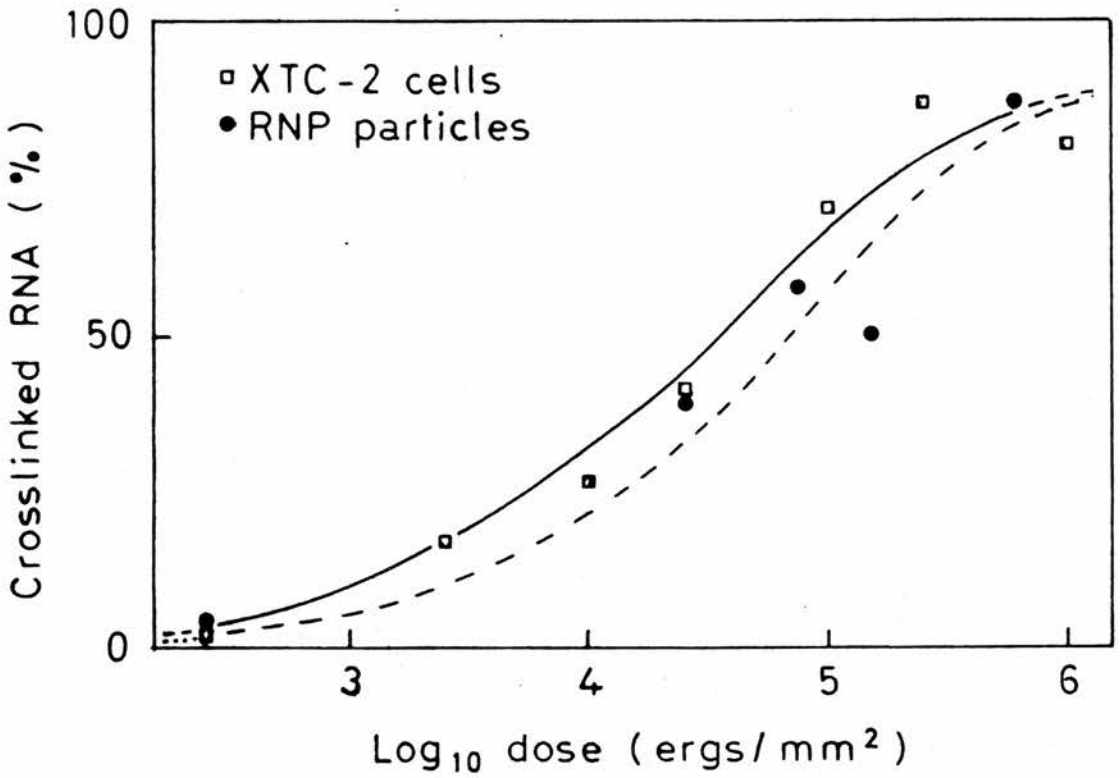

FIGURE 5. Dose response curve for UV-crosslinking of RNA to protein in XTC-2 cells and in RNP particles derived from previtellogenic ovary.

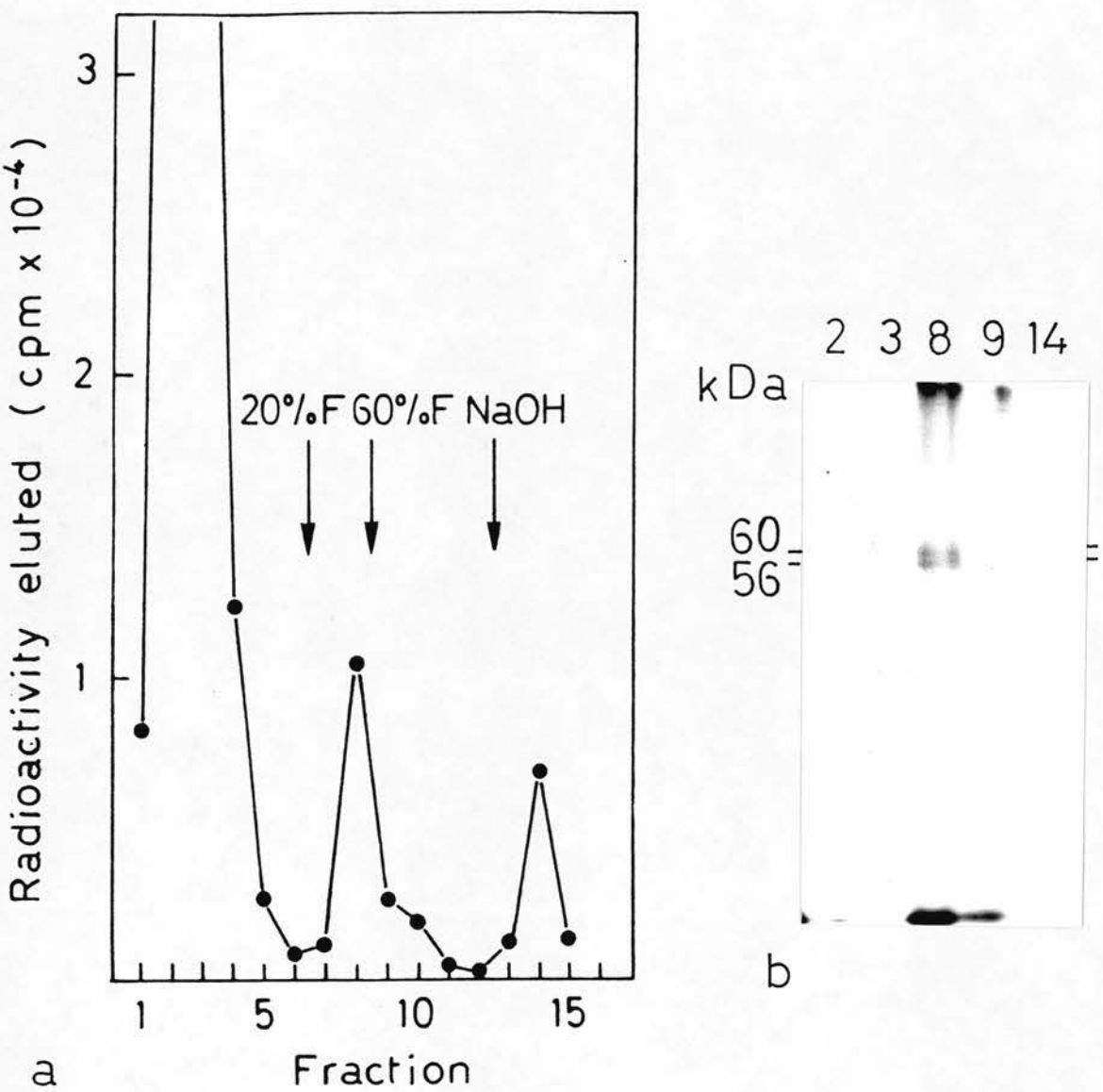

FIGURE 6. (a) Elution of phospholabel from an oligo(dT) column after labelling of mRNP particles (60-70S) from previtellogenic oocytes with ^{32}P-γ-ATP and UV-induced crosslinking. Elution steps are with 20% and 60% formamide and the column is finally washed with 0.1M NaOH. (b) Autoradiograph after SDS-PAGE of eluted fractions 2, 3, 8, 9 and 14. The 60kD and 56kD phosphoproteins (pp60 and pp56) are indicated.

particle. Given that virtually all phosphoproteins are present in this fraction, 20% formamide is a sufficient denaturing condition to elute the mRNP particles; and since the flow-through of unbound material and NaOH washes have no phosphoproteins evident, the radioactivity present in these two peak points is likely to be present as free nucleotides rather than phosphoproteins.

RNase Dose Response Curve

The poly(A)-tail of mRNA hydrogen bonds to the oligo-(dT) in oligo-(dT)-cellulose. This allows the possibility of removal of phospholabelled material from a column with RNase. These RNase released fractions represent cross-linked mRNP fragments and can be quantitated through radioactive counting. By using RNase A alone, the sequence poly(A) is not hydrolyzed, giving the possible separation of proteins bound to poly(A) from proteins bound to sequences containing cytosine and uridine (pyrimidine) residues.

In the first study, we examined the elution of the entire mRNP particle with 20% formamide. Now we can separate the mRNP particle from its accompanying poly(A)-tail to examine both separately for their specific cross-linked proteins. However first we must determine the optimum incubation period for cleavage with 10µg of RNase A at 20^0C. This study was performed using [^{3}H]uridine labelled, cross-linked RNA released from oligo-(dT)-cellulose with RNase A after incubations of 5, 15 and 30 mins. RNase A released radioactivity was counted via TCA precipitation of 25λ and graphed versus time in Figure 7. It is evident that 30mins incubation with 10µg of RNase A at 20^0C is sufficient for the cleavage of mRNA from its bound poly(A)-tail.

Run 3

For this experiment, the 50-60S sedimenting fraction of the glycerol gradient used in Run 1 and shown in Figure 2 was analyzed. This study was conducted as described in the Experimental Strategy section above. Cerenkov counting of the affinity chromatography elutions are graphed in Figure 8a. There are three peaks with each resulting from a change in elution conditions. The first eight fractions were eluted in loading buffer and represent all unbound material. Fractions 9-11 contain the RNase A digested mRNP particles minus the poly(A)-tail, while fractions 12-15 were eluted in 20% formamide and contain the poly(A)-tail. Fractions 3, 4, 9, 12, 13 and 14 were examined by SDS-PAGE with the resulting autoradiograph presented in Figure 8c. There are no phosphoproteins present in any track other than fraction 9. This fraction, representing the RNase A released mRNP particle minus its poly(A)-tail, shows that the phosphoproteins pp56 and pp60 are tightly bound to the mRNA. Since the 20% formamide elution shows a complete absence of phosphoproteins, there must be no covalently cross-linked phosphoproteins present on the poly(A)-tail. However, these results do not eliminate the possiblity that

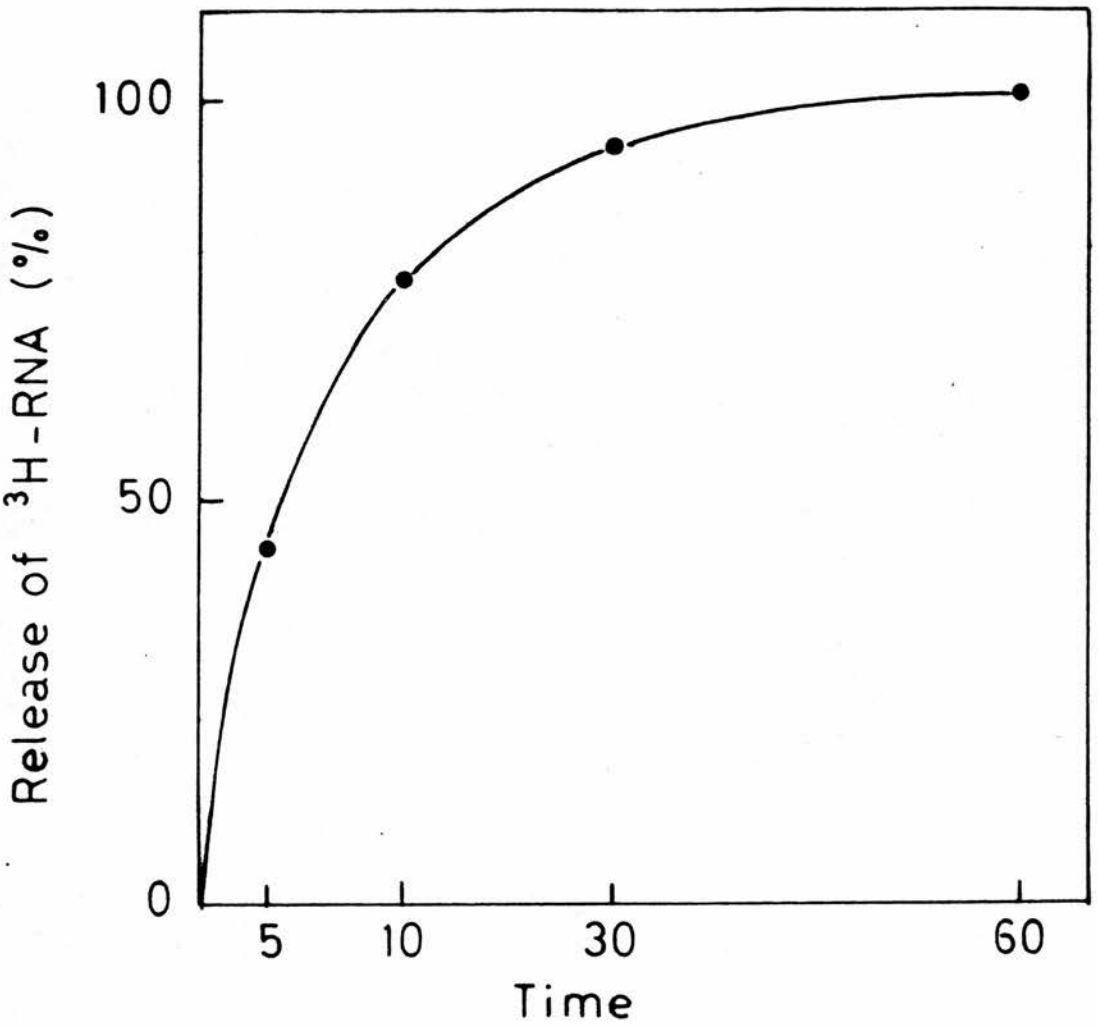

FIGURE 7. Release of ^{3}H-labelled RNA from mRNP particles bound to oligo(dT)-cellulose and treated with 10µg/ml RNaseA at 20^{0}C. The column was flushed at 5, 10 and 30 mins and cumulative radioactivity was calculated.

