Protein Kinase Activity Associated with Stored Messenger Ribonucleoprotein Particles of *Xenopus* Oocytes

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Abstract. As the oocytes of *Xenopus laevis* grow and develop they accumulate vast stores of mRNA for use during early embryogenesis. The stored mRNA is stabilized and may be prevented from being translated in oocytes by the binding of a defined set of oocyte-specific proteins to form messenger RNP (mRNP) particles. A key event in the interaction of protein with mRNA is the phosphorylation of those few polypeptides that bind directly to all classes of polyadenylated mRNA. In this study we show that the phosphorylating enzyme (protein kinase), in addition to its target phosphoproteins, is an integral component of the mRNP particles. This association extends through various stages in the formation and use of the mRNP particles. Examination of material from oocytes of an early developmental stage (early stage 1), when the level of accumulated mRNA is low, reveals an excess of protein particles free of RNA, sedimenting at 6–18 S, and containing protein kinase activity and mRNA-binding phosphoproteins. At stages of maximum rate of mRNA accumulation (stages 1 and 2), the phosphoproteins and kinase are found primarily in individual mRNP particles that sediment at 40–80 S. As ribosomes become abundant (stages 2 and 3), the mRNP particles tend to interact with ribosomal subunits, at least in vitro, to form blocked translation initiation complexes that sediment at 80–110 S. These results are compared with observation on stored mRNP in other developmental systems.

Of the messenger RNA synthesized and accumulated throughout oogenesis in *Xenopus laevis*, only ~5% is translated in oocytes, the remainder being stored as messenger RNP (mRNP)[1] particles for use during early embryogenesis (reviewed in reference 9). Although essentially the entire pool of $\sim 2 \times 10^{11}$ particles/oocyte is established before the onset of vitellogenesis (within stages 1 and 2), synthesis continues throughout much of the remainder of oogenesis (stages 3–6) to maintain the mRNP pool which might otherwise be depleted by RNA turnover (13, 17, 25). Nevertheless the bulk of mRNA appears to be stable over periods of several months (15). One function of the later phase of oogenesis (stages 2–6) is to accumulate the final pool of $\sim 10^{12}$ ribosomes. Thus by the end of oogenesis there is a massive stockpile of both mRNP particles and translation machinery to be mobilized after fertilization (reviewed in reference 28). Taking these observations into consideration, at least three possible functions can be assigned to the proteins that are bound to mRNA molecules in the mRNP particles: (*a*) to protect mRNA from degradation; (*b*) to prevent premature translation of mRNA; and (*c*) to provide components eventually to be used as translation factors.

1. *Abbreviations used in this paper*: HB, homogenization buffer; mRNP, messenger RNP; poly(A)$^+$, polyadenylated; poly(U), polyuridylic acid; pp, phosphoprotein.

The association of protein with mRNA in oocytes has been shown to confer ribonuclease resistance on the RNA (7) and so the suggested function of stabilization seems reasonable. As for inhibition of translation, mRNA recovered by deproteinization of mRNP particles is translated in vitro as efficiently as is mRNA extracted from polysomes (6); yet, addition of oocyte mRNP proteins to globin mRNA blocks its translation (23). Therefore the second suggested function also seems reasonable. However, the third suggested function is still open to question, for no systematic comparison of oocyte mRNP proteins with known translation factors has, as yet, been made.

Most of the protein mass of the mRNP particles is comprised of four polypeptides (60, 56, 54, and 50 kD) present in roughly equimolar amounts (7). Recently, we have shown that the 60- and 56-kD polypeptides are heavily phosphorylated at developmental stages when mRNA translation is maximally repressed (10). Phospholabeling of the 60-kD phosphoprotein (pp60) enables its isolation and characterization. It transpires that in in vitro binding assays, pp60 is the most effective polyadenylic acid binding protein from the oocyte mRNP particles (20). Formation of RNP complexes in vitro, using pp60 and globin mRNA, results in inhibition of translation; whereas dephosphophorylation of pp60 destabilizes the mRNA/protein complex making the mRNA accessible for translation (20).

Here we examine the association of protein kinase activity

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with the mRNP particles of *Xenopus* oocytes and show that phosphorylation of some mRNP proteins is initiated before their binding to mRNA. Although continuing phosphorylation is a feature of stored mRNA that accumulates as free particles, we demonstrate that phosphorylation, per se, does not preclude the binding of mRNP particles to ribosomes to form nontranslating complexes.

Materials and Methods

Phospholabeling of mRNP Particles

Pre- or early vitellogenic ovary was obtained from immature, female *Xenopus laevis*. For labeling in vivo, a single ovary was incubated in Barth's solution for 6 h at 18°C in the presence of [^{32}P]phosphate (500 µCi/ml; Amersham International, Amersham, UK) as described previously (20). After thorough washing in Barth's solution, the ovary was suspended in homogenization buffer (HB; 8% glycerol in 50 mM NaCl, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5) and homogenized with 20 strokes of a hand-operated teflon/glass homogenizer. The homogenate was clarified by centrifugation at 10,000 *g* for 10 min at 0°C and 1 ml of the supernatant was layered on a 32-ml gradient of 15–40% glycerol made using the same buffer. Centrifugation generally was at 17,000 rpm for 16 h at 2°C using an MSE 6 × 38-ml swing-out rotor. 1.5-ml fractions were collected and samples were analyzed for acid-precipitated radioactivity.

Unlabeled ovary was homogenized and fractionated as described above and individual gradient fractions were used for in vitro phospholabeling. To each 0.5 ml of gradient fraction containing 5–25 µg RNP we added 4 µCi (2 pmol) of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham International) and the mixture was incubated for 1 h at 18°C.

Labeled or unlabeled RNP material contained in glycerol gradient solution was either used directly for column chromatography and nondenaturing gels, fixed for CsCl gradient analysis by addition of neutralized formaldehyde to 3.6%, or precipitated with 2 vol ethanol for SDS-PAGE. Occasionally, RNP fractions were stored for periods of up to 1 wk at −70°C.

Labeling and Analysis of Aminoacyl and Peptidyl tRNA

Ovary from mature animals was treated with 0.2% collagenase to release individual oocytes (10). Stage 3 oocytes were selected and labeled for 24 h at 18°C in the presence of 50 µCi/ml [^{35}S]methionine (800 Ci/mmol; Amersham International). The oocytes were then washed free of label and incubated for a further 2 h with or without 50 µg/ml puromycin (Sigma Chemical Co., St. Louis, MO). Samples of 150 oocytes were then suspended in 0.5 ml HB and sonicated (four 5-s bursts at setting 5 of a W185 Sonifier [Branson Sonic Power Co., Danbury, CT]). Supernatants were prepared by centrifugation at 10,000 *g* for 10 min at 0°C and layered directly, or after addition of 5 U/ml calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN), onto glycerol gradients and centrifuged as described above. Gradient fractions of 0.4 ml were analyzed for aminoacyl- or peptidyl-labeled tRNA by precipitation with cetyltrimethylammonium bromide as described previously (8).

Affinity Column Chromatography

Columns of 1 ml polyuridylic acid- (poly[U]) Sepharose or of Sepharose 4B (both Pharmacia Fine Chemicals, Piscataway, NJ) were equilibrated with HB. After application of glycerol gradient fractions containing ~50 µg of RNP, the columns were washed twice with 0.6 ml (void volume) of HB and then the bound material was eluted successively with 20% formamide (2 × 0.6 ml) and 60% formamide (2 × 0.6 ml), both made up in HB. Formamide was removed from the column fractions by dialysis against HB and the dialyzed fractions were labeled with [γ-^{32}P]ATP as described above. In some instances dialyzed column fractions were added together before phospholabeling.

Gradient fractions containing mRNP phospholabeled in vivo were applied to poly(U)-Sepharose or to oligo(dT)-cellulose (Pharmacia Fine Chemicals) columns and eluted stepwise with HB containing 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50, and 1 M NaCl. Finally, residual bound material was eluted with H_2O. Elution peaks were collected in 2 ml buffer, precipitated with 2 vol ethanol, and analyzed by SDS-PAGE and autoradiography.

