Different Forms of Soluble Cytoplasmic mRNA Binding Proteins and Particles in *Xenopus laevis* Oocytes and Embryos

Mary T. Murray, Georg Krohne, and Werner W. Franke

Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg 1, Federal Republic of Germany

Abstract. To gain insight into the mechanisms involved in the formation of maternally stored mRNPs during *Xenopus laevis* development, we searched for soluble cytoplasmic proteins of the oocyte that are able to selectively bind mRNAs, using as substrate radiolabeled mRNA. In vitro mRNP assembly in solution was followed by UV-cross-linking and RNase digestion, resulting in covalent tagging of polypeptides by nucleotide transfer. Five polypeptides of ~54, 56, 60, 70, and 100 kD (p54, p56, p60, p70, and p100) have been found to selectively bind mRNA and assemble into mRNPs. These polypeptides, which correspond to previously described native mRNP components, occur in three different particle classes of ~4.5S, ~6S, and ~15S, as also determined by their reactions with antibodies against p54 and p56. Whereas the ~4.5S class contains p42, p60, and p70, probably each in the form of individual molecules or small complexes, the ~6S particles appear to consist only of p54 and p56, which occur in a near-stoichiometric ratio suggestive of a heterodimer complex. The ~15S particles contain, in addition to p54 and p56, p60 and p100 and this is the single occurring form of RNA-binding p100. We have also observed changes in the in vitro mRNA binding properties of these polypeptides during oogenesis and early embryonic development, in relation to their phosphorylation state and to the activity of an ~15S particle-associated protein kinase, suggesting that these proteins are involved in the developmental translational regulation of maternal mRNAs.

TRANSLATIONAL regulation of gene expression has recently been accumulating as a primary regulatory mechanism in eukaryotic systems (Hunt, 1988; Kozak, 1988; Brawerman, 1989), with two major categories of mechanisms being distinguished: those affecting mRNA stability and those involving specific proteins interfering with the translational activity of mRNAs. A classic example of regulation at the translational level is presented by the maternal mRNAs in early development. In certain invertebrates and vertebrates, several mRNAs are transcribed and stored during oogenesis which are required for, and translated in, blastula and postblastula stages of embryogenesis (Woodland et al., 1979; Lee et al. 1984; Stick and Hausen, 1985). A characteristic of these stored maternal mRNAs is their immediate and effective translational repression during oogenesis and their sharply regulated activation at defined time points after fertilization (for review see Davidson, 1986). The probably best studied vertebrate in this respect is the African clawed toad, *Xenopus laevis* (Woodland, 1974; Richter, 1987), whose oocytes accumulate and store translationally blocked ("masked") maternal mRNAs that are however translatable in vitro, indicating that translation is not controlled by alterations of the mRNA (e.g., Ballantine et al., 1979; Davidson, 1986; Wolin et al., 1987). It is widely assumed that proteins associated with these maternal mRNAs mediate the translational repression (Spirin, 1966; Richter, 1987; Kandror and Stepanov, 1988) although the details of the molecular mechanisms of maternal mRNP assembly and mobilization have not been elucidated.

In *Xenopus* oocytes, major protein components of mRNP particles have been identified (Darnbourgh and Ford, 1981) and in part characterized with regard to their capacity to bind, on filters, radioiodinated mRNA (Richter and Smith, 1983; Dearsly et al., 1985; Kick et al., 1987; Richter, 1987). It has also been reported that when proteins released by high salt buffer from such mRNP are allowed to bind to rabbit globin mRNA, they form complexes translationally inactive upon microinjection into oocytes (Richter and Smith, 1984), and similar results have been obtained with in vitro translation systems (Kick et al., 1987). This has been taken to indicate that some mRNA-associated proteins mediate translational repression of maternal mRNAs.

To elucidate the mechanisms of maternal mRNA storage as mRNP we have identified, in oocytes and early embryos, the soluble cytoplasmic proteins that can bind mRNAs and assemble mRNPs in vitro from radiolabeled mRNA, using fixation of the assembled complexes by UV-cross-linking and tagging by RNase treatment and nucleotide transfer to polypeptides, which were then analyzed by gel electrophoresis. Using this assay, we have identified three distinct forms of soluble mRNA-binding proteins and particles, and we show that their mRNA binding is influenced by protein phosphorylation.