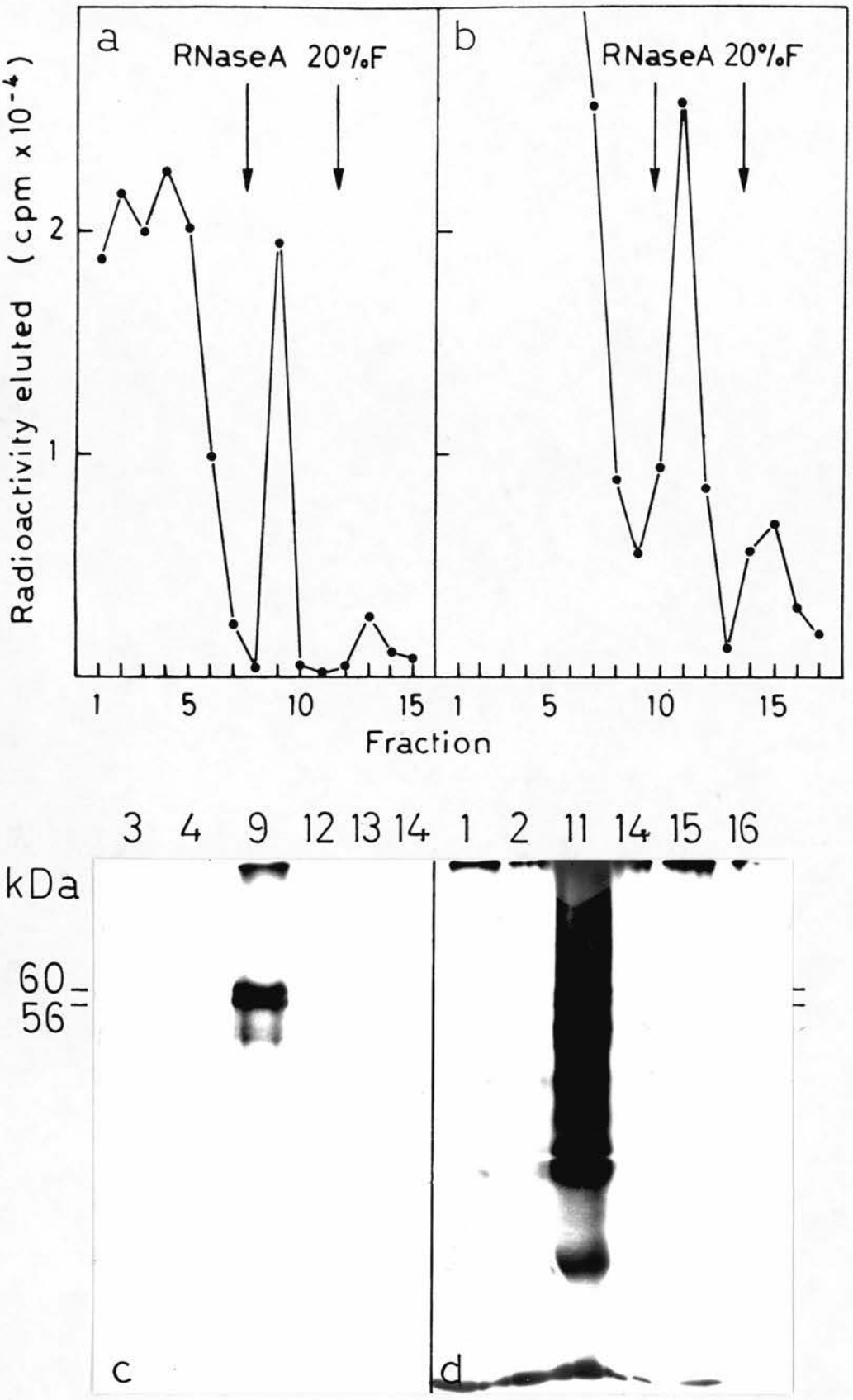

FIGURE 8. Elution of phospholabel from oligo(dT) columns after labelling of mRNP particles with ^{32}P-γ-ATP and UV-induced crosslinking. Elution steps are with 10μg/ml RNaseA for 30min at 20^{0}C and with 20% formamide. (a) 50-60S gradient fraction from previtellogenic oocytes. (b) 80-90S gradient fraction from stage I-IV oocytes. (c) Autoradiograph after SDS-PAGE of eluted fractions 3, 4, 9, 12, 13 and 14 from (a). (d) Autoradiograph after SDS-PAGE of eluted fractions 1, 2, 11, 14, 15 and 16 from (b).

other non-phosphorylated proteins are binding this sequence.

Run 4

Cross-linking studies were also conducted on more mature ovary -- containing a greater preponderance of ribosomes from the glycerol gradient of oocyte stages I-IV. 80-90S sedimenting material, containing the mRNP particles in this gradient, was analyzed as before and once again the quantitated radioactivity of the oligo-(dT)-cellulose fractions was graphed (Fig. 8b). Unbound material was collected in tubes 1-9; RNase A treated material in tubes 10-13; and 20% formamide elutions in tubes 14-17. Samples 1, 2, 11, 14, 15 and 16 were subjected to SDS-PAGE with the accompanying autoradiograph shown in Figure 8d. Once again, pp56 and pp60 are eluted with RNaseA digestion, but not any other treatment. However, many other lower molecular weight proteins are visible in the track of fraction 11. These proteins are presumably ribosomal in origin, for rRNA may appear bound to the column when in fact these ribosomes are probably binding to the poly(A) portion of the mRNP particle. By other experiments, we have shown that rRNA does not bind directly to oligo-(dT) (not shown). The formation of mRNP-ribosome complexes, inactive in translation, has been discussed previously (Cummings and Sommerville, 1988).

As in Run 3, labelled phosphoprotein is detected mainly in RNase A elutions, with no signals present in the 20% formamide elution of the poly(A)-tail of the mRNA. Thus, in Stage I-IV oocytes, as in PV ovary, phosphoproteins are only bound to the heterogeneous sequence of the mRNA, not its poly(A)-tail; and although there may be proteins covalently cross-linked to the poly(A)-tail, if so, they are not phospholabelled. Finally, in continuing this comparison of the autoradiographs of PV material and stage I-IV oocytes, it is observed that PV material is much simpler to use for these studies than stage I-IV oocytes.

Detection of Poly(A) Bound Protein

In order to detect additional cross-linked proteins, not phosphorylated under the in vitro labelling conditions employed, larger amounts of material (equivalent to an initial 50µg of RNP rather than the 10µg used in phospholabelling) were cross-linked and analysed by SDS-PAGE, this time the separated proteins being visualized by staining. Figure 9 again shows the elution of pp56 and pp60 from oligo-(dT)-cellulose after incubation with RNase A. On the stained gel an additional protein, of 72kD, is now seen to be released by denaturation with 20% formamide (Fig.9). These observations support the interpretation that pp56 and pp60 are bound to sequences other than poly(A) and that p72 is bound primarily to poly(A) and released along with poly(A) on denaturation of the oligo(dT):poly(A) hybrids. It is almost certain that this 72kD protein is the same as the one detected earlier by binding to poly(A)-Sepharose (Fig.3).

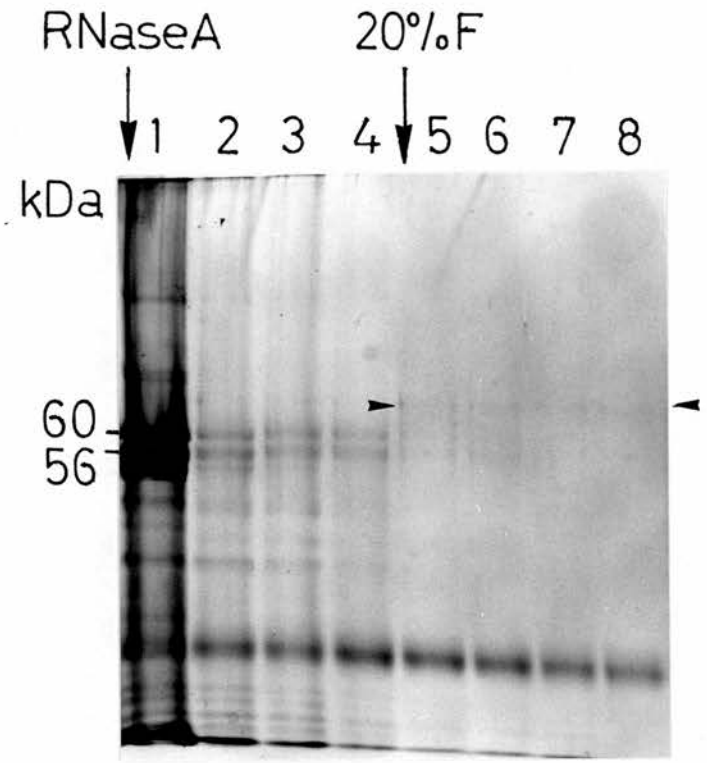

FIGURE 9. Detection of a 72kD protein bound to the poly(A) sequence. Elution of material from mRNP particles bound to oligo(dT)-cellulose with RNase A (fractions 1-4), then with 20% formamide (fractions 5-8). After SDS-PAGE, the gel was stained with Coomassie Brilliant Blue. The position of the 72kD protein is indicated with arrows.

To confirm that poly(A) does elute under these conditions, stage I oocytes were labelled with different ^{3}H-nucleosides. After cross-linking of mRNP particles and oligo(dT)-column chromatography, eluted fractions were analysed for radioactivity. As can be seen in Table 1, labelling with ^{3}H-adenosine showed a large bias towards radioactivity retained on the column after RNase A treatment and released on denaturation with 20% formamide. If the average length of the heterogeneous sequence of mRNA molecules is 1,200 nucleotides and the average poly(A)-tail length is 60 nucleotides (Cabada et al, 1977), one would expect nearly all of the label as ^{3}H-cytidine or ^{3}H-uridine to be released after RNase A treatment but only 83% (60 x 100/(0.25 x 1,200)+60) of the label as ^{3}H-adenosine to be released. The results shown in Table 1 are strongly biased in this direction.

CELLULAR LOCATION OF pp56 DURING OOGENESIS

Polyclonal antibodies have been raised against the oocyte mRNP phosphoproteins, pp60 (Dearsly et al., 1985) and pp56 (Cummings et al., 1989). Each antibody is monospecific for its own antigen unless mRNP particles are treated with non-ionic detergent before isolation of the proteins, in which case cross-reaction does occur (Cummings et al., 1989).

In taking extracts from oocytes and embryos at different stages of development, separating the proteins by SDS-PAGE and immunoblotting with anti-pp56, it is seen that pp56 is expressed throughout oogenesis and embryogenesis (Fig.10). After an initial increase from stage I to stage III oocytes (at which time the amounts of maternal mRNA are accumulating, Golden et al, 1980) the level of pp56 per oocyte or embryo, as detected by immunoblotting, remains fairly constant (Fig.10). The constant level of pp56 expression probably reflects maintenance of the number of mRNA molecules in the maternal pool. In order to detect any movement of this pool of mRNP particles between different cell compartments, immunostaining was undertaken using freeze-sectioned ovary reacted with anti-pp56 and tagged with FITC-labelled anti-rabbit IgG.

Small (stage I) oocytes in the sections are immunostained fairly evenly in the cytoplasm (Fig.11a). However, larger oocytes (stage II) show localization of immunostaining around the nuclear periphery (Fig.11b). By stage III, the immunostaining material becomes distributed more towards the cortex of the oocyte (Fig.11c). These changes in distribution of pp56 are similar to changes described for the location of mRNA sequences as detected by in situ hybridization (Capco and Jeffrey, 1982) and probably relate to the interaction of mRNP particles with newly-formed ribosomes (Cummings and Sommerville, 1988) and the interaction of mRNP-ribosome complexes with the cytoskeleton (Cervera et al., 1982; Howe

TABLE 1 Elution of RNA sequences from oligo(dT)-cellulose after binding of polyadenylated RNA from stage I oocytes labelled in vivo with different ^{3}H - nucleosides.

	Percentage Radioactivity Eluted	
^{3}H Nucleoside	RNase A	20% formamide
Adenosine	64.5%	35.5%
Cytidine	92.9%	7.1%
Uridine	94.5%	5.5%

FIGURE 10. Immunoblotting of protein extracted from oocytes, embryos and Xenopus culture cells with anti-pp56. Each track contains protein from 5 oocytes or embryos or from $5x10^5$ culture cells. Autoradiograph shows binding of ^{125}I-labelled protein A.

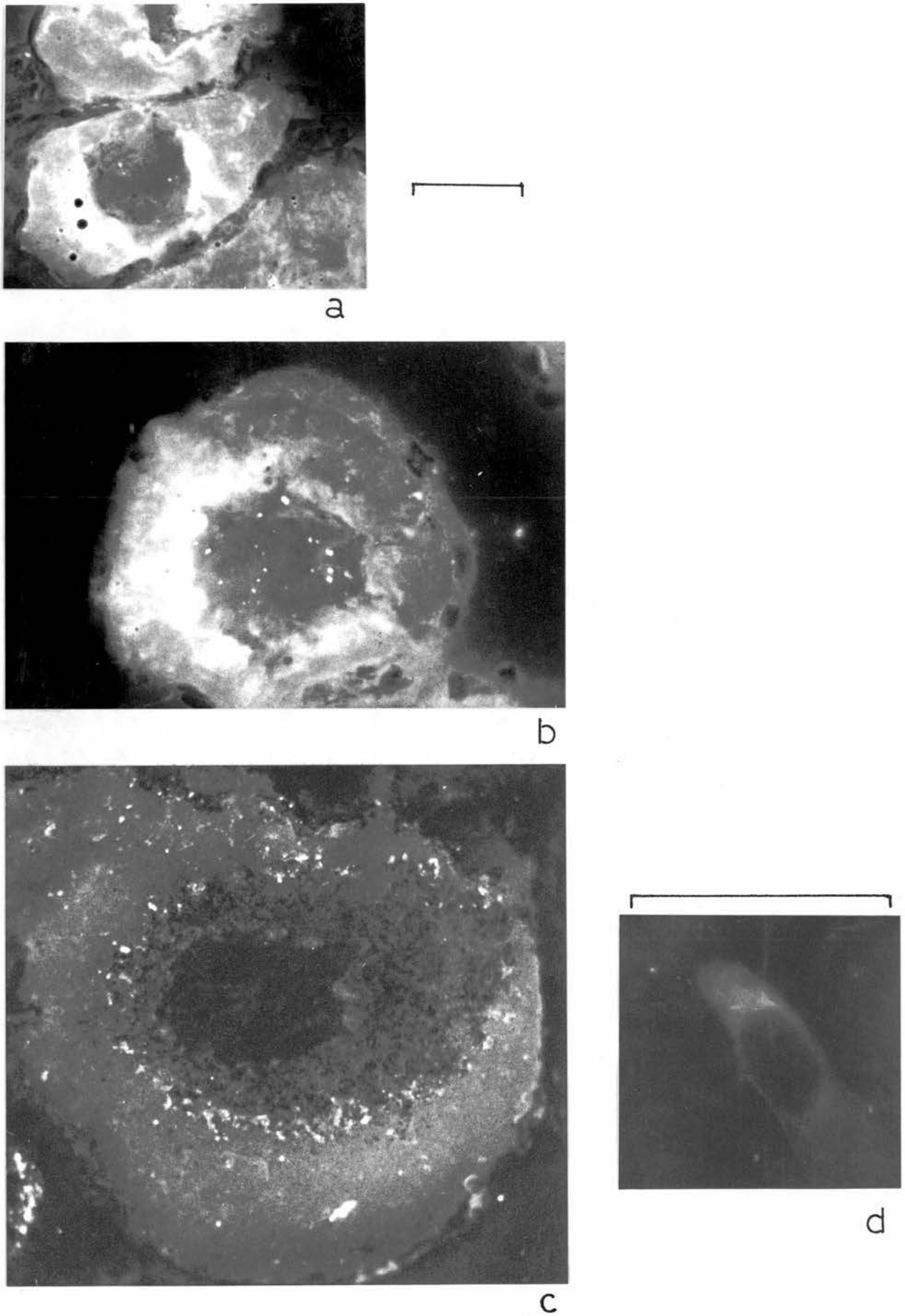

FIGURE 11. Immunostaining of freeze-sectioned ovary and XTC-2 cells using anti-pp56 and FITC-labelled anti-rabbit IgG (a) stage I oocytes; (b) stage II oocytes; (c) stage III oocytes; (d) XTC-2 cells. Bars represent 50μm.

and Hershey, 1984). Specific maternal mRNA sequences, such as Vg-1 mRNA whose protein product plays a role in determining the polarity of embryos, have been shown to migrate towards the cortex in stage III oocytes (Melton, 1988; Yisraeli and Melton, 1989).

DETECTION OF THE mRNA-BOUND PROTEIN pp56 IN CULTURE CELLS

XTC-2 cells are derived from metamorphosing tadpoles and so represent cells at the very end of development (Pudney et al., 1973), whereas XP cells are derived from adult tissue. Immunoblotting of proteins extracted from both types of culture cell with anti-pp56 reveals a single, reactive protein of 56kD (Fig.10). Thus pp56 would appear to be expressed not only throughout development but also in adult tissues. The location of pp56 in XTC-2 cells, as visualized by immunostaining, is fairly even throughout the cytoplasm, although occassionally a fibrillar distribution can be discerned (Fig.11d). Therefore in somatic cells, as in oocytes, interaction with cytoskeletal elements may be significant. That pp56 (and pp60) are bound to polyadenylated mRNA sequences also in culture cells can be demonstrated by UV-cross-linking followed by SDS-PAGE. As with oocytes, cross-linked proteins released from oligo(dT) only after RNase A treatment are detected in the range of 50-60kD (not shown). Immunoblotting from gels reveals that anti-pp56 reacts with proteins in the size range of 56-60kD (Fig.12). The fuzziness of bands in these blots probably reflects incomplete digestion of covalently linked RNA and hence distortion of protein migration.