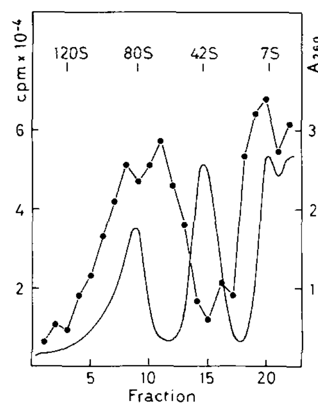


Figure 1. Separation of ribonucleoprotein particles by centrifugation of ovary homogenate through glycerol gradients. Previtellogenic ovary was labeled in vivo for 6 h in the presence of [^{32}P]phosphate. After centrifugation, the gradient was scanned at 260 nm and fractions were acid precipitated to determine phospholabeling. The distinction between absorbance (——) and phospholabeled (●——●) peaks is evident. Sedimentation rates (*S*) for marker particles are indicated.

Density Gradient Centrifugation

Samples (100 µl) containing fixed phospholabeled RNP were layered on 5.5-ml preformed gradients of 6–48% CsCl in 3.6% neutralized formaldehyde, 0.5% Brij-58 (Serva Fine Chemicals, Heidelberg, Federal Republic of Germany) 0.1 M potassium phosphate, pH 7.2. After centrifugation at 38,000 rpm for 16 h at 18°C using an MSE 3 × 6-ml swing-out rotor, 0.3-ml fractions were collected and analyzed for density, absorbance, and acid-precipitated radioactivity.

Electrophoresis and Autoradiography

Electrophoresis of polypeptides through SDS–12% polyacrylamide gels was performed as described previously (21).

For electrophoresis of native RNP structures, glycerol gradient fractions were layered directly on 5% polyacrylamide gels and were subjected to electrophoresis using 50 mM Tris-glycine buffer, pH 8.3. Material was transferred from the gel onto nitrocellulose and strips corresponding to gel tracks were incubated either in the presence of antiserum directed against the 60-kD mRNP protein followed by [^{125}I]protein A as described previously (10), or in the presence of 10 µCi/ml of [γ-^{32}P]ATP in HB. After thorough washing in HB the strips were air dried.

After electrophoresis, gels were stained with 0.1% Coomassie Blue R250 in methanol/acetic acid/water (4:1:5), destained in methanol/acetic acid/water (2.5:1.0:6.5), and dried on a heated evacuating drier (Bio-Rad Laboratories, Richmond, CA). Molecular masses of proteins were determined using protein standard mixtures (Sigma Chemical Co.).

After drying, the gels or filter strips were exposed for 2–4 d at −70°C in contact with Kodak X-Omat S film and an intensifying screen.

Results

Both Protein Kinase and Target Phosphoproteins Are Integral Components of mRNP Particles

Messenger RNP particles are recovered from homogenates of *Xenopus* previtellogenic ovary by sedimentation through glycerol gradients. On labeling the ovarian tissue with [^{32}P]phosphate for 6 h before homogenization, the sedimentation pattern of incorporated phospholabel (Fig. 1) is seen not to reflect the major peaks of RNA-containing particles: the 80-S monosome peak; the 42-S peak containing stored 5-S RNA and tRNA (12); and the 7-S peak containing stored 5-S RNA (12). Rather, phospholabeling occurs over a broad range of sedimentation (40–120 S) with a distribution identical to that of particles containing polyadenylated (poly[A]$^+$) RNA; i.e., as bimodal with peaks at 65 and 90 S (Fig. 1; cf. reference 7). In addition, a second major phospholabeled fraction sediments at 6 S–18 S as particles free of poly(A)$^+$RNA (cf. reference 7) and, as we show later, like protein ag-

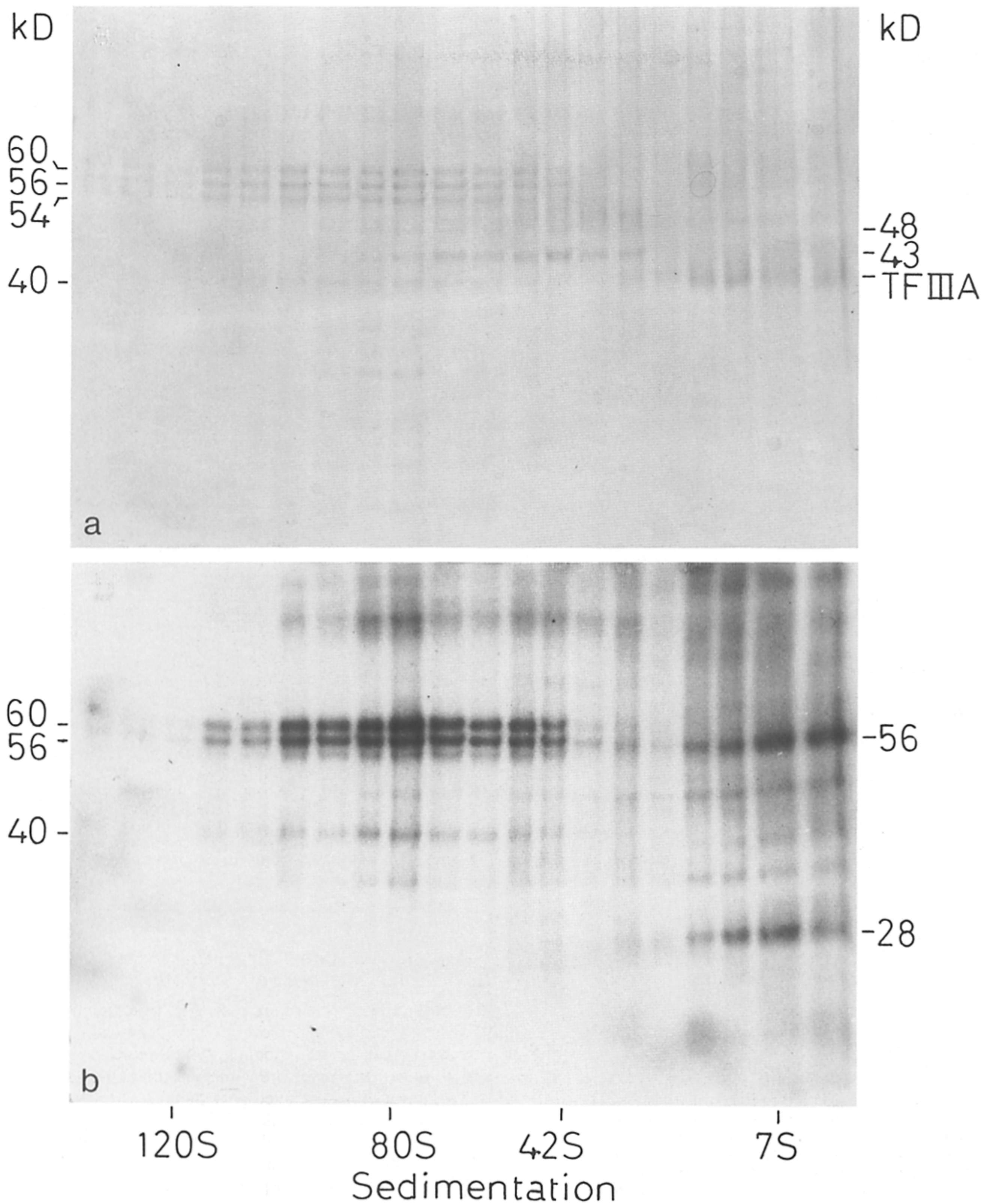
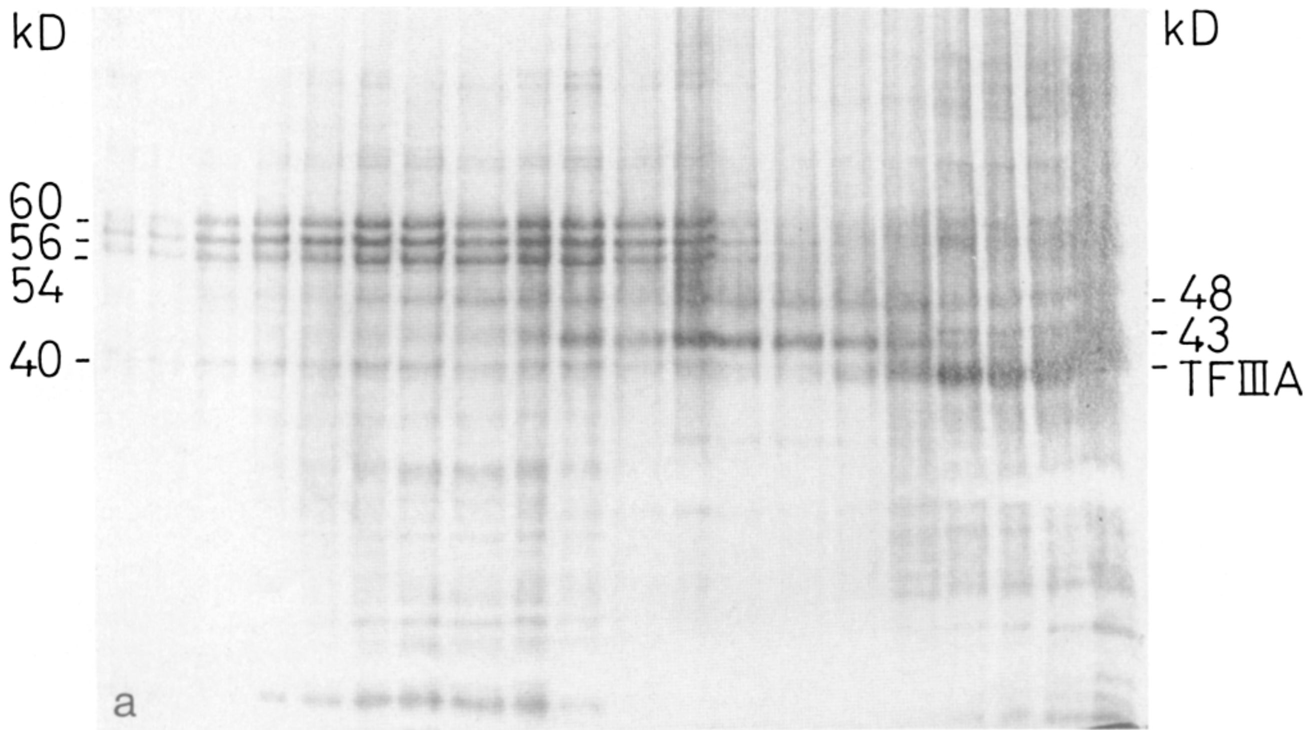