Materials and Methods

Animals, Oocytes, and Embryos

Procurement of *Xenopus laevis* adults, in vitro fertilization of eggs, handling of embryos, and dissection of oocytes were as described (Benavente et al., 1985). Oocytes were staged according to their diameter (Dumont, 1972) and embryos according to the Normal Table (Nieuwkoop and Faber, 1967).

Preparation of Radiolabeled mRNA and Protein Extracts

mRNA transcribed from the *Xenopus laevis* lamin LI cDNA (Krohne et al., 1987) was used in all experiments, although similar results were obtained with mRNA of the nucleolar protein NO38 (Schmidt-Zachman et al., 1987), and the histone binding proteins N1 and nucleoplasm (Kleinschmidt et al., 1986; Dingwall et al., 1987). The transcription reaction (Melton et al., 1984) was modified, being carried out in a final volume of 50 μl with 200 μCi α^{32}P-CTP (800 Ci/mmol; New England Nuclear, Du Pont de Nemours, Bad Homburg, FRG), 0.5 mM ATP, GTP, and 5-bromo UTP (5 br-UTP;[1] Pharma-Waldhof GmbH, Düsseldorf, FRG), and 24 nM CTP. After ethanol precipitation, the lamin LI mRNA (Krohne et al., 1987) was polyadenylated (20 μl reaction volume) with 1 U *Escherichia coli* PolyA polymerase according to the supplier's protocol (Pharmacia, Freiburg, FRG) for 10 min at 37°C, purified by extraction of proteins with phenol and chloroform and precipitated with ethanol, dissolved in sterile water (treated with 0.1% DEP; diethylpyrocarbonate), and used within 5 d. For RNA radiolabeled exclusively during polyadenylation, one-twentieth of a nonradiolabeled transcription reaction was polyadenylated with 50 μCi α^{32}P-ATP (800 Ci/mmol; New England Nuclear).

All solutions and glassware were freed of RNase contamination by sterilization and/or treatment with DEP. S100 extracts were prepared essentially as described (Dignam et al., 1983) and stored at −70°C. No change in mRNA binding was noted with long-term storage (up to 1 yr); however, repeated freezing and thawing was avoided.

Stage VI oocytes were dissected in sterile 5:1 medium (83 mM KCl, 17 mM NaCl, 10 mM Tris-HCl, pH 7.2) containing 0.5 mM PMSF, the germinal vesicles placed on ice, and the ooplasmic material used for preparing the cytoplasmic low-speed supernatant (LSS) fraction: Ooplasms were homogenized by repeated pipetting through a 200-μl pipette tip, followed by two rounds of centrifugation at 12,000 *g* for 10 min at 4°C, and the final supernatant was taken with minimal lipid. The final assay volume for the nuclei and cytoplasmic fractions was 25 μl. For sedimentation analyses, 50 oocytes were dissected, and each fraction was incubated in a final volume of 225 μl, and 200 μl were applied to the glycerol gradient. Glycerol gradient sedimentation of RNPs was performed as described by Cummings and Sommerville (1988), except in 4.2-ml gradients and in a rotor (SW60; Beckman Instruments Co., Fullerton, CA) at 55,000 rpm for 90 minutes, and 200 μl fractions collected with a displacement apparatus linked to a UA-5 absorbance monitor at 254 nm (ISCO, Lincoln, NE). Normal polyribosome preparations were spun in parallel.

Freshly prepared ovary S100 extract was concentrated threefold by vacuum dialysis against buffer D (20 mM Hepes, pH 7.9, 0.1 M KCl, 0.2 mM DTT, 0.2 mM EDTA, 1 mM PMSF; Dignam et al., 1983), and 500 μl (i.e., ~50 mg protein) was applied to a 13-ml 5–30% glycerol gradient in buffer D, centrifuged at 36,000 rpm for 18 h in a rotor (SW40; Beckman Instruments), and 400 μl fractions taken. For reference, BSA (4.3S), catalase (11.3S), and thyroglobulin (16.5S) were applied to a parallel gradient.