One further expectation might be that mRNA with bound pp56 should occur primarily in the pool of ribosome-free mRNP particles in culture cells. That this is indeed the case is shown by immunoblotting fractions taken from sedimentation of XTC-2 cell homgenates through glycerol gradients. Here, anti-pp56 reacts primarily with particles sedimenting between 10S and 40S, presumably representing ribosome-free mRNP (Fig.13), although some reaction is seen with a 56kD component in ribosome (80-90S) fractions.

Therefore in its occurence and cellular distribution, the mRNA-bound phosphoprotein pp56 can not be considered to be unique to oocytes and early embryos. Although predominant in maternal mRNP, pp56 (and probably also pp60) is found also in free mRNP pools of adult somatic cells. These proteins may well have a role in the general stabilization of non-translating mRNA sequences in all cell types.

PROTEIN KINASE ASSAYS

Protein Kinase Activity In mRNP Particles Is Of The Casein Kinase II Type

Under a wide range of ionic conditions protein kinase, the phosphorylating enzyme,

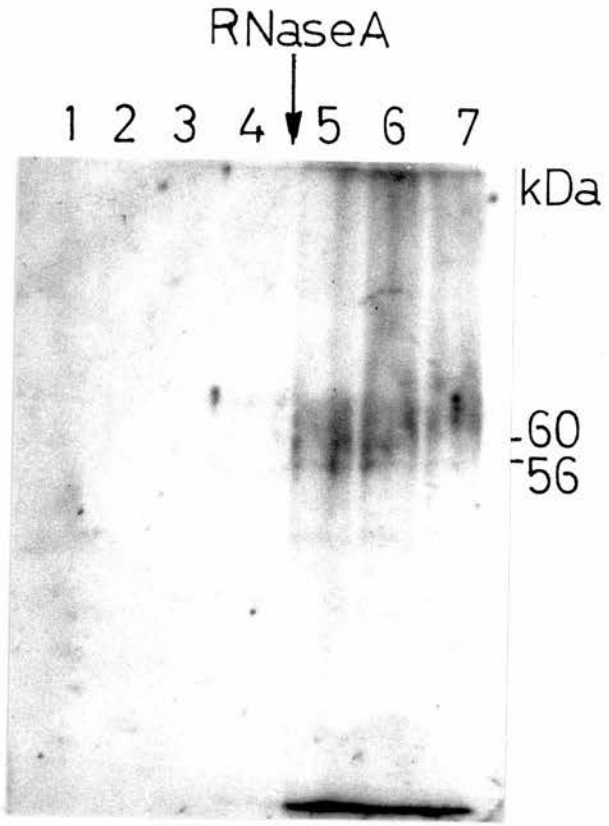

FIGURE 12. Immunoblotting with anti-pp56 of UV-cross-linked mRNP proteins from XTC-2 cells. Proteins released from oligo(dT)-cellulose were separated by SDS-PAGE and then immunoblotted using anti-pp56. Autoradiograph shows binding of ^{125}I-labelled protein A. The positions of 60 and 56kD marker proteins are shown. There is evidently cross reaction of anti-pp56 with pp60 in this preparation.

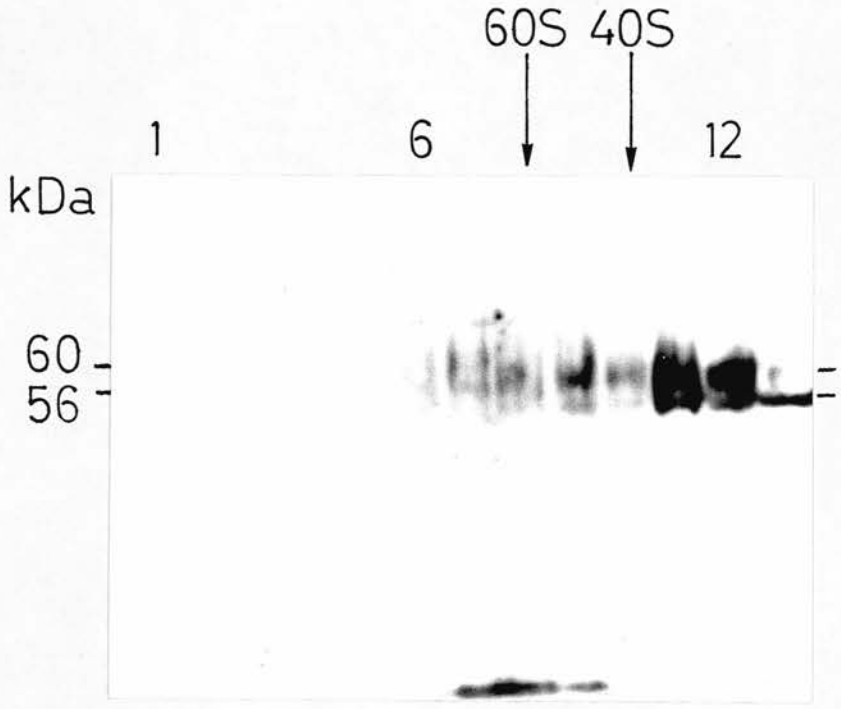

FIGURE 13. Immunoblotting with anti-pp56 of RNP particles from XTC-2 cells separated by glycerol gradient centrifugation. XTC-2 cells (approx. $5x10^5$ cells) were homogenized in a buffer containing 0.3M KCl, 0.5% sodium deoxycholate(DOC) and 500 units/ml HPRI. An SN^{10} is then run in a glycerol gradient made up in the same buffer. This treatment dissociates monosomes into 60S and 40S subunits but preserves polysomes (Woodland, 1974). mRNP particles should be released or bound to 40S ribosomal subunits. Autoradiograph shows binding of ^{125}I-labelled protein A. The positions of 60 and 56kD marker proteins are shown. After DOC treatment, anti-pp56 cross reacts with pp60.

remains an integral component of oocyte mRNP particles (Cummings & Sommerville, 1988). The function of protein kinase in the mRNP particle is suggested in Figure 14. In this diagram, one sees an mRNA binding phosphoprotein attached to a segment of mRNA. When phosphorylated, this protein blocks the translation of its attached mRNA. This phosphorylation is catalyzed by protein kinase and results from the transfer of the terminal phosphate from either ATP or GTP to a suitable amino acid side chain in the target phosphoprotein. This reaction is accelerated through the presence of kinase activators, namely cations such as Mg^{2+}, Mn^{2+} and polyamines. Protein phosphatase catalyzes the release of the phosphate from the binding protein, and may permit the subsequent translation of the message. Phosphatase activity may therefore act in competition with protein kinase activity. Kinase activity is blocked through the action of kinase inhibitors, such as haemin, heparin, polyanions and dinucleoside polyphosphates. It is postulated that the accessibility to sites of phosphorylation in the substrate proteins are modified through protein-polyion interaction (Hara & Endo, 1982).

The stimulation of kinase activity by divalent cations and polyamines, the inhibition by low levels of heparin, haemin and polycations, cyclic AMP independence, and the ability to use GTP, all point to the enzyme being of the casein kinase II type (Hathaway & Traugh, 1979; Cohen, 1985; Edelman et al., 1987). A further property of casein kinases is their natural preference for target proteins which are acidic, such as casein or phosvitin, rather than basic, such as histones or protamines. It is interesting to note, therefore, that of the wide range of charged forms of the major mRNA-binding proteins pp60 and pp56, it is mostly the acidic forms that are phosphorylated (Cummings et al, 1989).

Separation Of Protein Kinase Activity From mRNP Phosphoproteins

Supernatants from stage II oocytes homogenized in HB were mixed with Oligo-(dT)-cellulose in RNP Buffer 3, shaken for 30min. on ice and centrifuged to remove the supernatant. 60% formamide in Elution Buffer was added, after several washes in RNP Buffer 3, to displace the bound mRNP particles. This eluted sample was then dialyzed in KLB, which contains EDTA to destabilize the mRNP particle, mixed with heparin/Sepharose and eluted through a gradient of increasing salt concentration. Each fraction was divided into two aliquots with 0.5µg/ml heparin added to one. Reaction Mix 1, which contains B-casein and ^{32}P-γ-ATP, was incubated with each fraction to measure the phosphorylation of B-casein by kinase. Assay samples were stopped with cold ATP, TCA precipitated on Whatman disks and scintillation counted. Measured radioactivity was graphed and presented in Figure 15. Measured casein kinase activity is presented as pmol of ATP converted per minute. One sees that the only major peak of casein kinase activity elutes in 1M NaCl and this activity is almost completely sensitive to low concentrations of heparin. In fact, low concentrations of heparin (0.5µg/ml) only give a

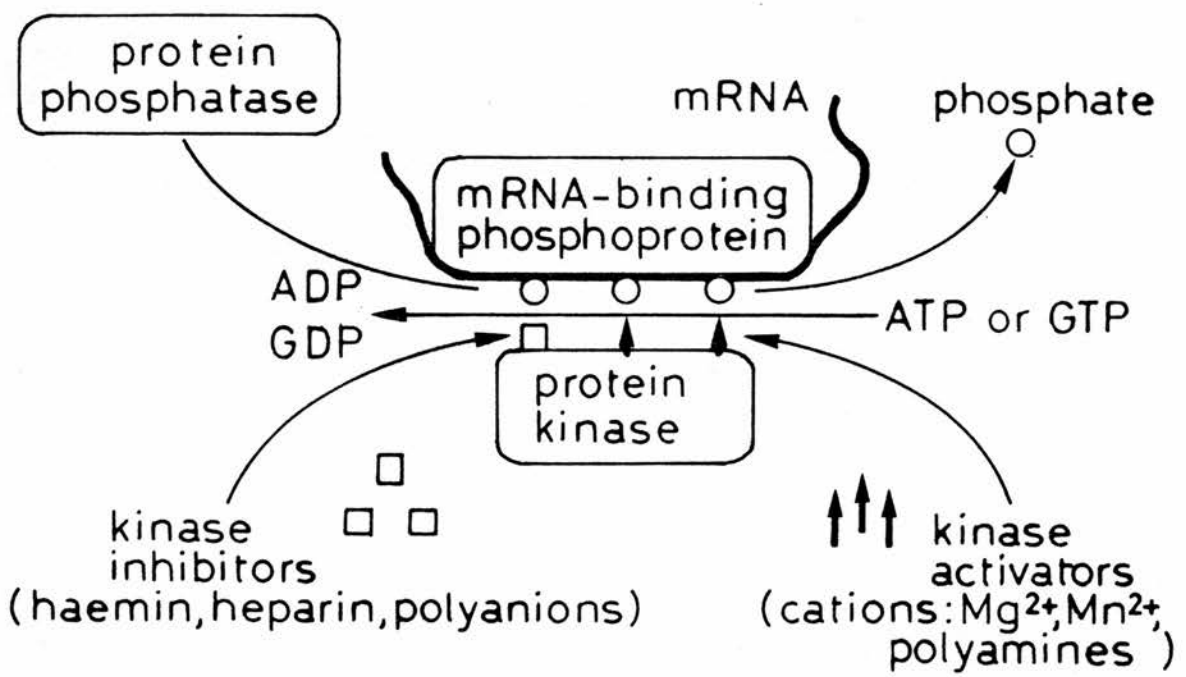

FIGURE 14. Scheme describing the properties of the mRNP-bound protein kinase (Cummings and Sommerville, 1988). The stimulation of kinase activity by divalent cations and polyamines and inhibition by low levels of heparin, haemin and polycations catagorizes the enzyme as a casein kinase II. This enzyme can use either ATP or GTP as phosphate doner. Dephosphorylation of mRNP phosphoproteins is effected by protein phosphatase 2A (Kick et al, 1987).

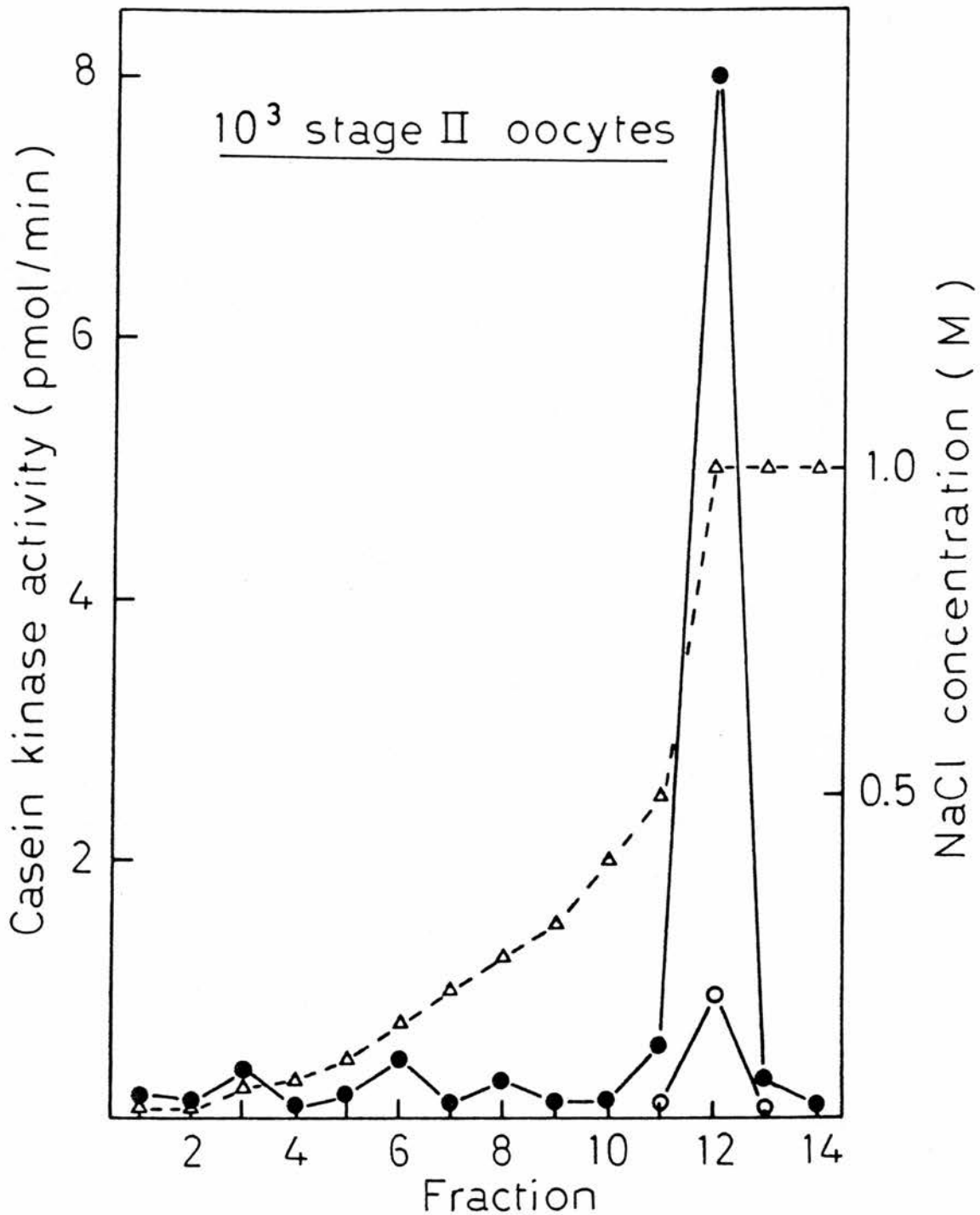

FIGURE 15. Elution of casein kinase activity from the mRNP-bound enzyme transferred to heparin-Sepharose CL-6B. Enzyme activity (closed circles) is eluted with 1M NaCl. Activity is estimated in a standard phosphorylation of B-casein using ^{32}P-γ-ATP as substrate. Less that 10% of this activity is resistant to 0.5μg/ml heparin (open circles).

residual 15% activity. Other characteristics of the protein kinase activity, namely dependence on ATP concentrations and time course of the phosphorylation reaction are shown in Figure 16. Standard assay conditions were with 30μM ATP incubated at 20^0C for 10min. Heparin resistant activity was subtracted from total incorporation in most instances.

mRNP particles from stage II oocytes selected through affinity chromatography on oligo-(dT)-cellulose were in vitro labelled with ^{32}P-γ-ATP and destabilized in EDTA prior to column chromatography on heparin/Sepharose. Fractions were sequentially eluted as above through a gradient of increasing salt concentration and then examined by SDS-PAGE. The autoradiograph proves that, of the mRNP proteins phospholabelled in vitro, most does not bind to the column (Fig. 17a). In fact, more than 95% of the label in the major phosphoproteins pp60 and pp56 elutes at less than 0.1M NaCl, whereas only 3% elutes in 1M NaCl.