Figure 2. Distribution of proteins in glycerol gradient fractions and identification of mRNP proteins phospholabeled in vivo. (*a*) Stained gel. (*b*) Autoradiograph. Previtellogenic (stage I) ovary was labeled as for Fig. 1. Sedimentation rates for the fractions are indicated as are the molecular masses of major proteins. Transcription factor IIIA (*TFIIIA*) has a mass similar to the 40-kD protein of mRNP particles, but unlike the mRNP protein is never phospholabeled.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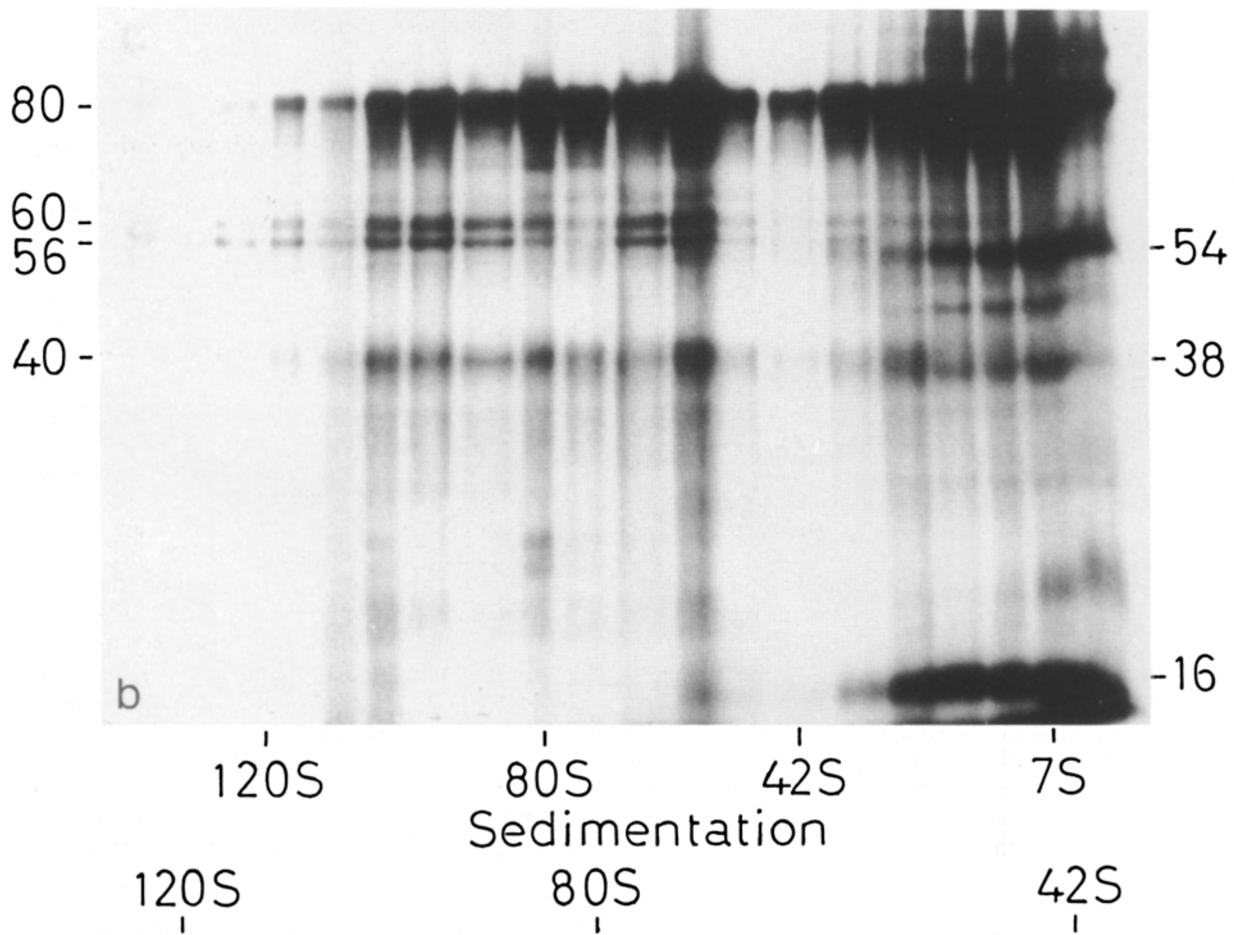
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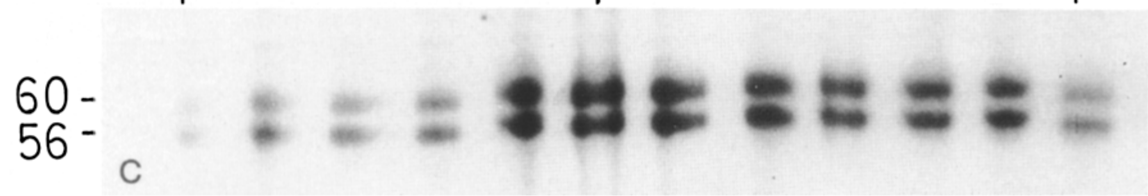
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gregates apparently free of any RNA. Over 90% of the acid-precipitated phospholabel recovered from the gradients is incorporated into protein, mostly contained in mRNP particles (see below).

The polypeptide constituents from gradient fractions are shown in Fig. 2 *a*. Over the range of 40–120 S, by far the most abundant proteins are the mRNP proteins of 60, 56, and 54 kD (see also references 7, 10). Around 42 S, the two major proteins (at 48 and 43 kD) associated with stored 5-S RNA and tRNA (12) are obvious, whereas at 7 S the most abundant protein is that at 40 kD (TFIIIA; reference 12) associated with stored 5-S RNA. Ribosomal proteins are less obvious due to the relatively low concentration of ribosomes in stage 1 oocytes.

The in vivo labeling pattern (Fig. 2 *b*) shows the 60- and 56-kD constituents of mRNP particles to be phospholabeled over the whole range of sedimentation classes. These major phosphoproteins are referred to as pp60 and pp56 (10, 20). pp56 is also a major phosphoprotein in the particles sedimenting at 6–18 S. In general, the extent of phospholabeling is proportional to the amount of mRNP protein in each fraction, although shorter term incubation (3 h) results in relatively more labeling of the 40–80-S class of particles compared with the 80–120-S class. (The nature of such variations in labeling pattern is considered in more detail in the next section.)