Conditions of mRNA Binding Assay

The optimal conditions for the protein-mRNA binding assay were empirically determined from conditions used in similar assays (Greenberg, 1980; Hamm et al., 1987; Leibold and Munro, 1988). 1.0–2.5 × 10^6 cpm of mRNA was mixed with S100 extract (50 or 100 μg) or other oocyte fractions, in incubation buffer (2 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 100 mM KCl, 2–4 U/μl RNasin) in a final volume of 10 μl in microtiterwells, incubated for 1 h on ice, and then exposed to 312 nm UV light (filter removed; K. Benda, Wiesloch, FRG) at a minimal distance, for 30 min on ice. When designated, heparin was added (5 mg/ml final concentration; porcine intestine grade IA; Sigma, Deisenhofen, FRG) before UV exposure. The mixture was digested in 10 mM Tris-HCl (pH 7.5) with 500 U/ml RNase T1 and 0.34 mg/ml RNase A (Boehringer, Mannheim, FRG) for 30 min at 37°C, and the proteins were precipitated with 10% TCA, washed with 90% acetone, and separated by SDS-PAGE (10 or 12%). Autoradiographic exposure was usually overnight, using an intensifying screen and Kodak XAR film. In competition experiments bovine liver tRNA (Boehringer) or total ovary RNA were first mixed with the S100 extract at the final incubation conditions. For experiments using oocyte fractions, the final salt concentration was 100 mM (83 mM KCl and 13 mM NaCl).

Protein Isolation, Antibodies, Immunoblotting, and Immunoprecipitation

Isolation of p54 and p56 during preparation of the homooligomeric 14.5S ATPase particle has been documented elsewhere (Peters et al., 1990). *Xenopus* ovaries were homogenized in buffer A (5:1 medium containing 2 mM DTT, 0.2 mM $CaCl_2$, 0.5 mM PMSF). The 100,000 *g* 1-h supernatant was subjected to ion exchange chromatography, and bound proteins were eluted by increasing ionic strength in buffer A (60–500 mM KCl). Fractions containing particles of ~5–20S were pooled, precipitated with ammonium sulfate (80%), dissolved in 5:1 medium, and analyzed by sucrose gradient centrifugation (5–30%). The ~6S fraction contained primarily p54 and p56, which bound radiolabeled mRNA in the mRNA binding assay.

The polypeptides of the ~6S peak were separated by SDS-PAGE (Fig. 4, lane *I*), polypeptide bands containing p56 and p54 were excised, and the protein was eluted from the gel and used for immunization of guinea pigs as described (Benavente and Krohne, 1986). For immunoblotting, proteins were separated by SDS-PAGE, transferred to nitrocellulose, and the filter blocked with 1% BSA, 0.05% Tween-20 in PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4). This was probed with p54/p56 antisera at dilution 1:20,000, and immunocomplexes were detected using ^{125}I-labeled protein A and autoradiography.

For isoelectric focusing experiments, five unfertilized eggs were disrupted in concentrated sample buffer, urea was added to 8 M, and the total proteins were separated in the first dimension by isoelectric focusing (O'Farrell, 1975) and then SDS-PAGE (10%). Protein analysis by NEPHGE required removal of yolk platelets: stage II oocytes or unfertilized eggs were homogenized in buffer D containing aprotinin (2 μg/ml) and leupeptin (0.5 μg/ml), and the supernatant from two successive 12,000 *g* spins for 10 min each at 4°C was taken. An aliquot equivalent to one oocyte or egg was used for NEPHGE; a 10% gel was used for the second dimension (O'Farrell et al., 1977). BSA and skeletal muscle actin were added as isoelectric references, and nitrocellulose filters stained with Ponceau S (Sigma).

For immunoprecipitation of ~15S material, the IgG fraction of p54/p56 antibodies bound to cyanogen bromide-activated Sepharose (Pharmacia) was used. Guinea pig antibodies against *Xenopus* lamin LI were used as control.

For phospholabeling of the 15S protein particle material, 4.5 μl of fraction 18 (Fig. 5) was incubated with 2 μCi γ^{32}P-ATP (>5,000 Ci/mmol; Amersham Buchler, Braunschweig, FRG) at 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT for 30 min at 20°C in 10 μl. The sample volume was then increased to 200 μl in buffer D containing aprotinin (2 μg/ml) and leupeptin (0.5 μg/ml), and 50-μl aliquots immunoprecipitated overnight at 4°C in buffer B (0.1% SDS, 0.5% deoxycholate, 1% NP-40, 20 mM methionine, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA) plus 0.5 mM ATP. The Sepharose-antibody complex was washed three times with buffer B, and immunoprecipitated polypeptides dissolved in concentrated sample buffer.