Another pool of stage II, oligo-(dT) isolated mRNP particles were destabilized in EDTA, mixed with heparin/Sepharose and sequentially eluted through an increasing concentration of NaCl. These fractions from the heparin/Sepharose column were subjected to an assay, containing B-casein and ^{32}P-γ-ATP, which measures the phosphorylating capacity of the endogenous kinase. Each fractions was incubated with Reaction Mix 2. Reactions were stopped with cold ATP and subjected to SDS-PAGE with the resulting autoradiograph presented in Figure 17b. Protein kinase activity is only strongly evidenced in the higher salt, 1M NaCl, elutions. Thus, chromatography on heparin-Sepharose provides a simple step in separating protein kinase activity (bound to the column up to 1M NaCl) and the mRNP particles (eluted from the column in low salt).

However, phosphorylation of endogenous mRNP proteins appears to differ from phosphorylation of B-casein in one respect, the identity of the phospho-amino acids. In B-casein, casein kinase II phosphorylates mainly threonine residues and this phosphorylation is alkali-resistant (Mulner-Lorrillon et al., 1988). To compare the mRNP proteins with B-casein, both were phospholabelled together in vitro with the mRNP-bound kinase, separated by SDS-PAGE and autoradiographs made from the gel before and after incubation in 1M NaOH. Whereas phospholabelling of B-casein is largely unaffected by this treatment, most of the label is washed out from pp60 and pp56 (Fig. 17c). This indicates that serine residues, rather than threonine residues, are the targets of kinase activity in mRNP particles, at least in the in vitro labelling reaction described here. Analysis of hydrolysed mRNP phosphoproteins confirms that the major phospholabelled amino acid is serine (Sommerville, unpublished).

mRNA-Bound Casein Kinase Activity Through Early Development

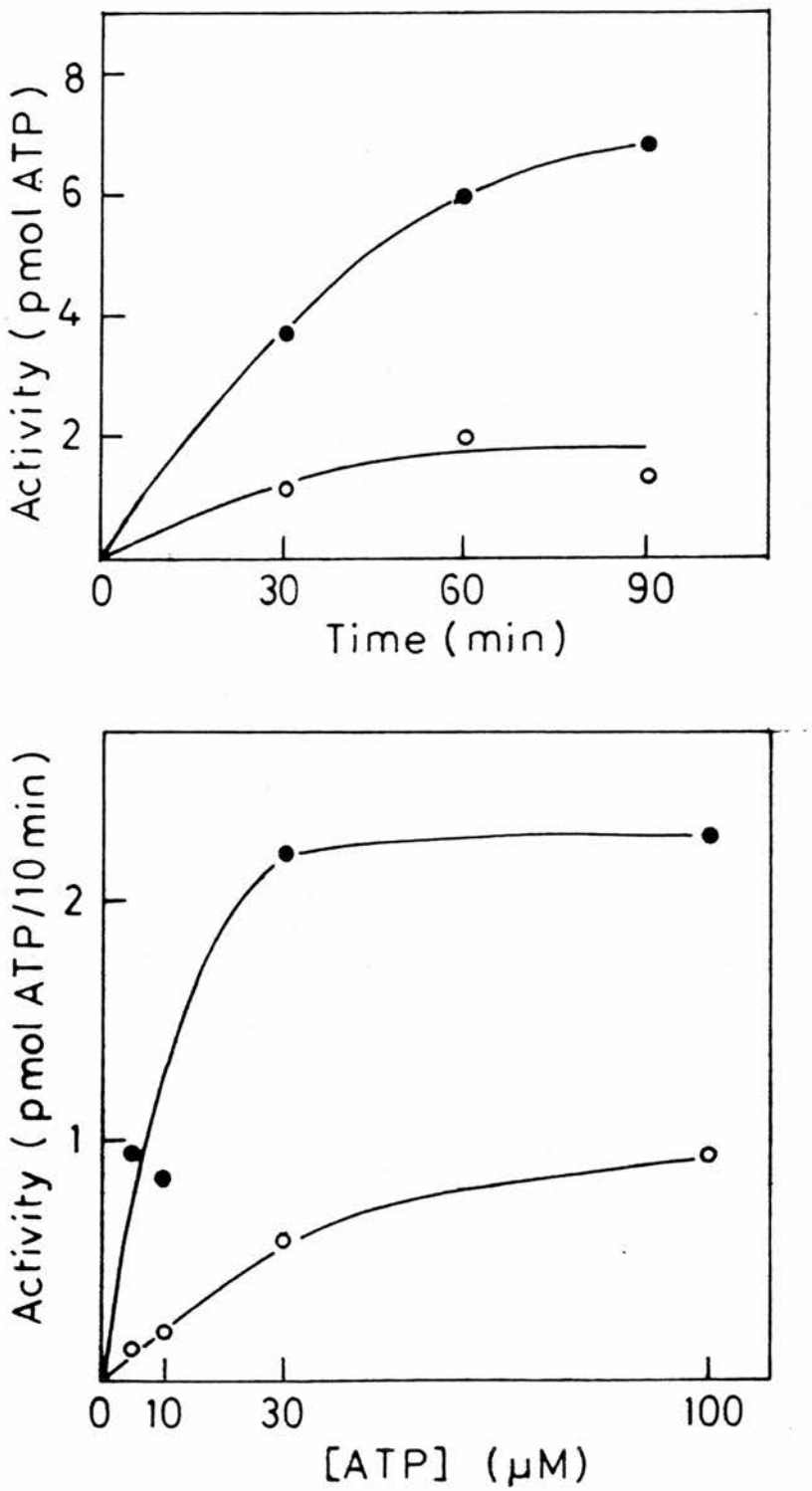

FIGURE 16. Conditions for determination of casein kinase activity. (a) Time course of phosphate transfer from ^{32}P-γ-ATP to B-casein in the absence (closed circles) and presence (open circles of 0.5μg/ml heparin. The reaction was judged to approach linearity over at least the first 10min. (b) Effect of increasing ATP concentration on activity recorded in 10 min in absence (closed circles) and presence (open circles) of heparin. Standard assays were run at 30μM ATP. All incubations were at 20^{0}C.

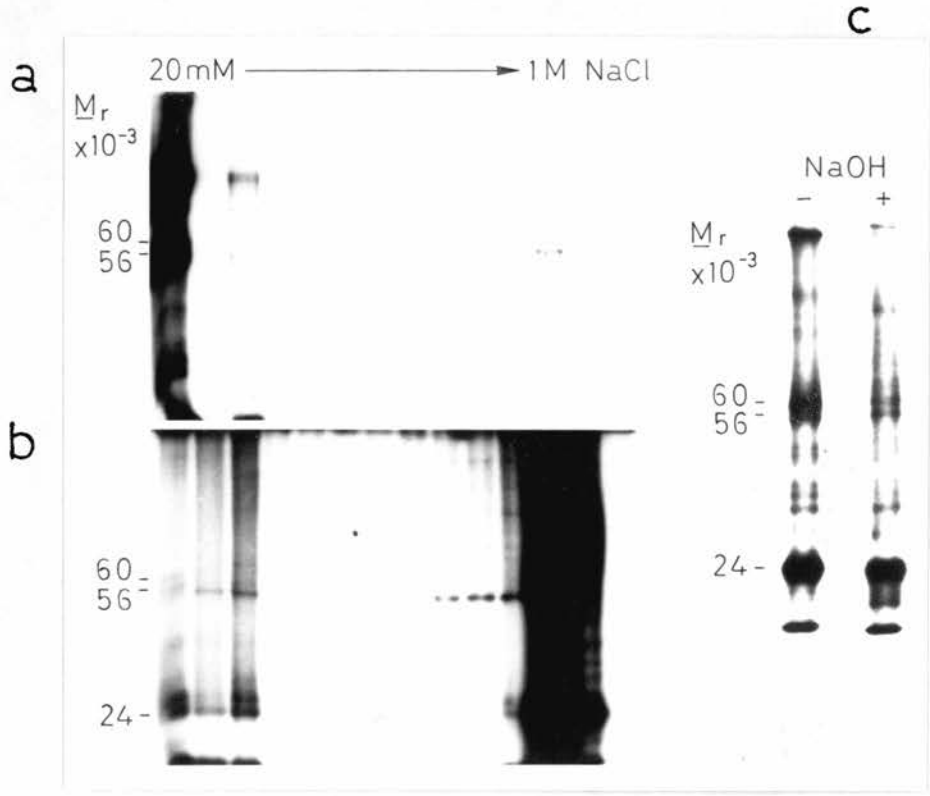

FIGURE 17. Analysis by SDS-PAGE and autoradiography of fractions eluted from heparin-Sepharose CL-6B phospholabelled either before chromatography (a) or after chromatography (b). For (a), mRNP particles were phospholabelled in vitro with ^{32}P-γ-ATP and the endogenous kinase. Nearly all of the phospholabelled mRNP proteins elute in the first fraction. In (c), the gel track containing the peak of kinase activity (fraction 12 in (b)) was washed with 1M NaOH at 55^{0}C for 2hr (Mulner-Lorrillon et al., 1988), re-exposed and compared with the original autoradiograph.

mRNP particles were selected from homogenates of sets of twenty-five oocytes or embryos by binding to oligo-(dT)-cellulose. The particles were eluted in a buffer containing 60% formamide, dialysed to remove the formamide and partially dissociated by addition of EDTA. Although the oocyte mRNP particles are stable over a wide range of salt concentration (0-1M NaCl), they are destabilized in the presence of 5mM EDTA. Casein kinase was separated from the other mRNP components by binding to, and elution from, heparin-Sepharose through a gradient of increasing salt concentration. Eluted fractions were assayed for the capacity to phosphorylate B-casein using ^{32}P-γ-ATP as a substrate both in the presence and in the absence of 0.5µg/ml heparin, as described in the methods. Each assay sample was TCA precipitated and scintillation counted for quantitation of heparin-sensitive kinase activity. The results from four independent assays carried out on the range of oocyte and embryo stages are shown in Figure 18.

It can be seen that protein kinase activity increases through previtellogenesis to reach a maximum in stage III oocytes; thereafter the level remains constant through to fully-grown oocytes. Again, these changes are very similar to the pattern of accumulation in growing oocytes of total polyadenylated RNA (Golden et al., 1980). Therefore the amounts of mRNA-bound protein kinase in oocytes, follows the amount of mRNA itself and, as demonstrated earlier (Dearsly et al., 1985), also the amounts of pp60 and pp56. Expressed otherwise, the stoichiometry of RNP particles-RNA:phosphoproteins:protein kinase appears to be maintained throughout oogenesis.

During early embryogenesis, however, dramatic changes occur in the level of protein kinase activity (Fig. 18). The rapid drop occurring during cleavage stages is coincidental with mobilization of mRNA into polysomes (Woodland, 1974). Since there is little net loss of mRNA during this period, it is presumed that protein kinase is lost from maternal mRNP particles as they are incorporated into polysomes. A relatively low level of kinase activity is maintained through to the tadpole stage, with apparently a small recovery of activity after gastrulation. This later effect could be significant, relating to the increased formation of zygotic mRNA after mid-blastula (Newport & Kirschner, 1982) with a slight accumulation of free mRNP before the polysome concentration reaches its maximum (Woodland, 1974).

It should be emphasized that mRNP-bound protein kinase activity is never completely depleted, being detected in all types of active cell tested. The absolute amount may well correspond to the size of the polysome-free mRNA pool.

Endogenous Inhibitors Of The mRNP Phosphorylation Reaction

As has been reported previously (Cummings & Sommerville, 1988), inhibitors of the mRNP phosphorylation reaction are present in crude mRNP fractions derived from vitellogenic

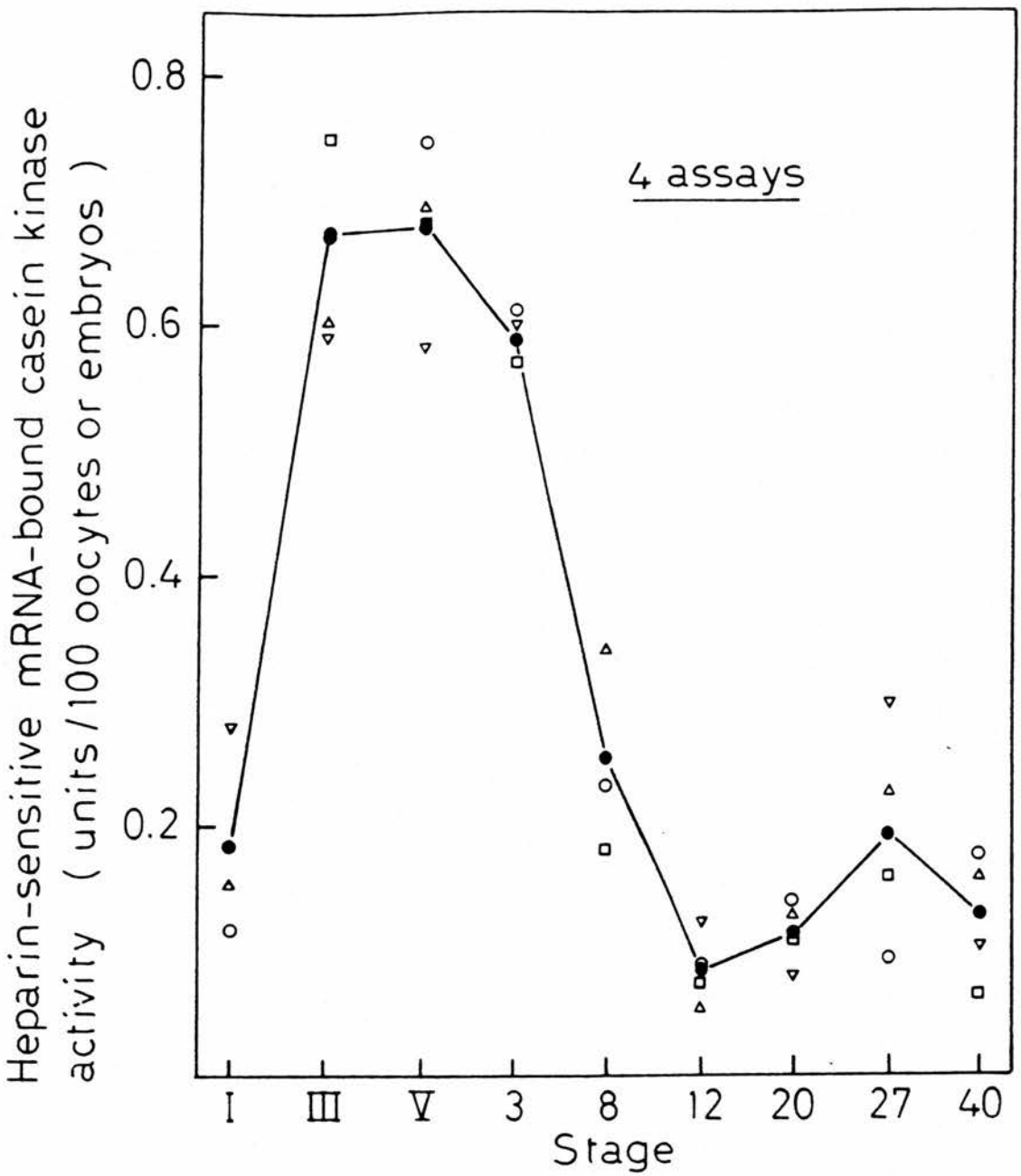

FIGURE 18. Assay of heparin-sensitive, mRNA-bound casein kinase activity isolated from oocytes and embryos at different stages of development. Four independent assays, each using material from 25 oocytes or embryos, were performed on material from each stage (open shapes). Average values (closed circles) are also plotted.