On incubating individual gradient fractions in the presence of [γ^{32}P]ATP, in addition to phospholabeling of pp60 and pp56, a third major phosphoprotein of 80 kD (pp80) appears also to be associated with mRNP particles (Fig. 3). However, as we have demonstrated before (20), pp80 appears to be less tightly bound to the mRNA (see also Fig. 5) and so is less relevant to the present study. Again the pattern of phospholabeling, this time in vitro (Fig. 3 *b*), reflects the distribution of mRNP particles and indicates that the protein kinase responsible for phosphorylation of mRNP proteins is itself present in mRNP particles of all sizes. Although some variation in the relative extent of in vitro labeling is seen between fractions (Fig. 3 *b*), this is most probably due to cosedimenting inhibitors of protein kinase activity found in oocytes of slightly later developmental stage. (The ovary used for this experiment contained some oocytes approaching stage 2.) Generally, ovary containing exclusively stage 1 oocytes gives a more even distribution of kinase activity (Fig. 3 *c*).

To confirm that the phosphoproteins are indeed bound either directly or indirectly to RNA and are not merely cosedimenting aggregates of protein (as are the 6–18 S particles), material from the 60-S region of the glycerol gradient was phospholabeled, fixed with formaldehyde, and analyzed on CsCl density gradients. After centrifugation to equilibrium, practically all of the label (i.e., in pp80, pp60, and pp56; Fig. 4, *inset*) is found to be contained in a single peak with a modal density of 1.36 g/cm^3 (Fig. 4 *a*). This value represents an RNP complex with an average protein/RNA mass ratio of 4:1 as already described for the mRNP particles of *Xenopus* oocytes (20, 23). Since very little of the label occurs at the density position of isolated phosphoprotein (1.25 g/cm^3; reference 20), it can be assumed that the phosphoproteins sedimenting in the range of 40–80 S are indeed constituents of the mRNP particles.

The protein kinase activity is present in the same RNP particles as the phosphoproteins; this is confirmed by electrophoresis of whole particles from the 40–50-S region of glycerol gradients in nondenaturing, low-concentration acrylamide gels. Material from the gel was transferred electrophoretically to nitrocellulose and identical tracks were either immunolabeled using antibody directed against pp60 (10) or incubated in the presence of [γ^{32}P]ATP. Autoradiography revealed that immunological detection of pp60 and in vitro phosphorylation of protein were coincident in single bands of identical electrophoretic mobility (Fig. 4 *b*).

Finally, the presence of both phosphoproteins and protein kinase in RNP particles that contain poly(A)$^+$RNA was checked by affinity chromatography of RNP particles on columns of poly(U)–Sepharose. As shown previously, poly(A)$^+$RNA and tightly associated proteins are bound by poly(U)–Sepharose (30) or oligo(dT)–cellulose (7, 22) and eluted only under relatively stringent conditions of denaturation (>25% formamide at 18°C). This separation was performed using glycerol gradient fractions sedimenting at 60–70 S. Material collected as flow through (0% formamide) and after elution with 20 and 60% formamide were dialyzed to remove the formamide and then incubated in the presence of [γ^{32}P]ATP. Phospholabeling was restricted to the material most tightly bound (i.e., eluting with 60% formamide) and was exclusive to the mRNP phosphoproteins pp60 and pp56 (Fig. 5 *a*, lane *3*). Therefore their cobinding and coelution indicate a natural cohesion between poly(A)$^+$RNA, the mRNA-associated phosphoproteins, and protein kinase. The control experiment, using Sepharose 4B in place of its poly(U) form, confirmed that the mRNP particles did not chromatograph in the resin but eluted, both kinase and phosphoproteins, in the flow through fraction (Fig. 3, lane *1'*).

Although isolated (denatured and renatured) forms of pp60 and pp56 do not autophosphorylate (not shown), we have not excluded the possibility that protein kinase activity derives from these phosphoproteins themselves. The tightness of binding of pp60 and pp56 to poly(A)$^+$ RNA is emphasized by binding of phospholabeled mRNP particles to either poly(U)–Sepharose or oligo(dT)–cellulose and eluting proteins with increasing concentrations of NaCl. As can be seen from Fig. 5, *b* and *c*, pp60 and pp56 are not released at NaCl concentrations of up to 1 M, but are readily released along with poly(A)$^+$ RNA in nucleic acid denaturing conditions (no salt). Again, although several other mRNP proteins are released earlier, for instance pp80 at 0.5 M NaCl, protein kinase activity elutes with pp60 and pp56 (not shown). Thus we have so far been unable to separate protein kinase activity from its major target phosphoproteins, pp60 and pp56.

Figure 3. Distribution of proteins in glycerol gradient fractions and identification of mRNP proteins phospholabeled in vitro. (*a*) Stained gel. (*b*) Autoradiograph. Gradient fractions from a homogenate of previtellogenic (stages 1 and 2) ovary were incubated for 1 h in the presence of [γ^{32}P]ATP. (*c*) Autoradiograph showing phospholabeling of the major mRNP phosphoproteins in fractions derived from earlier stage ovary (exclusively, stage 1). Sedimentation rates for the fractions and molecular masses of the major proteins are indicated.

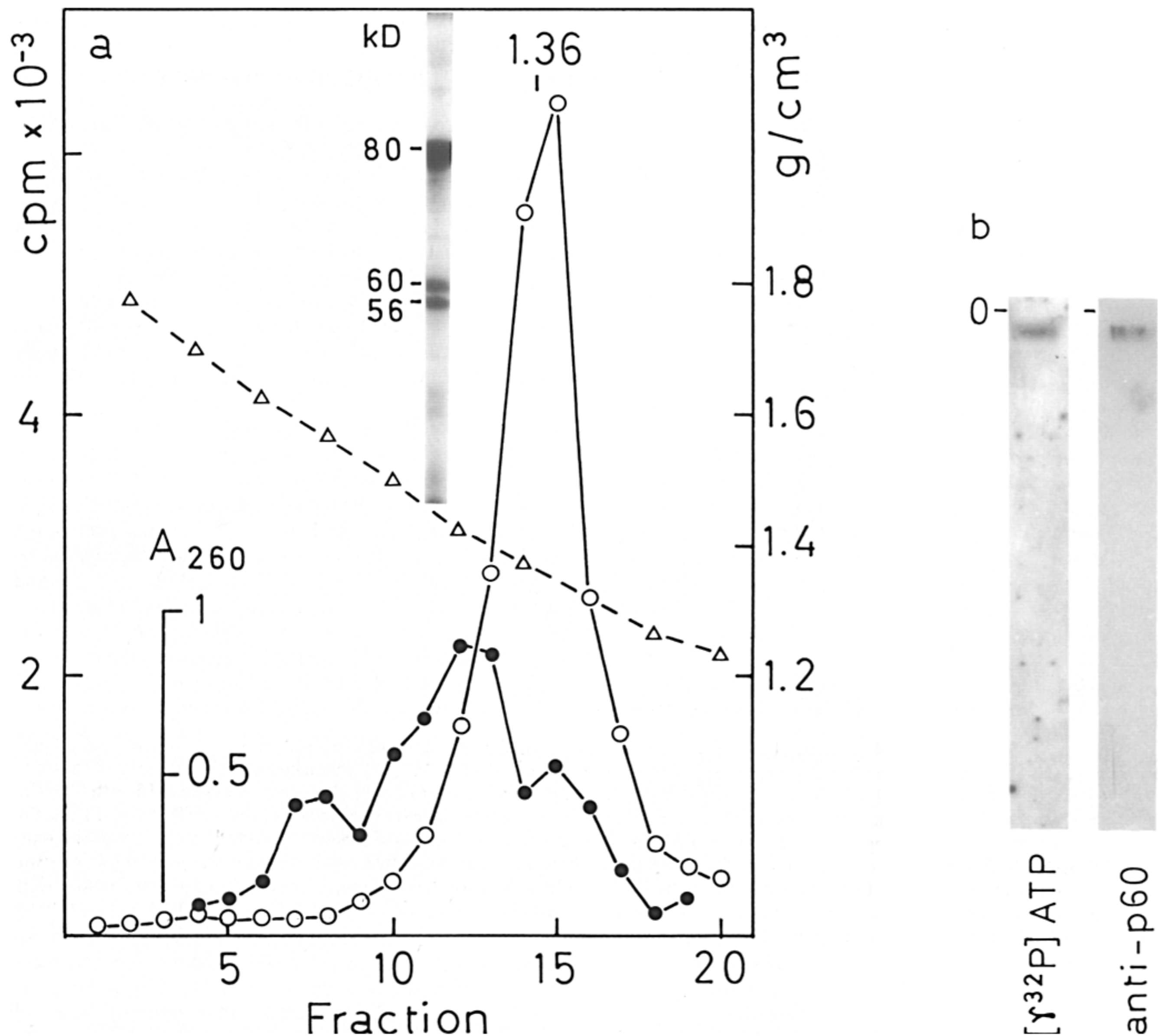

Figure 4. Characterization of mRNP particles by CsCl density gradient centrifugation and by electrophoresis. (*a*) Presence of in vitro-phospholabeled protein in a single peak corresponding to the density of mRNP particles. Particles used were from the 60-S region of a glycerol gradient and derived from stage 1 oocytes. Autoradiograph (*inset*) shows phosphoprotein composition of this material. (○) Radioactivity. (●) Absorbance at 260 nm. (△) Density. (*b*) Coelectrophoresis of protein kinase activity and mRNP protein. Particles sedimenting at 40–50 S were subjected to electrophoresis on nondenaturing gels and transferred to nitrocellulose. Strips of the transfer were incubated in the presence of [γ^{32}P]ATP or immunostained using anti-p60 and [^{125}I] protein A. Autoradiograph is shown.