Phosphatase Treatment and Protein Kinase Assay

Protein fractions containing 4.5S and 6S or 15S material (7.5 μl of fractions 6 and 16, Fig. 5) were incubated with either calf intestinal phosphatase (Boehringer; 20 U), or an equal volume of phosphatase dilution buffer (50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM ZnCl, 1 mM spermidine) for 30 min at 37°C in the presence of aprotinin and leupeptin. For less extensive phosphatase treatment, 15S material (4.5 μl of fraction 18, Fig. 5) was incubated with 0, 2, or 10 U in the presence of 1 mM ATP at 20°C for 30 min. For protein kinase activity, 5 μl of 15S material (fraction 18, Fig. 5) was incubated with 2 μCi γ^{32}P-ATP at 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT (10 μl final volume) for 30 min at 20°C, in the presence or absence of 0.1 mg/ml heparin. For the influence of protein kinase activity on protein–mRNA binding, 7.5 μl of fraction 13 (Fig. 5) was first incubated at either optimal (10 mM $MgCl_2$, 10 mM ATP) or suboptimal (2 mM $MgCl_2$, 1 mM ATP) conditions at 20°C for 30 min and then tested for mRNA binding on ice. Heparin (0.1 mg/ml) was added at the beginning or end of the 20°C incubation period.

1. *Abbreviations used in this paper*: 5-brUTP, 5-bromoUTP.