oocytes and can be separated from the mRNP particles themselves by chromatography on poly(U)-Sepharose. Particle-free supernatants (SN^{100}) were tested for inhibition of protein kinase activity by addition to a standard in vitro phosphorylation reaction containing mRNP particles isolated from previtellogenic oocytes. Phospholabelling of mRNP proteins by the associated protein kinase was compared after incubation in the presence of supernatants derived from the range of oocyte and embryo stages. Supernatants derived from 25 oocytes or embryos were each added to 12µg of mRNP particles plus 0.5µCi of ^{32}P-γ-ATP and the mixture was incubated at 20^0C for 1hr. The 12µg of mRNP is equivalent to the content of about 25 full grown oocytes (Rosbash & Ford, 1974; 100ng of mRNA/oocyte is equivalent to 0.5µg mRNP, Cummings & Sommerville, 1988).

Inhibition of mRNP phospholabelling became apparent with addition of supernatants from stage III oocytes and increases further with addition of supernatants from stage V oocytes (Fig.19a). Similar levels of inhibition are maintained with supernatants from all embryo stages tested, although phosphorylation of pp88 (a protein phosphorylated in vitro but only weakly associated with mRNA) becomes more refractile to inhibition with addition of supernatant from early tadpoles (stage 40, Fig. 19a). Obvious from the autoradiograph (Fig.19b) is the presence of additional protein kinase activity, acting mainly on low molecular weight proteins, introduced into the phosphorylation reaction from the supernatants. The differrential inhibition of phosphorylation of pp60 and pp56 compared with pp88 indicates specificity of this effect, however it is not known to what extent kinase inhibitors play a part in regulating phosphorylation of mRNP proteins in vivo.

Endogenous Phosphatase Activity Through Early Development

Supernatants derived from oocyte and embryo stages were also tested for their content of phosphatase activity capable of dephosphorylating mRNP phosphoproteins. As with kinase inhibitors, there is little evidence for phosphatase activity in previtellogenic oocytes, but when phospholabelled mRNP particles are exposed to supernatants from later stages substantial dephosphorylation is seen in the absence of proteolysis (Fig.20a). In these experiments, supernatants from 25 oocytes or embryos were added to 12µg of mRNP which previously had been phospholabelled in vitro. It is interesting to note that the developmental pattern of dephosphorylation of pp56 and pp60 does not mirror the pattern for pp88 (Fig.20b). This effect is not due to differences in accessibility of phosphatase to mRNP phosphoproteins, for added calf-intestinal phosphatase gives similar close reponses with the pp60/56 and pp88 components of mRNP particles (Fig.20c). It is concluded that there is selectivity of endogenous phosphatases in their action on different mRNP proteins and that phosphatase activity is differentially expressed during early development.

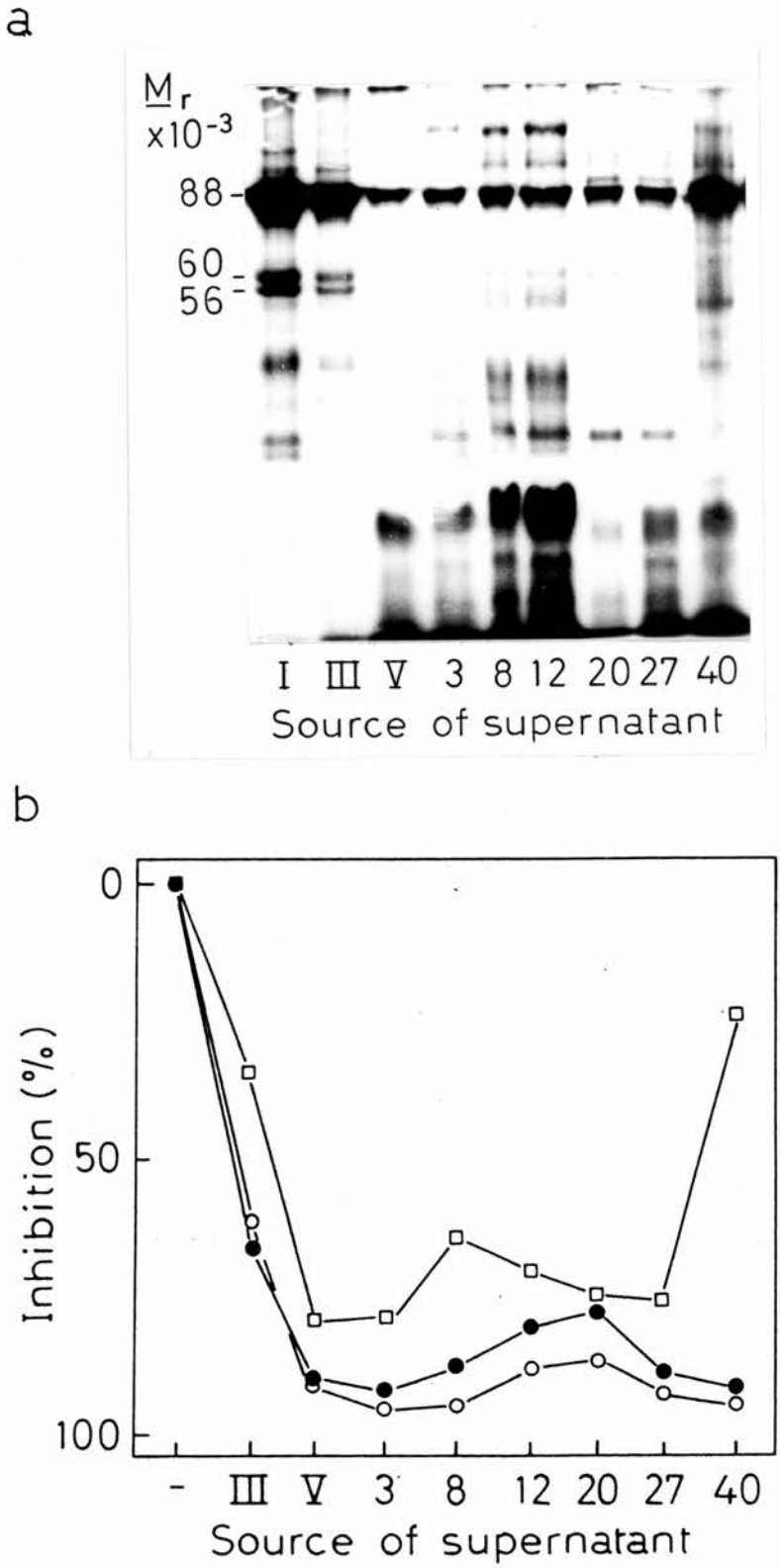

FIGURE 19. Inhibition of the in vitro phosphorylation reaction on mRNP particles by addition of supernatants prepared from different oocyte and embryo stages. Supernatants from 25 oocytes or embryos were each added to 12µg mRNP particles from stage II oocytes plus 0.5mCi ^{32}P-γ-ATP in standard phosphorylation buffer and incubated at 20^{0}C for 1hr. (a) Autoradiograph after SDS-PAGE of phospholabelled proteins. (b) Values from densitometer traces of autoradiographs showing inhibition of phosphorylation of pp60 (closed circles), pp56 (open circles) and pp88 (open squares).

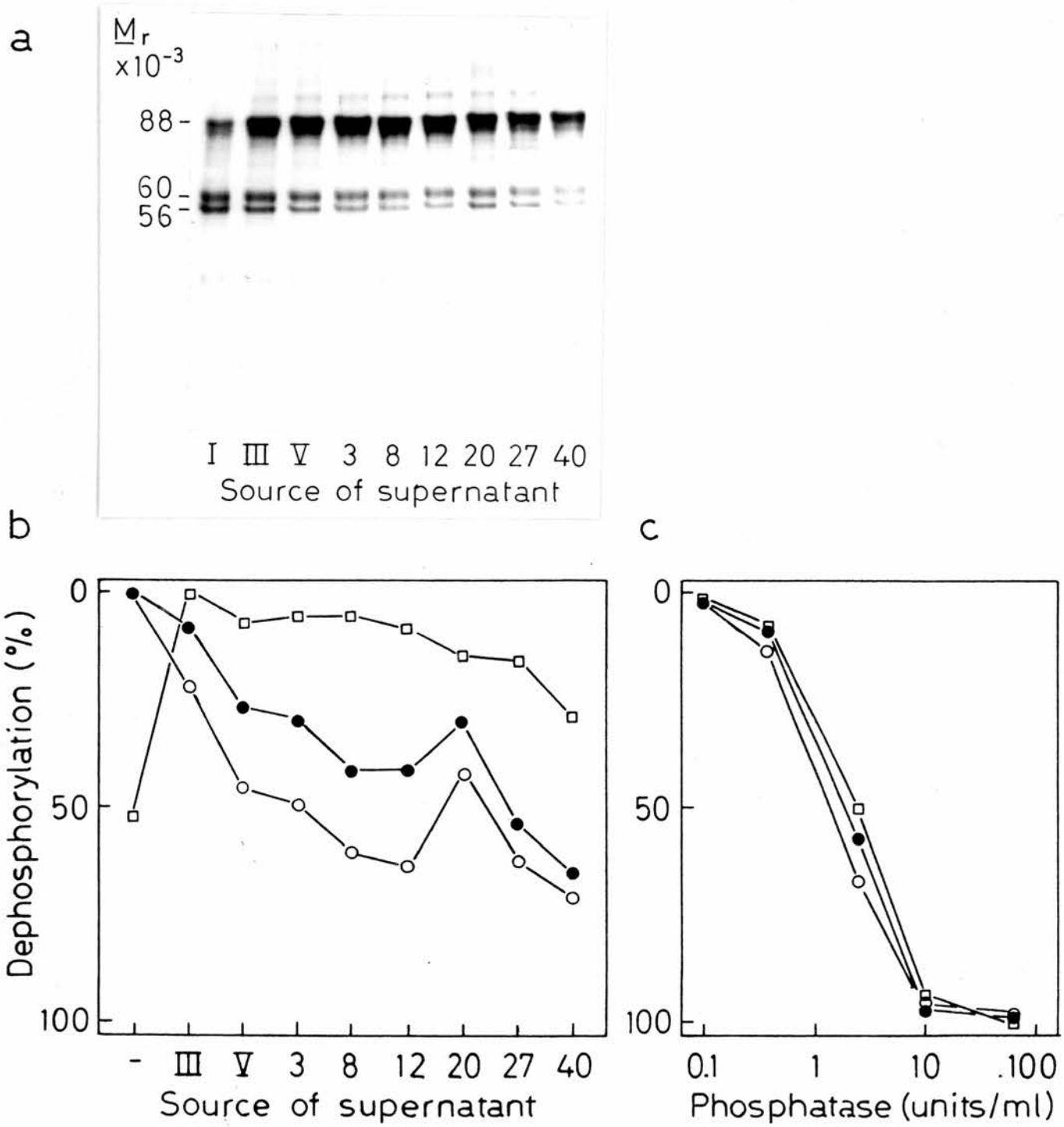

FIGURE 20. Assay for endogenous phosphatase activity through early development. mRNP particles isolated from stage I oocytes were labelled with ^{32}P-γ-ATP as described in Fig. 19 and then dialyzed and pelleted to remove free nucleotide. Aliquots of the resuspended pellet were then incubated with supernatants extracted from oocytes and embryos at different developmental stages. Stained gels show no loss in staining intensity of pp60 and pp56 (not shown). (a) Autoradiograph after SDS-PAGE of treated phospholabelled mRNP proteins. (b) Values from densitometer traces of autoradiographs showing dephosphorylation of pp60(closed circles), pp56(open circles) and pp88(open squares). (c) Dose response curves showing dephosphorylation of pp60(closed circles), pp56(open circles) and pp88(open squares) with increasing concentrations of added calf intestinal phosphatase.

CYCLIN STUDIES

Initial studies were conducted in an attempt to identify possible specific sequences, here the cyclin B1 message, masked by these phosphoproteins. Cyclin mRNA was chosen as a fairly typical maternal message but one whose expression is regulated through the cell cycle. Protein synthesis is a known requirement of interphase to induce mitosis. Oogenesis prepares the oocyte for the rapid translation of mRNA which occurs in fertilization and embryogenesis by amassing vast quantities of free mRNP particles, structural proteins, enzymes and ribosomes. Cyclin sequences are among the mRNA stored in the mature oocyte (Standart et al., 1987), and it has been shown that cyclin is the only newly synthesized protein required to induce mitosis (Murray and Kirschner, 1989). Here we examine where in the gradient this message appears and the possible role of the phosphoprotein pp56 in the masking of this message. Studies were conducted on total extracted RNA and immunoprecipitated RNA from both PV ovary and stage II oocytes. Initial experiments were carried out on slot blots. Slot blots work as a rough indication of quantitating mRNA, but they do not, however, eliminate the possibility of non-specific binding. It is only in gel electrophoresis and Northern blotting that this can be ascertained.

Initial Immunoprecipitation Studies

mRNP particles from sonicates and homogenates of PV ovary and stage II oocytes were selected through affinity chromatography on oligo-(dT)-cellulose. Bound in RNP Buffer 2, these particles were dissociated and eluted with 50-60% formamide in RNP Buffer 2. Dialyzed in ImmunoHB (RNP Buffer 2 plus 0.5% NP-40), mRNP particles could then be selectively immunoprecipitated, here anti-pp56 binding to the phosphoprotein pp56 on the mRNP particle, followed by precipitation through affinity binding on protein A - Sepharose or fixed S. aureus cell walls. Extracted RNA from mRNP particles eluted with 1% SDS in ImmunoHB and from unbound elutes could be bound to nitrocellulose in a slot blot apparatus and probed with the cyclin B1 ^{32}P-UTP radiolabelled antisense strand, JS23 or R3, to determine if cyclin message is bound to pp56. These initial blots were hybridized at 42^{0}C and washed in 2X SSC/0.1% SDS at 50^{0}C twice for 30min each.