Two advantages of isolation of mRNP proteins on affinity columns are that (*a*) the RNA-bound phosphoproteins pp60 and pp56 are obtained relatively free of less tightly bound phosphoproteins (e.g., pp80) and (*b*) they are separated from protein kinase inhibitors that are collected in the flow-through and 20% formamide fractions. The presence of kinase inhibitors is demonstrated by mixing the dialyzed eluates (Fig. 5 *a*, lanes *1*+*2*, *1*+*3*, and *2*+*3*). It is interesting to note that the conditions for kinase inhibition are enhanced by passage of the glycerol gradient fraction through poly(U)-Sepharose compared with the unfractionated material (Fig. 5 *a*, lanes *T* and *T'*) and its passage through Sepharose 4B (Fig. 5 *a*, lane *1'*). The basis of these effects is not resolved, although several potent kinase inhibitors have been identified.

Stored mRNP Particles Are Found in Two Distinct States

By using specific antibodies or phospholabeling as markers for mRNP proteins, we have been able to study their location in different cell fractions and their expression at different stages of oogenesis (10). All lines of evidence confirm that the mRNP particles sedimenting at 40–80 S consist of individual mRNA molecules of 10–40 S with about four times their mass of protein (7, 23). However, particles containing

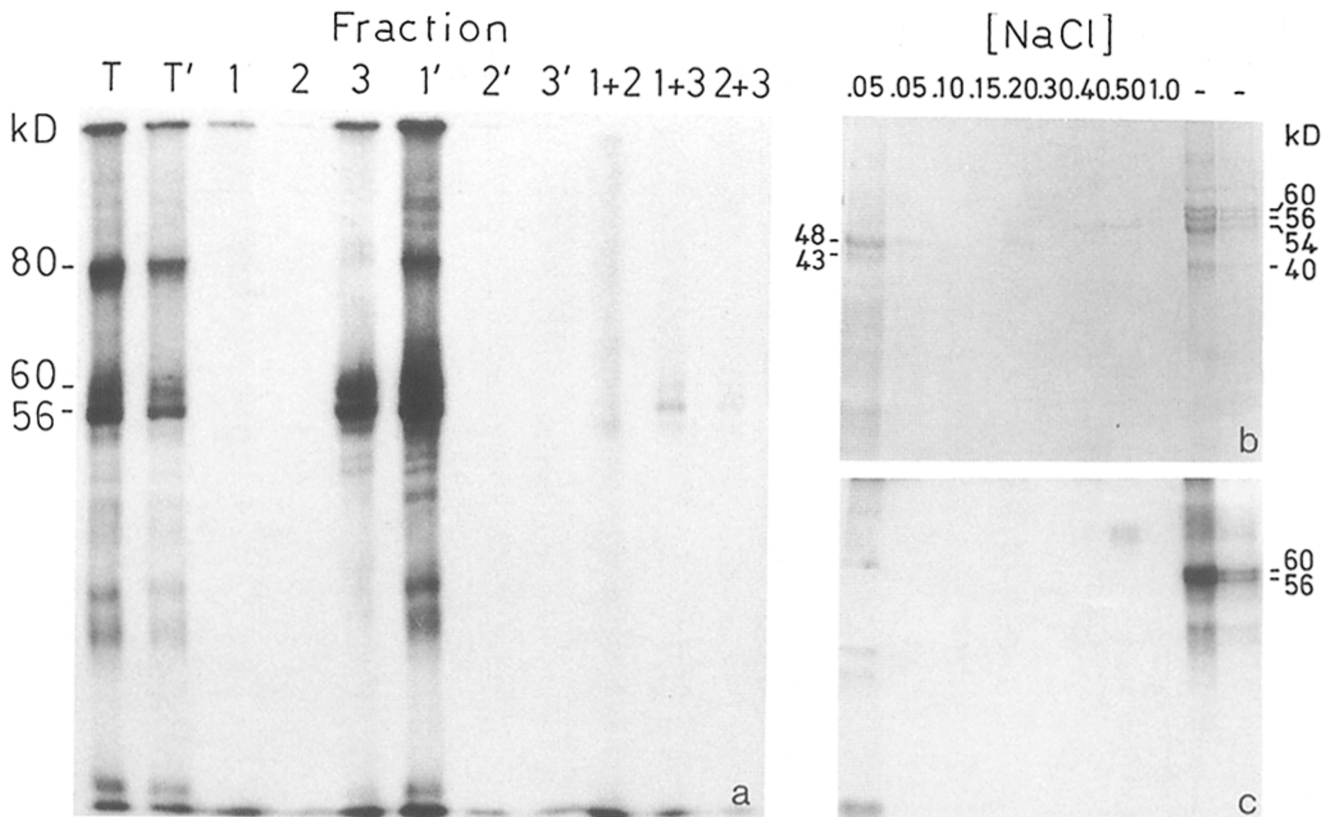

Figure 5. Characterization of mRNP particles by affinity column chromatography. (*a*) Coisolation of mRNP phosphoproteins and protein kinase by binding of particles to poly(U)-Sepharose. Samples of 50 μg (*T*) and 20 μg (*T'*) of unfractionated mRNP particles and fractions from 50 μg mRNP eluted from poly(U)-Sepharose with 0% (*1*), 20% (*2*), and 60% (*3*) formamide and from Sepharose 4B with 0% (*1'*), 20% (*2'*), and 60% (*3'*) formamide were dialyzed to remove formamide and phospholabeled in vitro with [$\gamma^{32}P$]ATP. Dialyzed fractions from poly(U)-Sepharose chromatography were also mixed in the order *1* + *2*, *1* + *3*, and *2* + *3* before in vitro labeling. Autoradiograph after SDS-PAGE is shown. (*b* and *c*) Salt stability of mRNP particles bound to oligo(dT)-cellulose. Particles from previtellogenic ovary, labeled in vivo, were collected as a fraction sedimenting at 50-70 S and eluted from a column of oligo(dT)-cellulose with buffer containing increasing concentrations of NaCl. Step elutions were with 50 (twice, unbound), 100, 150, 200, 300, 400, and 500 mM and 1 M NaCl then twice with H_2O (no NaCl). (*b*) Stained gel. (*c*) Autoradiograph.

mRNP proteins and sedimenting at <20 S and >80 S do not fit in with this interpretation.

The particles sedimenting at 6-18 S are the simplest to describe. They contain the major mRNP proteins, yet apparently lack RNA, having a buoyant density (1.26 g/cm^3) approaching that of pure, isolated phosphoproteins (1.25 g/cm^3; reference 20) and a very low absorbance/phospholabeling ratio (Fig. 6 *a*). Although they also have an associated protein kinase activity (Fig. 3 *b*), their pattern of in vivo phospholabeling (Figs. 2 *b* and 6 *b*) indicates that the key mRNA binding protein pp60 is underlabeled in these particles compared with pp56 and two additional polypeptides of 16 and 28 kD. In fact, in protein particles phospholabeled in vitro, it is the 54-kD mRNP protein, rather than pp56, that is a major labeled component (Fig. 3 *b*). The nature of such in vivo/in vitro differences will require more detailed analysis. The role of protein particles as precursors of the mRNP particles is suggested by the finding that in oocytes of early stage 1, when the pool of stored mRNA is very low, most of the phospholabeling detected is in the 6-18 S particles (Fig. 6 *b*).