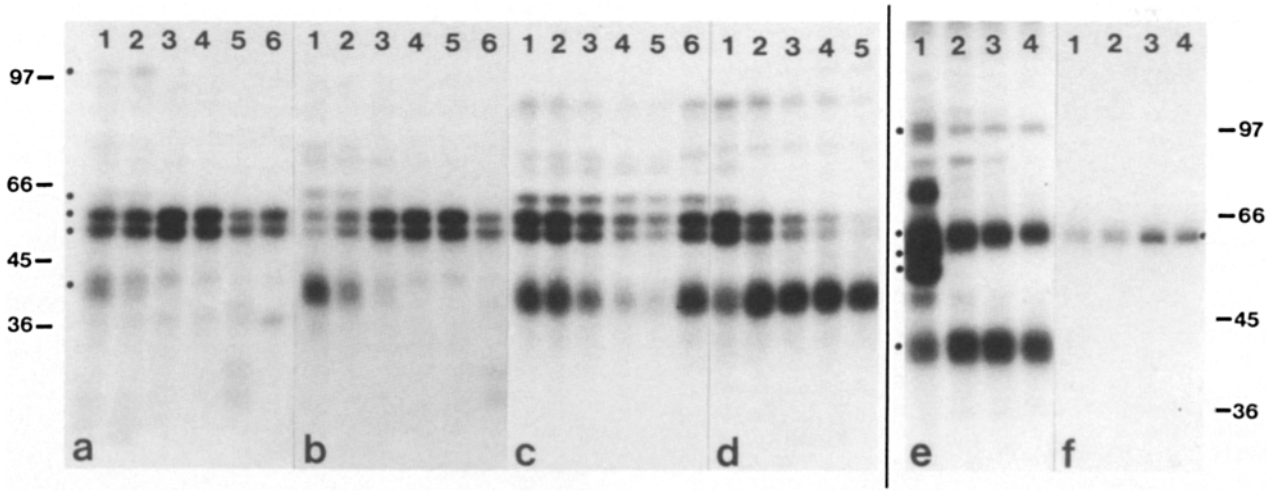

Figure 1. Detection mRNA binding proteins by UV-cross-linking to radiolabeled mRNA. Proteins binding in vitro synthesized, radiolabeled mRNA are detected by autoradiography after UV-cross-linking to covalently radiolabel the RNA binding proteins. Proteins of a 100,000 *g*, 1-h supernatant fraction (S100) from *Xenopus* ovary were incubated with radiolabeled mRNA transcribed from lamin Ll cDNA with α^{32}P-CTP and 5-brUTP in incubation buffer with 40–500 mM KCl on ice for 1 h. The resulting particles were exposed to 312 nm light for 30 min, digested with RNases A and T1, TCA precipitated, and separated by SDS-PAGE. (*a* and *b*) Influence of increasing ionic strength on the binding of proteins from ovary S100 extract to nonpolyadenylated mRNA. 100 μg of ovary S100 extract was incubated at increasing KCl concentrations (lane *1*, 40 mM; lane *2*, 100 mM; lane *3*, 200 mM; lane *4*, 300 mM; lane *5*, 400 mM; lane *6*, 500 mM) with 2 × 10^6 cpm of mRNA for 1 h on ice, without (*a*) or with (*b*) addition of heparin (5 mg/ml) before UV-cross-linking. (*c* and *d*) Competition of protein binding to nonpolyadenylated mRNA was assayed by addition of tRNA (*c*, lanes *1–5*: 0, 1.0, 5.0, 10, and 20 mg/ml), poly(A) (*c*, lane *6*: 20 mg/ml) or total ovary RNA (*d*, lanes *1–5*: 0, 0.25, 0.5, 1.0, and 2.0 mg/ml) to 100 μg of S100 extract at 100 mM KCl incubation conditions before adding the radiolabeled mRNA, followed by incubation for 1 h, addition of heparin (5 mg/ml) and UV-cross-linking. (*e*) The influence of polyadenylation on protein–mRNA binding was tested using mRNA having a nonradiolabeled poly(A) tail: increasing amounts of total ovary RNA (lanes *1–4*: 0, 0.25, 0.5, and 1.0 mg/ml) were added to ovary S100 extract (50 μg each) as in *d*, except that heparin was not added. (*f*) mRNA radiolabeled exclusively during polyadenylation was used as substrate to determine which proteins could be detected as binding directly to the poly(A) tail. 50 μg of S100 protein extracts from immature or mature ovaries, unfertilized eggs, and neurula (stage 15) embryos (lanes *1–4*) were assayed under standard conditions, without addition of heparin. The dots in *a–e* indicate polypeptides of 100, 60, 56, 54, and 42 kD (p100, p60, p56, p54, and p42), and in *f*, p60. The proteins were separated on 10% (*a–d*) or 12% (*e* and *f*) gels, and the horizontal bars to the right or left indicate the respective relative molecular mass standards for each (97, 66, 46, and 35 kD). No signal was detected in controls omitting UV exposure or when BSA was substituted for the ovary S100 extract.

Results

We initiated our study by examining the protein component of native *Xenopus laevis* maternal mRNP and found a common set of proteins associated with mRNPs, thus confirming Darnbourgh and Ford (1981) who identified a set of eight polypeptides (16, 22, 50, 52, 56, 59, 75, and 100 kD) in all sedimentation classes of poly(A)-rich mRNPs from immature ovaries. Moreover, in other authors' previous studies, mAbs raised against the 56-kD polypeptide (Richter and Evers, 1984) recognized 40–60S mRNPs, although some nonreactive 56-kD polypeptides were also found in faster sedimenting mRNPs. These antibodies were subsequentially used to select translationally regulated mRNAs from immature oocyte mRNPs (Crawford and Richter, 1987). In agreement with these data, phosphorylated ~54-, 56-, and 60-kD polypeptides have been identified as components of 40–120S mRNPs (Cummings and Sommerville, 1988), whereas in the earliest oogenic stages phosphorylated p56 was found primarily in 6–18S particles that also had protein kinase activity. Finally, experiments by Swiderski and Richter (1988), using microinjection of radiolabeled mRNAs into oocytes that was followed by UV irradiation of the oocyte homogenate, had shown a set of proteins, including polypeptides of 56 and 60 kD, bound both translationally competent (globin) and stored (G10) mRNAs, in addition to some unique proteins. On the basis of these thorough biochemical characterizations of *Xenopus* maternal mRNPs, we devised a general biochemical assay to detect possible soluble forms of such RNA-associating proteins by assembly of mRNP in vitro from soluble components.

Detection of Soluble mRNA Binding Proteins

To identify proteins of oocytes, eggs, and embryos that bind mRNA and assemble into mRNPs, radiolabeled substrate mRNA was incubated with proteins contained in the 100,000 *g*, 1-h supernatant ("soluble proteins," "S100 extract"; Dignam et al., 1983), and proteins bound to this mRNA were then detected by radioactive nucleotide transfer upon covalent UV-cross-linking (Greenberg, 1980), RNase digestion, and SDS-PAGE. For this assay, mRNA was labeled during transcription in vitro from *Xenopus laevis* lamin LI cDNA (Krohne et al., 1987) by incorporation of 5-brUTP and α^{32}P-CTP, and, when desired, was also polyadenylated using *E. coli* poly(A) polymerase. Identical results (see below) were obtained when other mRNAs were tested. Incorporation of 5-brUTP enhanced the cross-linking at least 50-fold over nonsubstituted substrate mRNA (not shown), facilitating the sensitivity of the mRNA binding assay. Pretreatment of the extracts with micrococcal nuclease had no effect on the