Cyclin Expression Detected In Glycerol Gradient Fractions

PV ovary was centrifuged and sedimented through a 15-40% glycerol gradient of 0.2M NaCl. Gradients were normally run at 50mM NaCl, for any higher salt concentration would dissociate the 42S peak, as is evidenced in Figure 21a. The concentration of 0.2M NaCl was purposely utilized in order to purify the fractions containing the mRNP particles. Material from the 42S peak, including all of the tRNA and 5S RNA, is now shifted to the 10S region of the gradient. After fractionation into 3ml aliquots, 1.5ml of each were in vitro labelled with ^{32}P-γ-ATP and separated on a protein gel. Presence of mRNP particles can

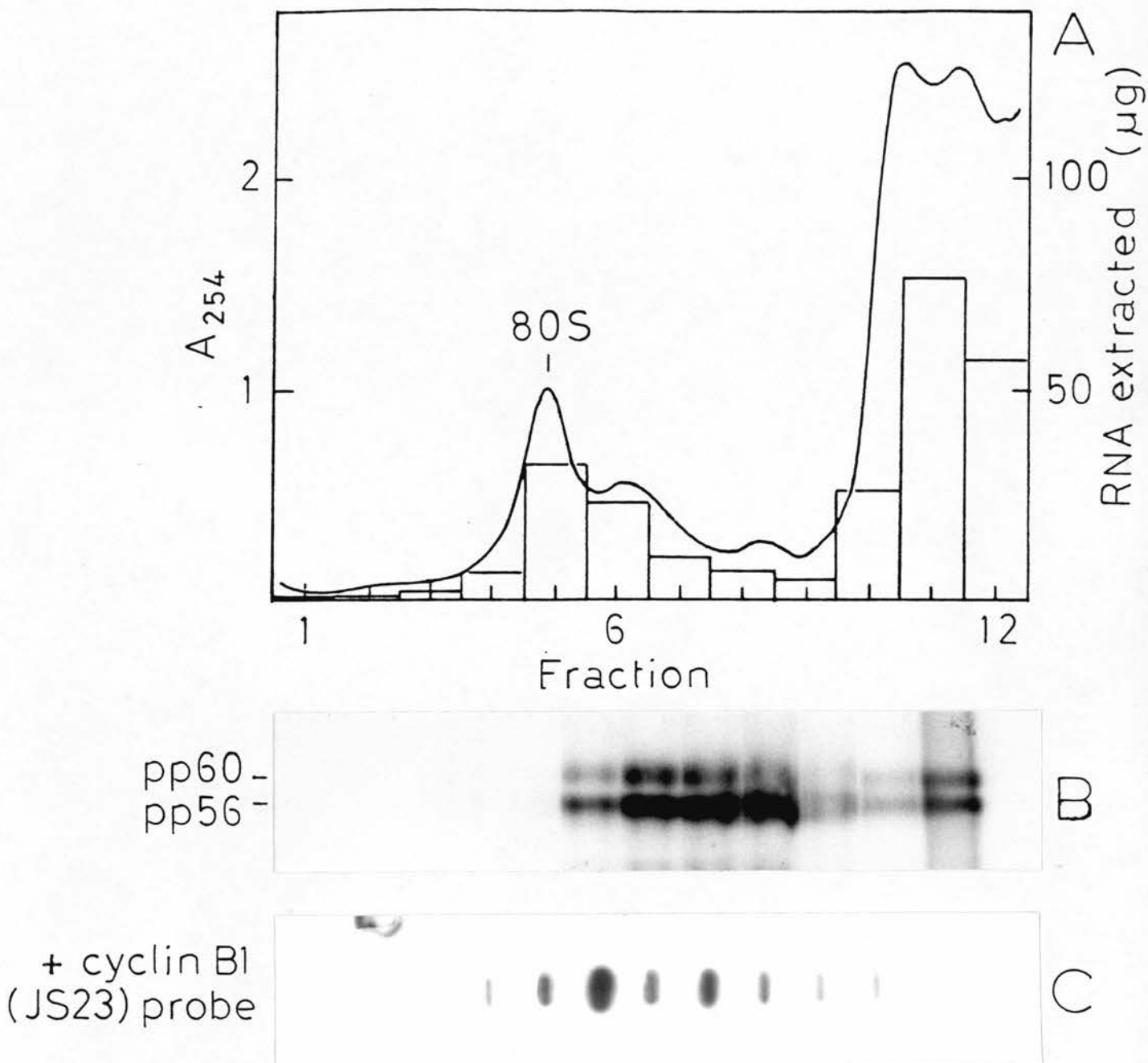

FIGURE 21. Hybridization of RNA extracted from glycerol gradient fractions of stage I oocytes with a labelled cyclin B1 RNA probe. (A) Gradient absorbance profile showing relative amounts of RNA extracted. (B) Autoradiograph showing distribution of phosphorylating mRNP particles in gradient fractions. (C) Autoradiograph showing distribution of hybidized cyclin probe.

be assayed through the prevalence of the heavily phosphorylated proteins, pp56 and pp60. From the accompanying autoradiograph (Fig.21b), one can see that the size distribution of mRNP particles is virtually unchanged by this increase in salt concentration, with most mRNP particles, as expected, contained between 30 and 80S. This autoradiograph also indicates a second major fraction of mRNP proteins around 10S. Now that we have identified the location of the mRNP particles, we must compare it with the location of the cyclin message.

The remaining 1.5ml of the gradient fractions were incubated with Proteinase K in 2% SDS, PIC extracted and ethanol precipitated as a means to purify the RNA from any extraneous material. 50λ of each sample were mixed with 50λ of denaturation solution, slotted onto nitrocellulose and probed with JS23 for the presence of the cyclin message. Again hybridization was conducted at 42^0C and washing at 50^0C. The resulting autoradiograph is presented in Figure 21c.

Stage II oocytes were also homogenized in RNP Buffer 2, sedimented through a 0.2M NaCl 15-40% glycerol gradient and fractionated into 3ml aliquots. In this gradient, ribosomes, and hence ribosomal RNA molecules, are much more prevalent. Again, the 42S material is displaced to the 10S position of the gradient. RNA was extracted from 1ml of each aliquot and blotted, probed with JS23 and washed as in the PV ovary. The autoradiograph of the hybridized slots is shown in Figure 22.

It is interesting to compare these two autoradiographs. In the PV ovary slot blot, prevalance of cyclin message is indicated in the fractions 3-9, with the strongest signals evidenced in the 5th and 7th gradient fractions, corresponding with the 80S and 60S portion of the gradient, respectively (Fig.21c). There is no indication of cyclin expression in the last few fractions of the gradient, fractions which contain high concentrations of tRNA and 5S RNA. This data indicates the possible potential for the incorporation of cyclin mRNA in mRNP particles. Further immunoprecipitation studies with anti-pp56 and anti-pp60 are necessary to confirm this. The stage II oocyte slot blot provides strong signals through the first 7 fractions of the gradient, with the most intense bands in fractions 3 (120S) and 6 (70S) (Fig.22). The 80S signal, which contains far more material than in the PV ovary, expresses a much weaker cyclin signal in this blot. The strong signal in the first few fractions indicates the possible attachment of message in these larger oocytes to the cytoskeleton, where it may be anchored and blocked until translation resumes. It is also interesting to note the possible expression of cyclin message in the 12th fraction. This signal is not, however, as intense as that for fractions in the faster sedimenting portion of the gradient.

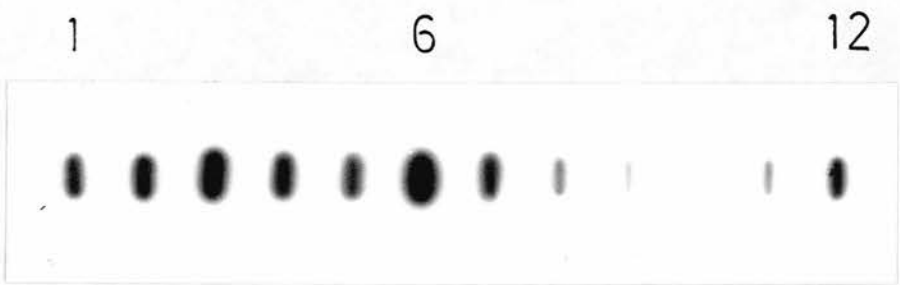

FIGURE 22. Hybridization of RNA extracted from glycerol gradient fractions of stage II oocytes with a labelled cyclin B1 RNA probe. Sedimentation was as for Fig. 21.

Northern Analysis of Gradient Fractions

As indicated earlier, for clear analysis of these fractions, the RNA must be separated by size through gel electrophoresis, Northern blotted and then probed with the radiolabelled cyclin. Here, 25λ of PV glycerol gradient fractions 5 and7 and stage II oocyte fractions 3, 6 and 12 were mixed with 24λ each of Blue Juice Mix, denatured and subjected to SDS-PAGE. RNA was diffusion blotted onto nitrocellulose, baked and probed with ^{32}P-UTP radiolabelled R3 Cyclin B1. This filter was hybridized at 42^{0}C and then washed in 2X SSC/0.1% SDS at 50^{0}C.

The resulting autoradiograph, displayed in Figure 23, shows cross-reaction with ribosomal RNA in PV fraction 5 and 7 and stage II oocyte fractions 3 and 6, but no cross-reaction in stage II oocyte fraction 12. These results show a binding pattern mimicking the content of rRNA, which is perhaps due to the shift to the R3 cyclin B1 probe. Although this subsequent analysis with R3 showed some cross-reaction with 28S and 18S ribosomal species, the JS23 probe, when used on extracted RNA from glycerol gradient fractions on slot blots, gave reactions not directly proportional to the amount of RNA present. The signals observed in JS23 analysis on RNP fractions derived from PV and stage II oocytes probably represent the true location of cyclin message. This hypothesis needs future confirmation through reprobing the Northern blot with the JS23 probe at a higher temperature. There is, however, some indication of a cyclin signal just below the 18S at 1.5 kilobases, as expected, in PV 5 and 7, stage II 3 and 6, and faintly in stage II 12. Again, reprobing is necessary.

Immunoprecipitation of Poly(A)$^-$ and Poly(A)$^+$ mRNA

After Northern analysis, it was decided to immunoprecipitate poly(A)$^-$ and poly(A)$^+$ fractions from oligo-(dT)-cellulose, as a means to better understand cyclin mRNA structure. PV ovary was sonicated in 500λ RNP Buffer 2 and bound to oligo-(dT)-cellulose. Six 500λ fractions were collected of unbound material, representing poly(A)$^-$ mRNA. Poly(A)$^+$ mRNA was then displaced from oligo-(dT) with 2 elutions of 500λ 60% formamide in RNP Buffer 2. This A$^+$ elution was then dialyzed against ImmunoHB. Fractions 1 and 2 were pooled to represent the A$^-$ mRNA. 200λ of each were reacted with 200λ ImmunoHB and 40λ anti-pp56, followed by binding to 25λ S.aureus Protein A. Three unbound fractions were collected, while bound material was eluted in a fourth fraction with 1% SDS in ImmunoHB. All fractions, after RNA extraction, were slot blotted, probed with R3 and washed in 2X SSC/0.1% SDS. However, conditions of hybridization and washing were more stringent, with both conducted at 60^{0}C. The resulting autorad is presented in Figure 24.

The loss of signal is evidenced in the first fraction of the A$^-$ reaction and may be due to the

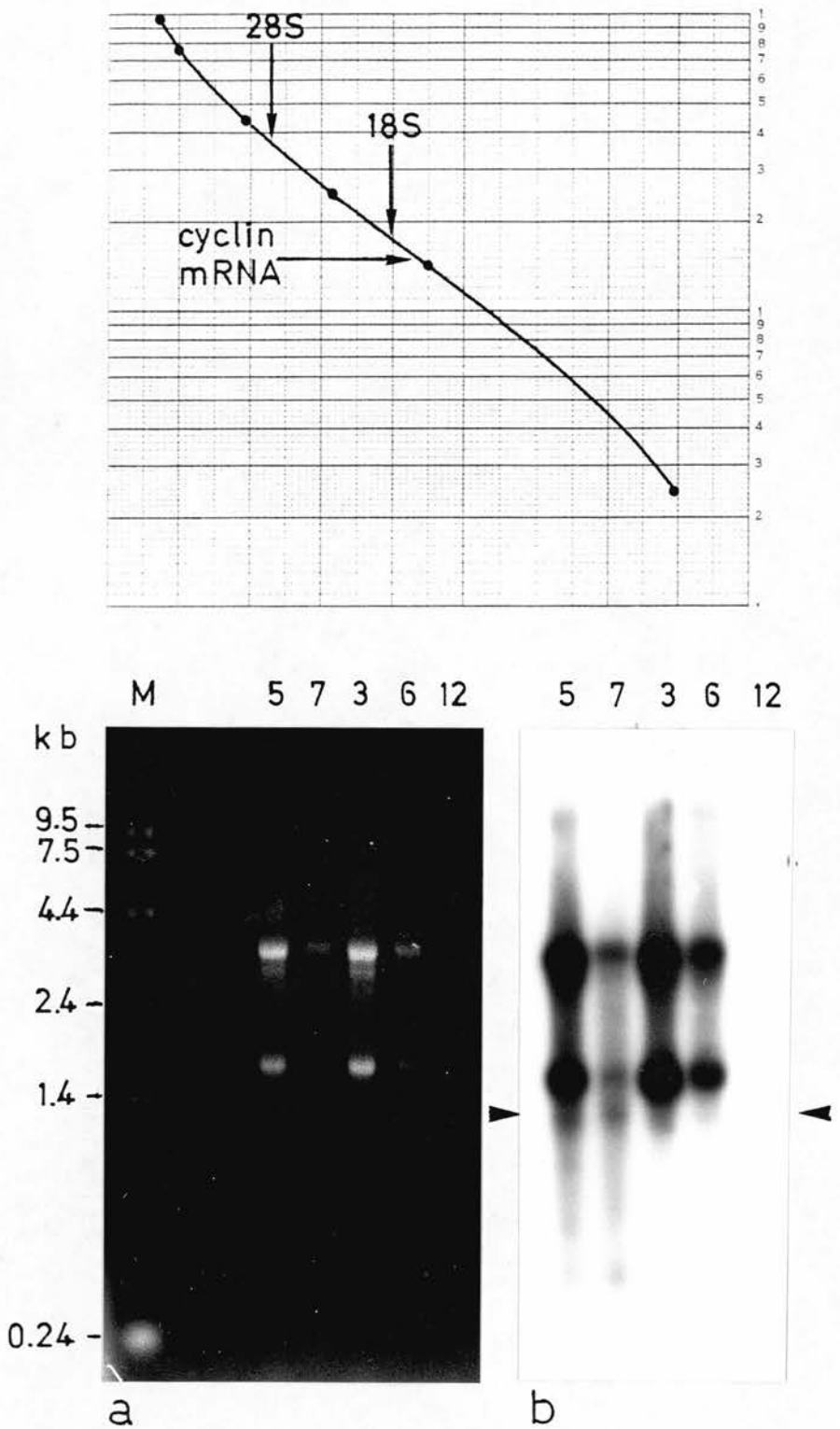

FIGURE 23. Hybridization of RNA separated on denaturing agarose gels with the cyclin B1 RNA probe. Samples corresponding to stage I fractions 5 and 7 and stage II fractions 3, 6 and 12 are shown. Marker RNA bands have lengths of 9.5, 7.5, 4.4, 2.4, 1.4 and 0.24 kilobases. (a) Gel stained with ethidium bromide. (b) Autoradiograph.

lack of presence of solubility because of a low NP-40 concentration, which itself prevents aggregation. Otherwise, the partitioning of signal between Poly(A)$^+$ and Poly(A)$^-$is not entirely convincing. Strongest signals are evidenced in fractions 2 and 4 of both the poly(A)$^+$ and poly(A)$^-$, thus indicating that not all cyclin message is binding to anti-pp56 and not all message is binding to oligo-(dT). Perhaps either the immunoprecipitation binding buffer system, or the hybridization binding buffer and conditions are not optimum for providing complete and specific binding. It is possible that the Poly(A)-tail length in cyclin mRNA is not long enough to ensure that message is not totally bound on passage through oligo-(dT)-cellulose. And again, perhaps this R3 probe is binding rRNA as in the Northern blot, thus explaining the poly(A)$^-$ signal in the fourth fraction. This blot was reacted at higher hybridization and washing conditions, 60^0C, but it was not analyzed with the JS23 probe. Reprobing is necessary.