Particles sedimenting at 80-110 S are too large to consist of free mRNP particles of the type described above, since few poly(A)$^+$ RNA molecules sediment faster than 40 S (7), yet they are too small to be polysomes. Their density characteristics demonstrate that they are not simply aggregates of two or more mRNP particles. Particles sedimenting at 90 S on glycerol gradients are phospholabeled in vivo, fixed with neutralized formaldehyde, and analyzed on CsCl density gradients. As can be seen in Fig. 7 *a*, both phospholabel and absorbance are mostly contained in a peak with a modal density of 1.46 g/cm^3, a value which is substantially greater than that of mRNP particles (1.36 g/cm^3; Fig. 4 *a*). This profile is changed by treating the particles, before fixation, with 10 mM EDTA which results in the dissociation of the particles into two components (Fig. 7 *b*): one has a low phospholabeling/absorbance ratio and a density corresponding to that of released ribosomal subunits (1.54 g/cm^3); the other has a high phospholabeling/absorbance ratio but a density (1.40 g/cm^3) still higher than that of mRNP particles. More complete dissociation is obtained by treating the particles with poly(U), which again results in the production of two components (Fig. 7 *c*). This time the first component (of density 1.56 g/cm^3) has a low phospholabeling/absorbance ratio

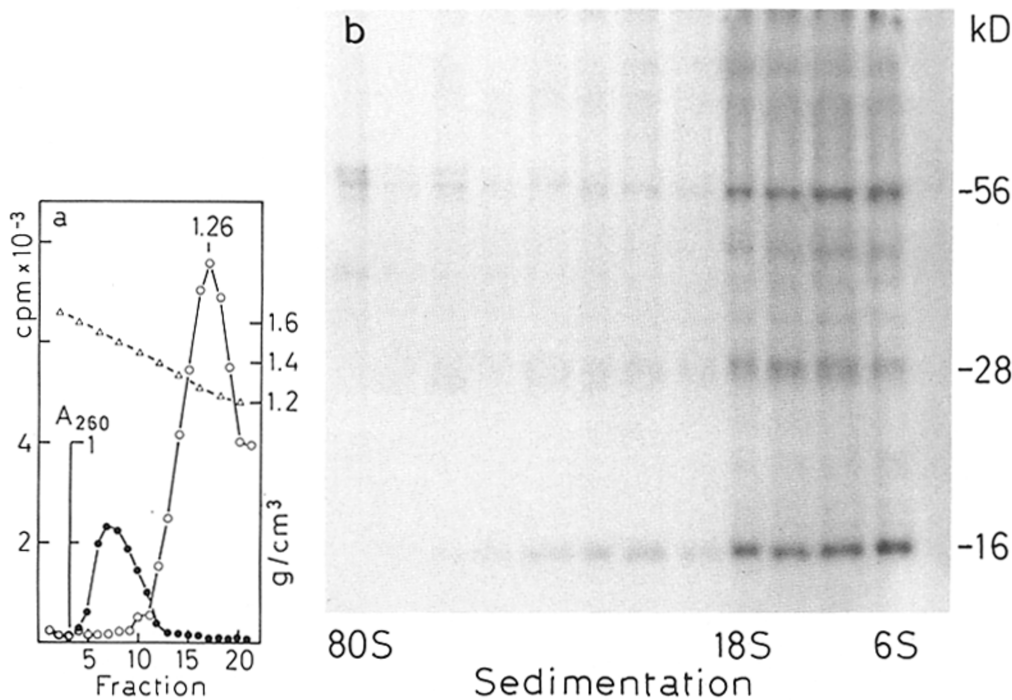

Figure 6. Characterization of protein particles that contain mRNP-type phosphoproteins. (*a*) CsCl density gradient analysis of in vivo phospholabeled material from the 10-S region of a glycerol gradient similar to the one shown in Fig. 2 *b*. (○) radioactivity. (●) Absorbance at 260 nm. (△) Density. (*b*) Gycerol gradient analysis of in vivo phospholabeled material derived from early stage 1 oocytes, showing most of the phospholabel in particles sedimenting at 6–18 S. Autoradiograph after SDS-PAGE is shown.

and corresponds to ribosomes (27) perhaps bound to poly(U). The second component (of density 1.36 g/cm^3) has a high phospholabeling/absorbance ratio and is identical in these respects to free mRNP particles. Excess poly(U) is mostly pelleted and in the bottom few fractions.

These interpretations are supported by examination of sedimentation profiles and identification of phosphoproteins contained in sedimentation classes, after treatment of 90-S particles with EDTA and poly(U). A convenient marker for identifying the location of 80-S ribosomes and the 40-S ribosomal subunit is the ribosomal protein S6 which is substantially phospholabeled in stage 3 oocytes (31). Treatment of phospholabeled 90-S particles (Fig. 7 *d*) with 10 mM EDTA results in the release of 60- and 40-S ribosomal subunits (Fig. 7 *e*). Most of the phospholabel now sediments between the subunits with a modal peak at ~56 S. The fact that this peak contains not only pp60 and pp56 but also some of the 40-S subunit protein S6 displaced to a faster sedimentation value (up to 60 S; Fig. 7 *e*) suggests a physical association of mRNP particles with the ribosomal 40-S subunit. Treatment of the 90-S particles with poly(U) results in the complete displacement of mRNP particles from the ribosomes (Fig. 7 *f*). The sedimentation properties of the released mRNP particles (as detected by both staining of polypeptides and autoradiography) vary between preparations with some of the material pelleting in an aggregated form and some sedimenting at ~30 S or less (Fig. 7 *f*).

Taken together, the results from density and sedimentation analyses are compatible with the view that 90-S particles represent an association of mRNP particles with ribosomes. The actual proportion of mRNP particles in this state varies with the number of ribosomes available: whereas in oocytes of stages 1 and 2, mRNP particles are in excess and mostly sediment at 40–80 S, by stage 3, ribosomes are in excess and most of the phospholabeled protein (including pp60, pp56, and S6) is contained in complexes sedimenting at 80–110 S (not shown). Homogenization of phospholabeled ovary containing a mixture of oocyte stages 1–3 results in the displacement of the phospholabeled mRNP particles from the 40–80-S region of gradients (as seen in stage 1 oocytes; Fig. 2) to, exclusively, the 80–110-S region of the gradient (typical of stage 3 oocytes; Fig. 8). It must be concluded that the free mRNP particles are capable of spontaneously associating with ribosomes. Other types of RNP particle, for instance 42- and 7-S particles as judged by the distribution of their protein components (p48, p43, and TFIIIA; Fig. 8 *a*) and also the 6–18 S protein particles as judged by their phosphoproteins (at 56, 28, and 16 kD; Fig. 8 *b*), do not substantially associate with ribosomes on homogenization. Experiments are in progress to determine conditions for the interaction in vitro of mRNP particles with ribosomes.

The 90-S Particles Represent Blocked Translation Initiation Complexes

It has been demonstrated recently that sea urchin eggs (but not embryos) contain a dominant translation inhibitor that causes accumulation of mRNA with 40-S ribosomal subunits in in vitro translation systems to form blocked preinitiation complexes (19). Diagnostic of this level of translation complex assembly is the presence of initiator tRNA charged with methionine (see reference 19). By labeling *Xenopus* stage 3 oocytes in vivo with [^{35}S]methionine, and precipitating from glycerol gradient fractions aminoacyl (and peptidyl) tRNA with cetyltrimethylammonium bromide, preinitiation complexes should be detected in a peak of radioactivity at 48 S or more (8, 19). In fact, such a peak is detected not at 48 S but at 90 S (Fig. 9 *a*) and with a distribution over the range