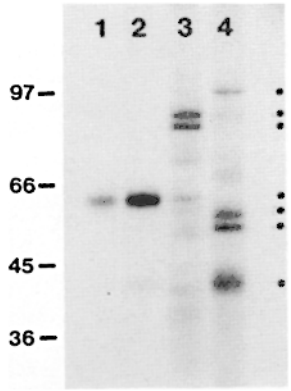

Figure 2. Compartmentalization of mRNA binding proteins. The germinal vesicle was removed from stage VI oocytes and a cytoplasmic low-speed supernatant (LSS) prepared. The substrate mRNA was radiolabeled only during in vitro transcription with inclusion of 7-methyl-GTP cap analogue. Nuclei (lanes *1* and *3*) or LSS (lanes *2* and *4*) from two oocytes were incubated with either mRNA having a nonradiolabeled poly(A) tail (lanes *1* and *2*) or nonpolyadenylated mRNA (lanes *3* and *4*) under standard conditions, immediately following their isolation in 5:1 medium. The dots indicate p100, p95, p90, p60, p56, p54, and p42 (10% gel). In Fig. 2 and 3, capped mRNA was used, although inclusion of cap analogue had no effect on mRNA binding of soluble ovary proteins.

protein binding detected, indicating that any RNA present had no effect on the protein binding studied.

To identify the proteins binding to the substrate mRNA and the forces involved, various conditions were tested. As seen in Figs. 1 and 2, a series of polypeptides (42, 54, 56, 60, 70, 75, 90, 95, and 100 kD) was transfer-radiolabeled as a result of binding to each of the tested mRNAs. Fig. 1, *a* and *b* also show the influence of increasing ionic strength (40–500 mM KCl) on the binding. A final concentration of 100 mM KCl resulted in the most complex protein binding pattern (Fig. 1 *a*, lane *2*), and was therefore chosen as the standard condition. When the effect of increasing ionic strength on the stability of the formed RNP particles was tested, marked differences were found: only the 54- and 56-kD polypeptides (p54 and p56) remained bound at KCl concentrations >100 mM, with p54 and p56 showing differential stabilities at higher ionic strengths (the ionic and other binding characteristics of these and other RNP complexes will be described in detail elsewhere; Murray, M., manuscript in preparation). The effect of adding heparin and spermidine as nonspecific polyanion and polycation competitors was also tested. Upon addition of heparin (Fig. 1 *b*), p54 and p56 as well as p60 were still bound, whereas binding of p100 was reduced. Addition of spermidine had only minor effects.

When the selectivity of the binding assay was examined by competition with other RNAs, tRNA had no specific effect, except at higher concentrations (10 and 20 mg/ml) that selectively reduced p42 binding (results with non-polyadenylated mRNA are shown in Fig. 1 *c*). Similarly, single-stranded DNA or total nonpolyadenylated RNA specifically competed p42 without affecting the other proteins' binding. Competition with either 20 mg/ml poly(A) (Fig. 1 *c*, lane *6*), 1 mM 7-methyl-guanosine triphosphate cap analogue, or 1 mM 5-brUTP (not shown) had no significant effect. However, when increasing amounts of total ovary RNA were used for competition (Fig. 1 *d*), p60 was the first protein whose binding was competed, while the other polypeptides with the exception of p42, were competed by increasing amounts of ovary RNA.

Competition with total ovary RNA was also studied with radiolabeled mRNA to which a nonradiolabeled poly(A) tail was added (Fig. 1 *e*): in this case, it did not diminish p60 binding but completely abolished the binding of p56 and p54, even at concentrations that did not affect their binding to non-polyadenylated mRNA (compare Fig. 1 *d*, lane *3*, and *e*, lane *2*). To investigate which proteins bind directly to the poly(A) tail, substrate mRNA radiolabeled solely during polyadenylation, i.e., in the poly(A) tail, was used (Fig. 1 *f*): significant labeling was found only in association with p60 (lanes *1–4*). Immunoprecipitation of protein radiolabeled after the binding assay with antibodies provided by John Sommerville (St. Andrews, Scotland; data not shown)