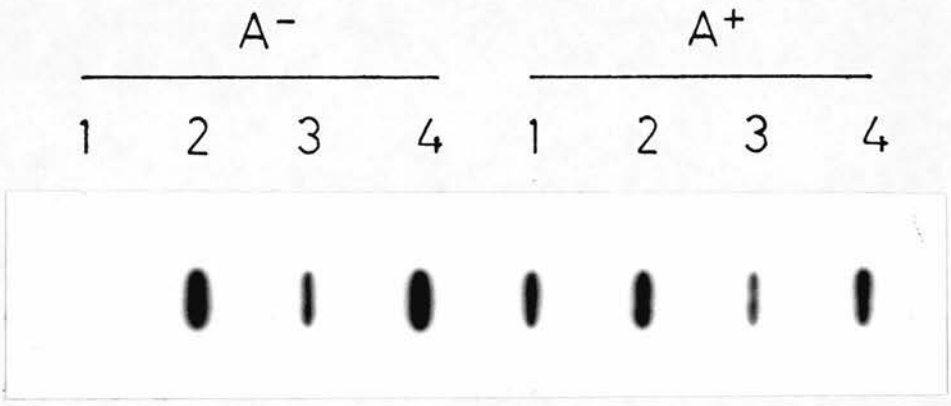

FIGURE 24. Hybridization with a cyclin B1 probe of RNA immunoprecipitated from poly(A)-minus and poly(A)-plus fractions of mRNP. Tracks 1-3 represent sequential elutions of unbound material from S.aureus-protein A-anti-pp56 while track 4 represents bound material eluted with 1% SDS. Autoradiograph of slot blotted RNA extracted from each fraction is shown.

DISCUSSION

Although relatively little research has been conducted on translation control, it is becoming increasingly more evident that regulation at this level is important to the general metabolism of cells (Hunt, 1985). Translation control is especially important in the regulation of gene expression during early animal development. In principle, translation control can occur at various steps in the assembly of mRNA into polysomes. In the past few years, specific examples have been cited to operate at the level of mRNA masking, in the formation of initiation complexes, in the elongation process and in the interaction of polyribosomes with cellular superstructures, which are the membranes, endoplasmic reticulum and cytoskeleton. Some of these regulatory steps are indicated in Figure 25.

Translation initiation consists of all steps leading to the interaction of mRNA with the ribosomal subunits and the initiation aminoacyl-tRNA. Aminoacyl-tRNA synthetases catalyze the formation of aminoacyl-tRNA -- the ester bonding of amino acids, corresponding to the anticodon of the tRNAs, to their respective tRNAs. The initiation aminoacyl-tRNA is always $tRNA^{Met}$. This $tRNA^{Met}$ binds eukaryotic initiation factor-2 (eIF-2), which transports the $tRNA^{Met}$ to the receptor site of the 40S small subunit of the ribosome, which itself interacts with the mRNA. This allows the anti-codon of the tRNA to interact with the AUG initiation codon of the mRNA. The initiation complex is then completed with the binding of the 60S large subunit of the ribosome.

Initiation factors, proteins bound transiently to ribosomes or mRNA, are important to the regulation of translation initiation. Eukaryotic initiation factors 4A, B and F (eIF-4A,B&F) bind to the 5'-cap structure of mRNA and are active as a helix destabilization complex which has the capacity to remove double stranded RNA structures from the 5'-end of mRNA molecules (Sonenberg, 1989). In order to function in helical unwinding, two of these factors, eIF-4B and eIF-4F probably have to be phosphorylated. Various protein kinases are involved, including protein kinase C, double-stranded RNA activated kinase and protease activated kinase II. At least some of these kinases are responsive to the presence of growth factors and mitogens. This effect will of course lead to the activation of protein synthesis. mRNA secondary structures, such as the 5'-stem loop, are themselves inhibitory for translation, probably acting by prevention of ribosome binding to the mRNA (Sonenberg, 1989). The activity of the cap-binding complex would remove such secondary structures.

The action of the mRNA-binding phosphoproteins described in this study is dependent for inhibition of translation on phosphorylation by casein kinase II. Another protein which

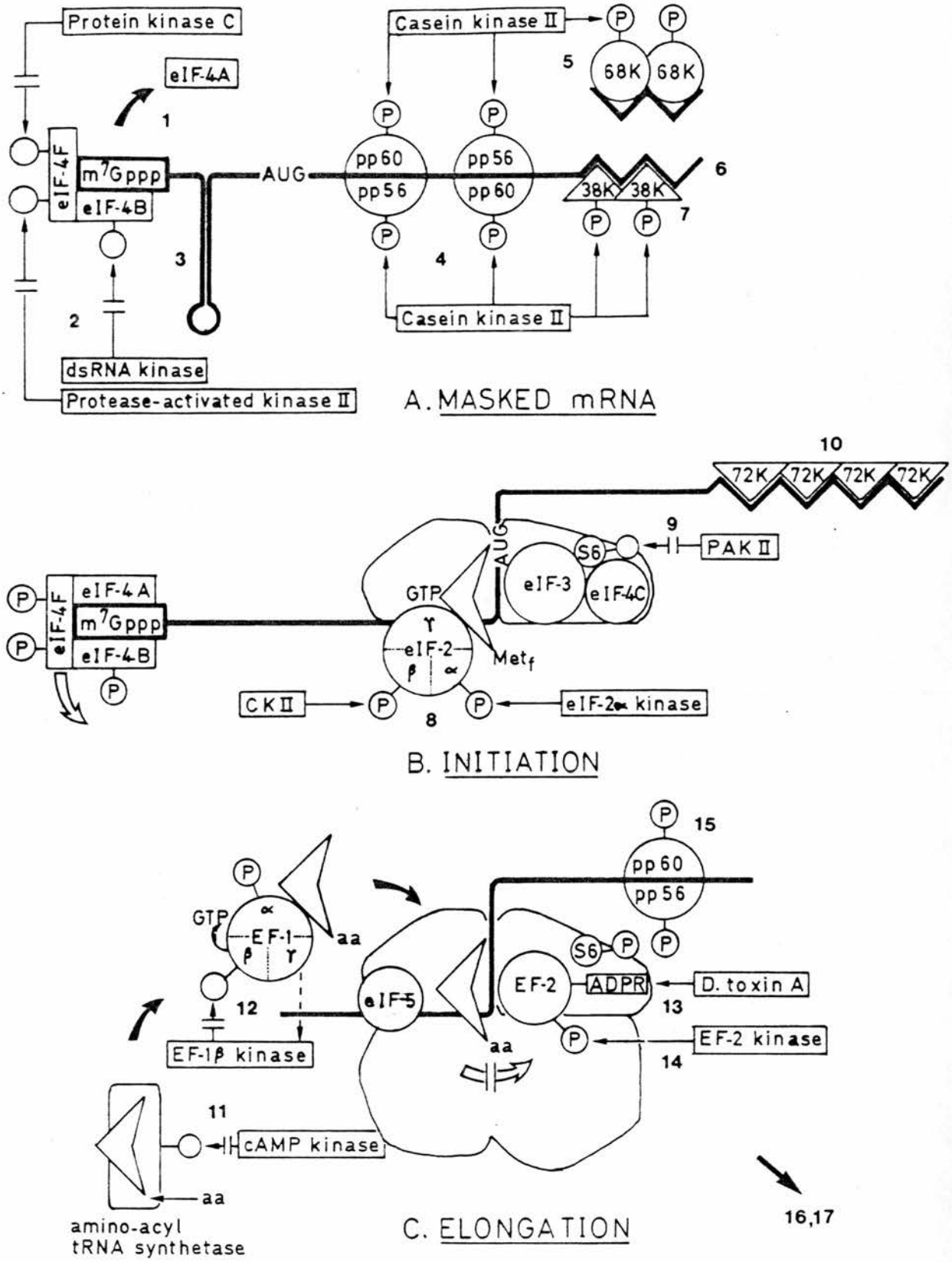

FIGURE 25. Scheme for the inhibition of protein synthesis at the level of mRNP formation, formation of initiation complexes and the elongation process.

A. Formation of mRNP particles.

1. Deficiency in eIF4A: Audet et al., 1987.

2. Lack of recycling or phosphorylation of eIF4B and eIF4F: Huang et al., 1987.

3. mRNA secondary structure: Lee et al., 1983; Sonenberg, 1989.

4. Phosphorylation of mRNP proteins: Kick et al., 1987.

5. Binding of inhibitor particles: Bag et al., 1980; Sarkar et al., 1981; Slegers et al., 1989.

6. Shortening of poly(A)-tail: Rosenthol et al., 1983; Paynton et al., 1988.

7. Binding of masking proteins to poly(A): DeHerdt et al., 1984.

B. Formation of initiation complexes

8. Phosphorylation of eIF2: Safer, 1983; Proud, 1986.

9. Lack of phosphorylation of S6: Taylor et al., 1985; Martin-Perez et al., 1986.

10. Non-availability of 72kD poly(A) binding protein: Baer and Kornberg, 1983; Sachs et al., 1986.

C. Elongation.

11. Lack of phosphorylation of S6: Traugh et al., 1989.

12. Lack of phosphorylation of EF1: Davydova et al., 1984.

13. ADP-ribosylation of EF2: Stitikov et al., 1984.

14. Phosphorylation of EF2: Ryazanov et al., 1988.

15. Retention of mRNA-binding phosphoproteins: Cummings & Sommerville, 1988.

D. Formation of superstructures.

16. Failure of polysomes to interact with membranes: Walter et al., 1981.

17. Failure of polysomes to associate with the cytoskeleton: Cervera et al., 1981; Van Venrooij et al., 1981; Howe & Hershey, 1984.

has a negative effect on translational ability is a 38kD protein which binds the poly(A)-tail of masked message in gastrulae of the brine shrimp, Artemia (DeHerdt et al., 1984). In addition to these proteins in Xenopus oocytes and Artemia gastrulae, there have been other inhibitory polypeptides described which interact with mRNA in a variety of cell types, including mammalian cells and sea urchins (Huang et al., 1987). Generally, the proteins in these cell types are inhibitory when they are phosphorylated and they all appear to be phosphorylated by casein kinase II. Another means of inhibiting translation, which may function in Xenopus, has been identified in Artemia. A separate inhibitory particle contains a short, U-rich RNA component (85-120 nucleotides), which may well act through base pairing with polyadenylated RNA. This U-rich RNA sequence and its associated proteins can be dissociated from the mRNP complex in low concentrations(1-5mM) of EDTA (Slegers et al., 1989). A formal possibility is that pp56 and pp60 are actually the proteins bound to this U-rich inhibitory particle; but since EDTA has been present throughout all cross-linking experiments conducted in this study, these inhibitory particles and all associated proteins would be dissociated from the polyadenylated mRNA sequence and not bound at the oligo-(dT)-cellulose selection stage.

Various claims have been made for the actual length of the poly(A)-tail being important for mRNA stability and translatability. Certainly at oocyte maturation, poly(A)-tail length changes. However, the significance of these changes are not fully understood. In this study, maturation stages (Stage VI oocytes) were not examined. Poly(A)-tail length on mRNA in oocyte stages examined here was predominantly 60-80 adenosine residues (Cabada et al., 1977). After fertilization, mRNA molecules in early embryos have poly(A)-tail lengths of 150-200 residues. Poly(A)-tail length was not considered to be a major factor in the regulation of translation of oocytes and embryo stages examined in this report.

The role of phosphorylation in the formation of translation initiation complexes is being intensively studied. Of particular interest is the phosphorylation of the α and B sub-units of eIF-2. Again, eIF-2B phosphorylation is catalyzed by casein kinase II and is inhibitory to translation. The eIF-2 α sub-unit is phosphorylated by its own eIF-2 α kinase. At the level of initiation, the S6 protein of the ribosomal small sub-unit is required to be phosphorylated for maximum translation of atleast certain mRNA species (Traugh et al., 1989). This positive effect of phosphorylation results from the activities of the mitogen stimulated S6 kinase and the protease activated kinase II mentioned earlier.

Translation elongation, step-wise addition of amino acids to the nascent polypeptide chain, requires supply to the translating ribosome of new, charged tRNA molecules and translocation of peptidyl tRNAs from the acceptor, amino-acyl site (A-site) to the peptidyl site (P-site). Transport of tRNAs to the ribosome is effected by elongation factor 1 (EF-1),

whereas translocation of peptidyl tRNAs between A and P sites is effected by EF-2. The elongation process can be potentially regulated at various steps. For instance, blockage might occur through lack of phosphorylation of amino acyl tRNA synthetases or lack of phosphorylation of EF-1 (Janssen and Moller, 1988). Alternatively, inhibition can occur through ADP ribosylation of EF-2 (Stitikov et al., 1984), or, as claimed more recently, phosphorylation of EF-2 (Davydova et al., 1984). It should be noted that inhibitory mRNA-binding phosphoproteins, such as pp56 and pp60 in Xenopus, can be retained, thus blocking the elongation of mRNAs (Cummings and Sommerville, 1988). Even though these mRNP particles are complexed with ribosomes and initiation has been stimulated, so long as these proteins remain phosphorylated, elongation will not proceed.

A final requirement of activated polysomes is that they should interact with the appropriate cellular superstructures. Elongation of translation of mRNA species encoding secretory proteins is stalled unless interaction occurs between the N-terminal signal peptide and the signal recognition particle (SRP-RNP complex). The SRP is responsible for the docking of the polysome on membrane receptors and the subsequent translocation of the polypeptide into the intra-molecular space (Walter et al., 1981). However, even polysomes which do not associate with the endoplasmic reticulum are believed to interact with structural components of the cytoplasm, namely the cytoskeleton (Cervera et al., 1981; Van Venrooij et al., 1981; Howe and Hershey, 1984). The elements believed to be most influential in anchoring ribosomes and translation factors are the intermediate filaments consisting of vimentin. It is interesting to note that the most prevalent intermediate filaments in amphibian oocytes consist, not of vimentin, but of cytokeratin (Franz et al., 1983).

As mentioned earlier, translation regulation is especially pertinent to developmental systems. The ooctyes of sea urchins, like Xenopus, produce and store maternal mRNA for its subsequent translation upon fertilization. The mass synthesis of protein from this maternal message does not occur until after fertilization, despite the presence of all necessary translation machinery throughout oogenesis. However, unlike Xenopus, in the sea urchin, the role of maternal mRNA masking proteins is believed not to be the most important factor in the inhibition of translation. Blockage occurs primarily at the level of formation of pre-initiation complexes, namely just after mRNA binding to the small ribosomal subunit. This blockage appears to be a result of eIF-4F binding in a functionally unavailable form due to modification by an as yet unidentified inhibitor of translation in the egg. It has been postulated that this egg inhibitor may prevent the normal recycling of eIF-4F. However, this eIF-4F modification is probably only one of several factors responsible for the regulation of mRNA utilization prior to fertilization (Huang et al., 1987).