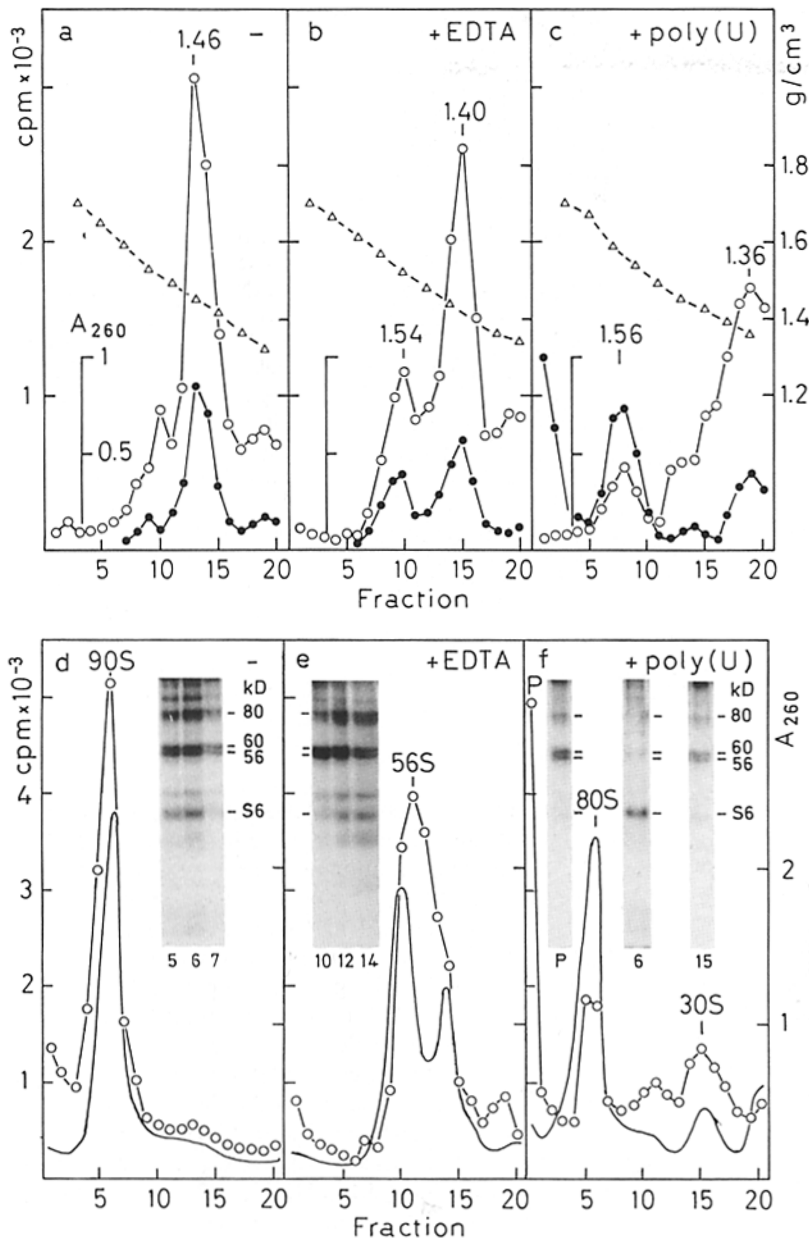

Figure 7. CsCl density gradient and sedimentation gradient analysis of in vivo-phospholabeled particles collected from the 90-S region of a glycerol gradient similar to the one shown in Fig. 2 *a*. (*a–c*) Density analysis of formaldehyde-fixed particles, particles treated with 10 mM EDTA for 15 min at 0°C before fixation, and particles treated with 20 μg polyuridylic acid, respectively. (○) Radioactivity. (●) Absorbance at 260 nm. (△) Density. (*d–f*) Samples treated as for *a–c* but unfixed and analyzed after sedimentation through glycerol gradients. (○) Radioactivity. (———) Absorbance at 260 nm. The phosphoprotein composition of peak fractions is shown as autoradiographs after SDS-PAGE (*insets*).

80–110 S similar to that of mRNP and ribosomal components (cf. Fig. 8). Incubation of oocytes for 2 h in the presence of 50 μg/ml puromycin results in a >80% inhibition of protein synthesis, presumably through the dissociation of translation complexes. This treatment, when applied to stage 3 oocytes prelabeled with [^{35}S]methionine, results in a significant reduction of labeled aminoacyl and peptidyl tRNA from the polysomal region of the gradient, but in little reduction from the 90-S peak (Fig. 9 *b*). The observation that the complexes sedimenting at 90 S (and containing ribosomes mRNP and charged $tRNA_{met}$) are refractile to puromycin treatment indicates that they are synthetically blocked. Thus the 90-S complexes can be considered to represent blocked initiation complexes (i.e., also containing the 60-S ribosomal subunit) rather than blocked preinitiation complexes.

We have already demonstrated that the block in translation in vitro of free mRNP particles can to some extent be removed through dephosphorylation of mRNP proteins (20). Addition of calf intestinal phosphatase, a potent agent for dephosphorylation of mRNP proteins (20), to homogenates of stage 3 oocytes labeled with [^{35}S]methionine results in a substantial drop of CTAB-precipitated label from the 90-S peak and a concomitant increase in the polysomal region. This shift is accompanied by a redistribution of some of the mRNP protein from 90 S to polysome regions of the gradient (not shown). Although these observations represent a structural redepolyment of components, they do not necessarily represent a functional reorganization of blocked initiation complexes into active polysomes. Nevertheless they do correspond with the observation that microinjection into oocytes of inhibitors (e.g., haemin or heparin) specific to the protein kinase that is responsible for phosphorylation of mRNP pro-

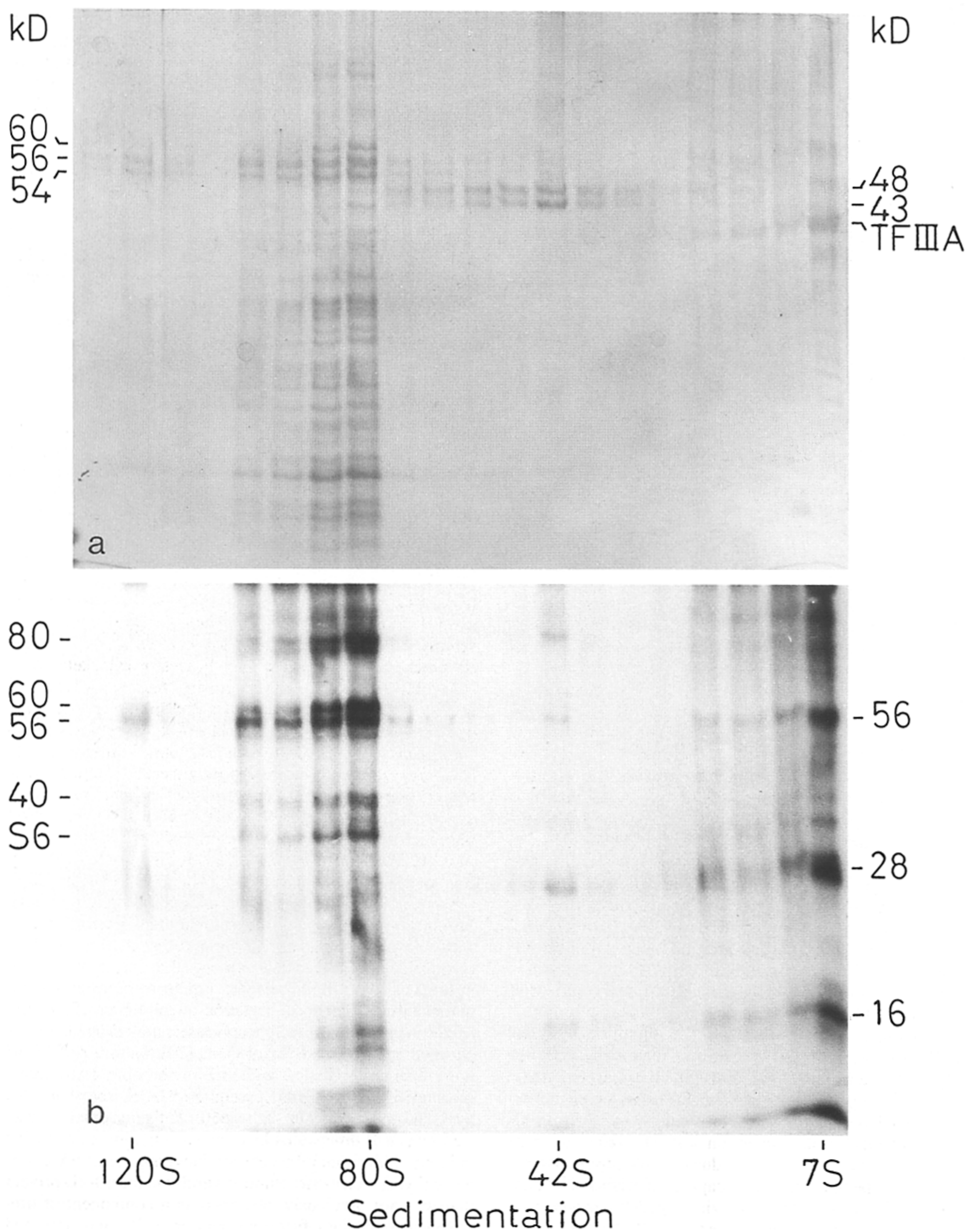

Figure 8. Glycerol gradient analysis of in vivo-phospholabeled material derived from ovary containing stage 1-3 oocytes. (*a*) Stained gel showing the distribution of particle proteins. (*b*) Autoradiograph.