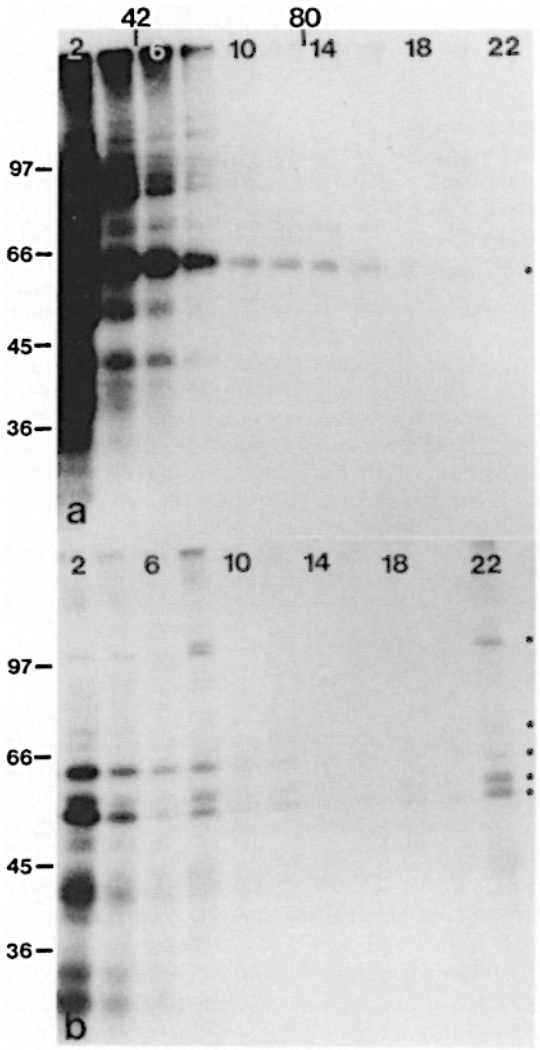

Figure 3. Sedimentation analysis of ribonucleoprotein particles formed in vitro. RNPs formed in vitro from radiolabeled mRNA with nuclear or cytoplasmic proteins were sedimented through glycerol gradients. Total proteins of germinal vesicles (*a*) or cytoplasmic LSS (*b*) from 50 stage VI oocytes were incubated for 20 h with substrate mRNA (an equal number of counts per minute of non- and polyadenylated), and centrifuged through 10–30% glycerol gradients. UV-cross-linking and the standard assay were performed on each fraction. Lanes 2–22 correspond to every second fraction of the gradient (from top to bottom). The positions of 42S and 80S (vertical bars in *a* from left) were determined in a parallel gradient. The dots in *a* indicate p60, and in *b* p100, p70, p60, p56, and p54.

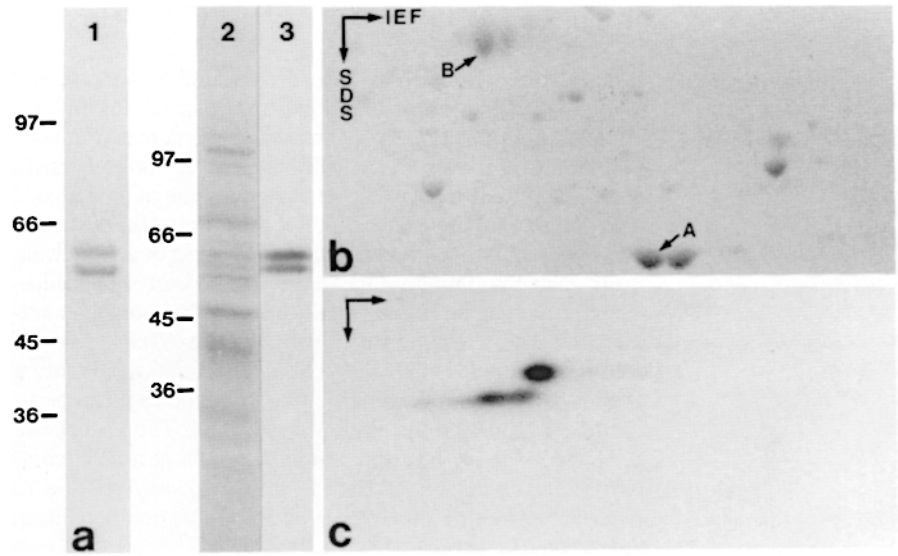

Figure 4. Antibodies to p54 and p56. Polypeptide composition of the 6S fraction used for isolation of p54 and p56, *a*, lane *1*: Coomassie blue-stained SDS-PAGE (10%). The specificity of the antibodies against p54 and p56 was tested by immunoblotting after separation of 50 μg of ovary S100 supernatant by SDS-PAGE (10%) (*a* lane *2*, Ponceau S-stained nitrocellulose; lane *3*, autoradiograph of ^{125}I-Protein A bound immunocomplexes), and after two-dimensional electrophoresis of total soluble proteins from unfertilized eggs (isoelectric focusing (IEF), first dimension; and SDS-PAGE (SDS; 10%), second dimension; *b*, Ponceau S-stained nitrocellulose; *c*, autoradiograph). The reference proteins BSA and actin are indicated in *b* by arrows (*B*, *A*).

showed that p60 corresponded to the 60-kD mRNP-polypeptide described by Dearsly et al. (1985). Our results not only showed that p60 had a high affinity for poly(A), but that it binds nonpoly(A) sites (which were not competed for by poly(A)) and that polyadenylation also had a stabilizing effect on these other nonpoly(A) binding sites of p60.