Artemia salina also store maternal mRNA in non-polysomal mRNP particles. Artemia provide an interesting model since they are cryptobiotic and their development is blocked at gastrulation, with gastrulae containing large stores of this non-polysomal mRNP. After reactivation of development, a rapid formation of polysomes is observed. This formation is due to the presence of both free mRNP particles and preinitiation complexes. The major factors inhibiting translation in this system are: 1) the presence of a specific poly(A) binding phosphoprotein of 38kD (DeHerdt et al., 1984) and 2) the presence of small inhibitor particles which interact with mRNP particles (Slegers et al., 1989). It is also known that Artemia use a similar phosphorylation/dephosphorylation mechanism to Xenopus, utilizing casein kinase II, to inhibit translation.

Nuclear and cytoplasmic RNP particles are evidently important to the development of sperm in Drosophila hydei and Drosophila melanogaster. It has been shown that the Xenopus anti-pp60 recognizes a distinct mRNP antigen in D. hydei and that the Y chromosomal loops provide chromatin compartments for distinct RNP populations, which most likely exert their function in post-meiotic stages during spermatogenesis. Anti-pp60 has been shown to cross-react with an RNP protein of 96kD, present both in the cytoplasm and the nucleus, which may be involved with the transport of pre-mRNP particles to the cytoplasm (Glatzer and Kloetzel, 1986). The Xenopus anti-pp56 also cross-reacts with Y chromosome loop RNP in Drosophila spermatocytes. Interestingly, the RNP structures recognized by anti-pp56 on the Y chromosome loops of Drosophila are different from those recognized by anti-pp60 (K.H.Glatzer, personal communication). The main conclusion is that these antigens have a function in Drosophila similar to that in Xenopus oocytes, that is in the storage of mRNA sequences.

In the amphibian oocyte system, previous work has identified a set of abundant phosphoproteins as the major inhibitory components. When phosphorylated, such proteins, particularly pp60 and pp56, interact strongly with mRNA and inhibit translation (Kick et al., 1987). Furthermore, the phosphorylating enzyme (protein kinase) has been shown to form an integral part of the mRNP complex in small oocytes (Cummings and Sommerville, 1988). The objectives of this study were: 1) to confirm that the phosphoproteins interact directly with mRNA sequences, 2) to identify the sequences bound to the phosphoproteins, 3) to assay the relative activities of the protein kinase throughout early development, when major changes in translational activity occur, and 4) to probe for a specific message, cyclin mRNA, in the RNP fractions. In addition to kinase activity, agents bringing about dephosphorylation, phosphatase activity and the presence of kinase inhibitors' were also assayed.

Cross-linking experiments provided direct evidence that phosphoproteins, namely pp56 and

pp60, are in direct contact with the mRNA sequences in mRNP particles. Through these studies, however, we have found no evidence that pp56 and pp60, negative regulators of translation when phosphorylated, bind to the poly(A) sequence in vivo. Their binding sites appear to be on the heterologous sequences of the mRNA. This is markedly different from the situation in Artemia. In Artemia, a 38kD phosphoprotein is found to bind the poly(A) sequence (DeHerdt et al., 1984). This protein is similar, but not identical to HD-40, a helical destabilizing protein, and HD-40, in turn, appears to be identical to eIF-4A. The proteins have yet to be sequenced for confirmation. Since HD-40 and eIF-4F would normally be associated with the cap structure, one might have to postulate that, in at least these mRNP particles, the 3' end meets the 5' end. In the Xenopus experiments, the only protein detected as being bound to the poly(A) sequence is a 72kD polypeptide, which is normally found on the poly(A)-tail of actively translating mRNA. On the basis of these results, it would appear that the major block occurs not at the poly(A) sequence, but rather the upstream heterologous sequence. From rough stoichiometric calculations, it would appear that one molecule of pp60 or pp56 binds every 35 nucleotides over the entire mRNA sequence, excluding poly(A)-tails.

The phosphoproteins pp56 and pp60 accompany maternal mRNA from oocytes through early embryos. The abundance of the phosphoproteins in mRNP fractions implies that they are bound to a major set of mRNA molecules. The protein kinase itself is part of the mRNP complex. Estimation of kinase activity, phosphatase activity and presence of kinase inhibitors through early embryogenesis (Fig.26) is consistent with the view that phosphorylation inhibits translation and dephosphorylation permits translation.

The results of the kinase studies were incorporated with previous phosphatase work to formulate a general scheme of formation of maternal mRNA and its incorporation into polysomes. In comparing the quantitated assay levels of kinase and phosphatase with the general schematic diagram of maternal mRNA and its incorporation into polysomes, we could roughly conclude that kinase activity decreases and phosphatase activity increases when translation occurs. However, translation is a complex process and other factors may be important, for instance, the apparent depletion of eIF-4A in Xenopus oocytes. This conclusion is based on the observation that microinjection of purified eIF-4A stimulates protein synthesis in oocytes two fold (Audet et al., 1987).

In PV ovary, vast amounts of mRNA are being synthesized, with much of this mRNA being stored as free mRNP particles. The relative activity of protein kinase starts low, but increases proportionately with the amounts of mRNA accumulated (Golden et al., 1980). Kinase inhibitor and protein phosphatase are relatively low in stage I oocytes. There are few ribosomes present, but close to 20% of those formed are present in polyribosomes.

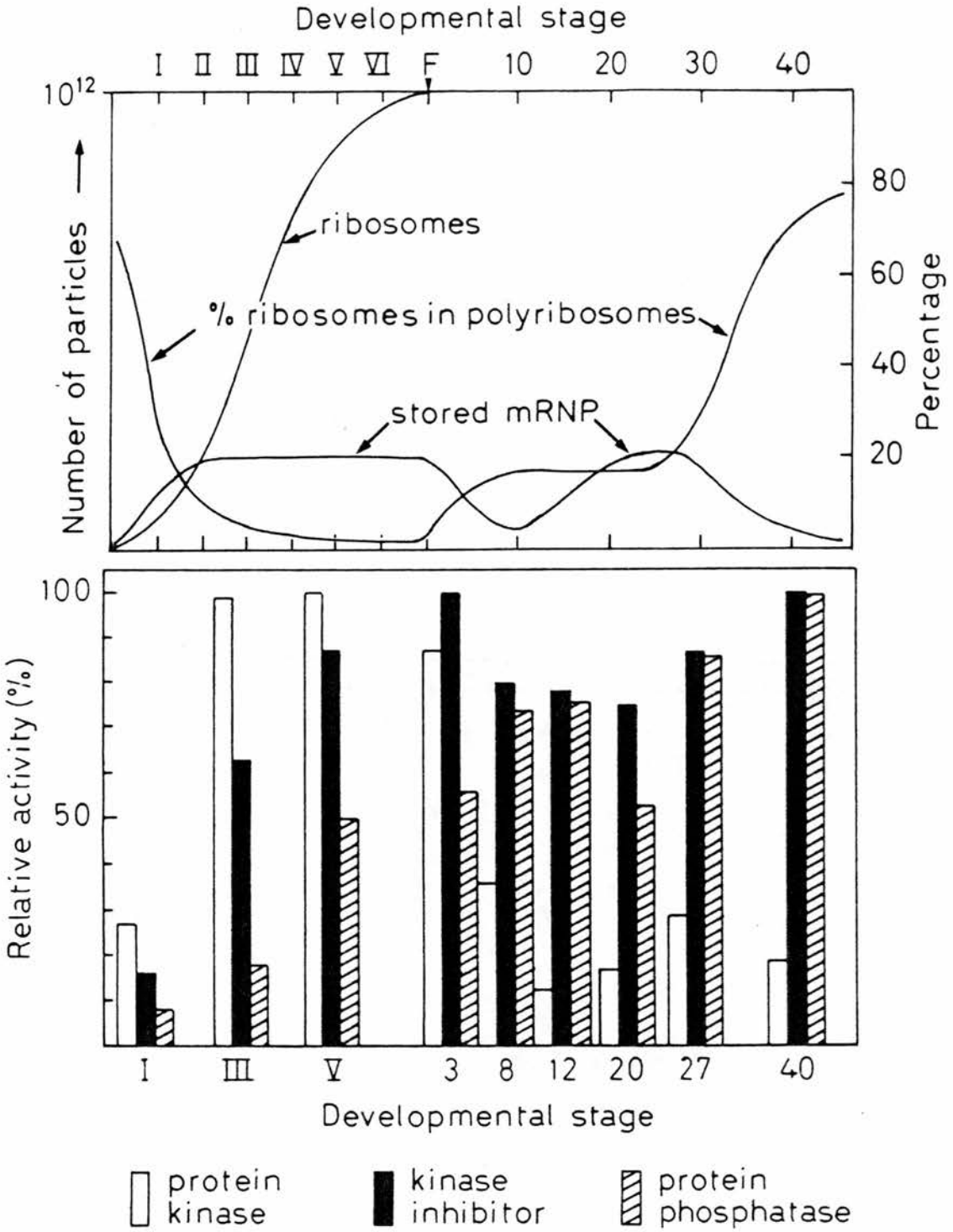

FIGURE 26. Summary of assays for protein kinase activity, presence of kinase inhibitors, and phosphatase activity operating on pp60/56 through early development compared with fluctuations in cellular pools of stored mRNP, ribosomes and polyribosomes. Data for pool sizes taken from Woodland (1974); Sommerville (1977); Golden et al (1980).

mRNP particles at this stage are mainly free and sediment in glycerol gradients between 30S and 80S.

In stage II oocytes, ribosomes start to accumulate in numbers equivalent to the pool size of mRNP particles, while the percentage of ribosomes in polysomes decreases (Fig.26). The distribution of mRNP particles in glycerol gradients now shifts towards aggregates sedimenting at 80-120S. We have demonstrated previously that these aggregates consist of 80S ribosomes and mRNP particles, apparently organized in a 1:1 ratio as blocked translation initiation complexes (Cummings and Sommerville, 1988). The pool size of mRNP particles plateaus at stage II and remains at this constant level until fertilization. It is interesting to note the coincidence between the capacity for phosphorylation, namely the dramatic increase in protein kinase activity coupled with a slight increase in kinase inhibitor activity and a minimal rise in protein phosphatase activity between stage I and stage III oocytes, and the accumulation of mRNA and ribosomes in inactive forms. Ribosomes, in particular, accumulate drastically between stage II and stage IV with the maximum (10^{12}) number of particles reached by fertilization, while the percentage of polyribosomes present remains at 1-2% through the last stages of oogenesis (A doubling in polysome number occurs, however, at maturation, Woodland, 1974). In accordance with these findings, kinase activity maintains its maximum level at stage III and V. These findings are reasonable given the plateau of stored mRNP particles and the accumulation of ribosomes. However, kinase inhibitor and protein phosphatase activity were found to increase as well, with a final relative activity of 85% and 55%, respectively, at stage V. Perhaps these findings are related to a balance between phosphorylation and dephosphorylation or even to a build-up of inhibitor and phosphatase in a non-available state.. Taken together, the accumulation of protein kinase with free mRNP particles and ribosome/mRNP complexes indicates an oocyte hindered, at least to some extent, from translation by phosphorylation of its bound proteins.

A very different situation arises post-fertilization. According to Woodland (1974), ribosomes become mobilized into polysomes after fertilization in two phases: through cleavage to blastula, the percentage of ribosomes in polysomes increases from 3% to 16%; this level remains constant until the end of neurula and between tailbud and feeding stages an increase to 76% occurs. It seems reasonable to assume that the first phase is associated with saturation of maternal mRNA with ribosomes and that the second phase is associated with the utilization of newly-synthesized zygotic mRNA. That the first phase is accompanied by a decline in mRNP-bound protein kinase activity is evident (Fig.26). Between embryo stage 3 and 8, protein kinase relative activity decreased from 87% to 12%, while kinase inhibitor activity decreased from 100% to 78% and protein phosphatase activity increased steadily from 55% to 75%. These results must be examined in relation to

the stored mRNP and polyribosome situation. The number of free mRNP particles declines drastically between fertilization and stage 10, with an accompanying increase in the percentage of ribosomes in polyribosomes, indicating translation of the previously inhibited maternal mRNA. It has been shown previously that free mRNP particles and mRNP/ribosome initiation complexes mask mRNA from translation while phosphorylated (Kick et al., 1987; Cummings and Sommerville, 1988). Between fertilization and stage 10, a marked increase in dephosphorylation in particles and complexes must occur, with our assay results confirming this theory.

By blastula (stage12), the embryo begins to manufacture its own zygotic mRNA, with a new rise in stored mRNP particles between stages 20 and 30, while the polyribosome content remains virtually the same until close to stage 30. A slight increase in kinase activity during neurula (stage 20) and stage 27 may represent short-term storage of some of the zygotic mRNA synthesized between mid-blastula and the end of neurula. Kinase inhibitor activity decreases slightly, while protein phosphatase activity decreases more significantly between stage 12 and 20. However, by stage 27, both have increased markedly again, corresponding to the decrease in the level of stored mRNP, the subsequent increase in the percentage of ribosomes in polyribosomes, and the translation of this zygotic mRNA. By stage 40, kinase inhibitor and protein phosphatase are maximally active, while kinase activity has decreased still further. Virtually no mRNA is stored in mRNP particles and polyribosomes occupy nearly 80% of the ribosomes in the embryo.

Taken together, the results in this report suggest that dephosphorylation of proteins bound to maternal mRNA occurs at early embyogenesis and that this transition is coincident with mobilization of the mRNA into polysomes. Whereas the individual pieces of evidence: loss of protein kinase activity from the mRNP particles; presence of protein kinase inhibitors; increase in phosphatase activity, are to some extent circumstantial, they are consistent with previously reported observations. For instance, in in vitro reconstitution experiments, translation of globin mRNA is inhibited by binding of phosphorylated pp60 but dephosphorylation leads to a weakening of binding and release of the mRNA for translation (Kick et al., 1987). Also, microinjection into oocytes of inhibitors of the mRNP associated protein kinase stimulates translation by recruiting more mRNA from the pool of stored mRNP particles (unpublished data).

Whereas the experiments reported here making use of cyclin RNA probes have merely indicated that this sequence is contained within the oocyte stored mRNP pool and is probably complexed with the phosphoprotein pp56, further work is required in this direction. A combination of assays for cyclin RNA, or some other specific, maternal mRNA sequence, and immunological localization of proteins associated with these mRNAs

would be valuable in plotting the distribution and compartmentalization of specific sequences in oogenesis and early development. Such studies should prove enlightening on the mechanisms of selective expression of maternal mRNA sequences.

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
DEPC	Diethyl pyrocarbonate
DOC	Deoxycholate
DTT	Dithiothreitol
HPRI	Human Placental Ribonuclease Inhibitor
kD	Kilodalton
2-ME	2-Mercaptoethanol
MOPS	Morpholinopropanesulphonic acid
mRNA	Messenger ribonucleic acid
mRNP	Messenger ribonucleoprotein
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SSC	Standard sodium citrate
PIC	Phenol/Isoamyl Alcohol/Chloroform
PV	Previtellogenic
UV	Ultraviolet

anti-p48	Antiserum raised against the tRNA-binding protein of Xenopus oocytes, used as a negative control.
Casein	Dephosphorylated forms from Sigma Chemical Co.
10K	10,000 rpm for 10 min in a Sorval HB4 rotor.
Collagenase	Sigma, Type IV.
λ	Lambda, sama as microlitre
Specific activities	^{3}H-uridine:26 Ci/mmol ^{32}P-gamma ATP: 2,000Ci/mmol
Tris	Tris-HCl, pH 7.5.

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