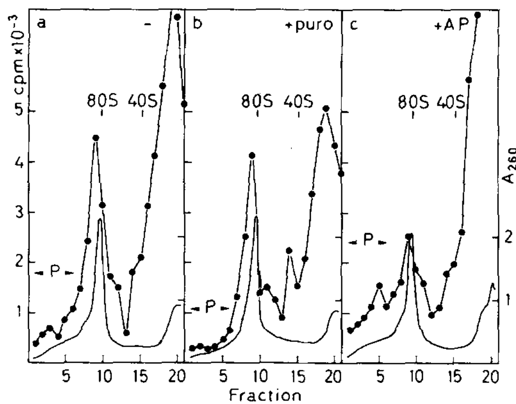

Figure 9. Distribution of [^{35}S]methionine-labeled aminoacyl and peptidyl tRNA in glycerol gradient fractions. Stage 3 oocytes were labeled for 24 h, washed free of label, and incubated for a further 2 h either without addition (*a* and *c*) or in the presence of (*b*) 50 μg/ml puromycin. After homogenization, 5 U/ml of calf intestinal phosphatase was added to *c*. After centrifugation, the gradients were scanned at 260 nm (——) and fractions were precipitated with CTAB for determination of radioactivity (●). The polysomal region of the gradient is indicated (*P*) as is the monosome peak (*80S*) and the position, in parallel gradients, of the small subunit (*40s*).

teins results in a two- to threefold increase in the rate of endogenous protein synthesis (Cummings, A., and J. Sommerville, unpublished observations).

Discussion

Protein kinase activity has been described previously to be associated with both free mRNP particles (2, 14, 24) and polysomes (5, 14) derived from a variety of cell types, and this association may represent a general feature important to the interaction of mRNP with ribosomes. A particular advantage of the *Xenopus* oocyte system is that phosphorylation of mRNP proteins is developmentally regulated and correlates with stages of repressed translation (10). Indeed the isolated phosphoprotein pp60 appears to be capable of regulating the translation of golbin mRNA in in vitro-assembled complexes (20). A further advantage is that stored mRNP particles are relatively abundant in oocytes, far outnumbering the polysomes, and even providing the major source of protein at early stags of oogenesis (Fig. 2 *a*).

In this study, we confirm that the protein kinase of stored mRNP particles in *Xenopus* oocytes is tightly associated with the particles and that high levels of phosphorylation are maintained in free mRNP particles. Furthermore, phosphorylated mRNP particles that accumulate from early oogenesis are not precluded from interaction with ribosomes, as these become progressively abundant during later oogenesis. Although this type of interaction is capable of arising spontaneously – for instance by homogenizing together early oocytes (stages 1 and 2) that contain excess free mRNP with later oocytes (stages 2 and 3) that contain excess ribosomes – it does appear to be analogous to the interaction that occurs with material from sea urchin eggs. Egg extracts, when added to a rabbit reticulocyte cell-free system, cause accumulation of a 48-S preinitiation complex containing initiator tRNA, globin mRNA, and the 40-S ribosomal subunit (19). Failure to bind the 60-S ribosomal subunit is believed to be due to a translation inhibitor, found in eggs but not in embryos, that blocks this binding or else prevents the 40-S subunit from migrating from the 5′ end of the mRNA to the initaition AUG codon (19). This block differs from that described here for *Xenopus* oocytes inasmuch as in oocytes, stable mRNP–ribosome complexes are found to sediment at 80–110 S (Fig. 8) and presumably contain also the 60-S subunit. In agreement with this interpretation, EDTA treatment of the 90-S complex results in the release of a 56-S complex that contains mRNP and the 40-S subunit (Fig. 7). Failure of the 90-S complex substantially to translate may be due to an inhibitory factor that prevents progress of the ribosome along the mRNA, or else lack of a necessary translation factor. We suggest that dephosphorylation of mRNP proteins may contribute to activation of translation, perhaps through conformational changes within the particle.

With regard to the biological significance of interaction of mRNP with ribosomes, it is interesting to note that a major change in the cytoplasmic distribution of mRNA occurs at stage 3 of oogenesis in *Xenopus*. Whereas in sections of stage 1 oocytes, in situ hybridization of [^{3}H]poly(U) reveals a uniform distribution of total poly(A)$^{+}$ RNA throughout the cytoplasm, in sections of stage 3 oocytes, a high concentration of poly(A)$^{+}$ RNA is detected in a perinuclear location (4). Whether or not distribution of mRNA around the nucleus relates to association of mRNP particles with newly formed ribosomes remains to be demonstrated. Indeed, assembly and storage of translation complexes may involve structural elements such as membranes or cytoskeleton.

The mRNP target phosphoproteins themselves have several interesting features. In free mRNP particles, pp60 and pp56 are each represented by ∼10 copies per mRNA molecule (7) and are present in equal amounts in mRNP particles of all size categories (Figs. 2, 3, and 8) and at different stages of development (10). Furthermore, pp60 and pp56 are virtually indistinguishable in two major respects. First, after phospholabeling in vitro, both pp60 and pp56 exhibit a wide range of isoforms that resolve on two-dimensional gel electrophoresis as a series of pairs of identical charge (Cummings, A., and J. Sommerville, unpublished observations). Second, protease digests of pp60 and pp56 generate practically identical cleavage patterns and distribution of phospholabeled serine residues among the fragments (Cummings, A., and J. Sommerville, unpublished observations). It is concluded that pp60 and pp56 are members of a family of structurally similar polypeptides that are subject to multiple modification. However, one major difference is evident: polyclonal antibodies raised against pp60 do not crossreact with pp56 (cf. reference 10) and antibodies raised against pp56 do not crossreact with pp60. Antigenic identity may well be due to differences in secondary modifications which influence mass rather than charge. Nevertheless pp60 is antigenically, as well as structurally, similar to the 60-kD protein that we have previously described as a component of pre-mRNP preparations from the oocytes of *Xenopus* (10) and *Triturus* (21), although the pre-mRNP protein is not as extensively phospholabeled in vivo presumably because of more stable phosphorylation (10).

Proteins analogous to the oocyte mRNP phosphoproteins have been described in other developmental systems. For in-

stance, stored mRNP particles isolated from cryptobiotic gastrulae of *Artemia salina* contain a protein kinase of the casein kinase II type (32) which has activities very similar to the *Xenopus* mRNP enzyme (Cummings, A., and J. Sommerville, unpublished observations). The major target phosphoprotein in *Artemia* mRNP particles is a 38-kD polypeptide (11) which, like pp60 (20), has a negative effect on mRNA translation when phosphorylated (11). In a completely different system (spermatocytes of *Drosophila hydei*) it is found that antibodies directed against pp60 cross react with RNP particles, even though located in the nucleus (16), indicating that similar phosphoproteins may operate in the development of both male and female gametes, in addition to extending their influence into early embryogenesis (Cummings, A., and J. Sommerville, unpublished observations).

We acknowledge that repression of translation is likely to be a multifactorial phenomenon. For instance, evidence has been presented that translation factor 4A is depleted in *Xenopus* oocytes; microinjection of factor 4A into the oocytes stimulates protein synthesis twofold (1). The mRNA-binding phosphoproteins described here are not obviously analogous to known translation factors. One possibility is that pp60 and/or pp56 are equivalent to the β-subunit of eIF2. However pp60 and pp56 do not chromatograph like eIF2 components. For instance, the phosphoproteins elute from heparin–Sepharose columns at lower salt concentrations (0.06–0.10 M KCl; Cummings, A., and J. Sommerville, unpublished observations) than does eIF2 (0.25–0.31 M KCl; reference 18). Nevertheless, the hypothesis that stored mRNA carries with it protein factors required for its eventual translation (29) remains an attractive possibility. Nor have we completely excluded the possibility that pp60 and pp56 are components of a small inhibitor RNP particle (3, 26) which itself is tightly bound to mRNA molecules and prevents their translation. Although small, 6–18 S particles containing at least pp56 are present throughout early oogenesis, these appear not to contain RNA. In this and earlier work (10, 21) we find a strong tendency of the 60- and 56-kD proteins to interact and even polymerise to form filaments in the absence of RNA, again raising the intriguing possibility of interaction of mRNA with cytoplasmic structural elements.

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