Despite the obvious selectivity of binding of these proteins for mRNA it has not been possible to demonstrate specificity for any particular mRNA. A variety of partial lamin LI mRNAs, generated by the use of unique restriction sites in the LI cDNA (see Krohne et al., 1989), yielded similar protein binding patterns. Alternatively, when other radiolabeled *Xenopus* mRNAs transcribed from cDNAs encoding the histone-binding proteins N1 (Kleinschmidt et al., 1986) and nucleoplasm (Dingwall et al., 1987) or the nucleolar protein NO38 (Schmidt-Zachman et al., 1987) were used, the same mRNA binding proteins were detected in the complexes formed. Moreover, when RNA transcribed from the Bluescript vector alone was tested, it was also bound by the same set of proteins. Since the oocyte is known to stably store a vast pool of poly(A)-rich RNAs containing interspersed repeat sequences (Anderson et al., 1982) which are nontranslatable (Richter et al., 1984), the seeming lack of specificity observed in our in vitro assay may reflect the biological group function of these proteins. Alternatively, it may of course also be that the assay lacks some component, e.g., one involved in nuclear RNP assembly, that may be necessary for specific mRNP assembly. At any rate, the described in vitro assay fulfills our intentions of a general biochemical screening test to detect soluble mRNA binding proteins.

Compartmentalization of RNA Binding Proteins in the Oocyte

To determine the cellular distribution of the mRNA binding proteins, stage VI oocytes were dissected, nuclei and ooplasms were manually separated, and nuclear lysates and cytoplasmic supernatants were used for the RNA binding assay. Exclusive binding of polyadenylated mRNA by p60 was detected (Fig. 2, lanes *1* and *2*), whereas p42, p54, p56, p90, p95, and p100 bound to non-polyadenylated mRNA (lanes *3* and *4*). This selective binding of p60 to polyadenylated LI mRNA at nonpoly(A) sites, together with the enhanced stabilization effect of polyadenylation on p60 binding outside of the poly(A) tail (see above), indicates that p60 can interact with multiple binding sites in mRNAs, including regions upstream of the poly(A) tail, that have conformations dependent on the polyadenylation state. Clearly, p60 is present in both the nucleus and cytoplasm, p90 and p95 are enriched in the nucleus, and p42, p54, p56, and p100 are primarily, if not exclusively localized in the cytoplasm (Fig. 2). The intracellular compartmentalization of p54 and p56 was independently confirmed by protein blotting with the antibodies described below.

To characterize the RNPs formed in vitro, they were centrifuged through a glycerol gradient: a mixture of non- and polyadenylated mRNA was incubated for 20 h with either the nuclear or cytoplasmic soluble proteins from 50 oocytes, followed by UV cross-linking of each gradient fraction and further processing. The result (Fig. 3 *a*) showed that p90 and p95 did not assemble into RNPs larger than 42S, whereas p60 was recovered in a range of different-sized RNPs sedimenting across the gradient, including particles of more than 80S. The selective appearance of p60 in fast-sedimenting RNPs may reflect multiple p60 association, either alone or in combination with other proteins that are indirectly bound and therefore not radiolabeled.

In contrast to the RNPs assembled with nuclear proteins, those formed with cytoplasmic proteins (Fig. 3 *b*) sediment throughout the gradient, with each of the major RNA-bound proteins present in every size class, resembling the common distribution of polypeptides in native poly(A)+ mRNP particles (Darnbourgh and Ford, 1981). The inclusion of proteins p54, p56, p60, p70, and p100 in RNPs of every size class may be taken to indicate that they form a general mRNP structure. Whether the widely variable sizes of the RNPs formed in vivo are due to incomplete assembly or to associations of additional components remains to be examined.

Purification of p54 and p56 and Production of Antibodies

The major soluble cytoplasmic proteins with mRNA binding

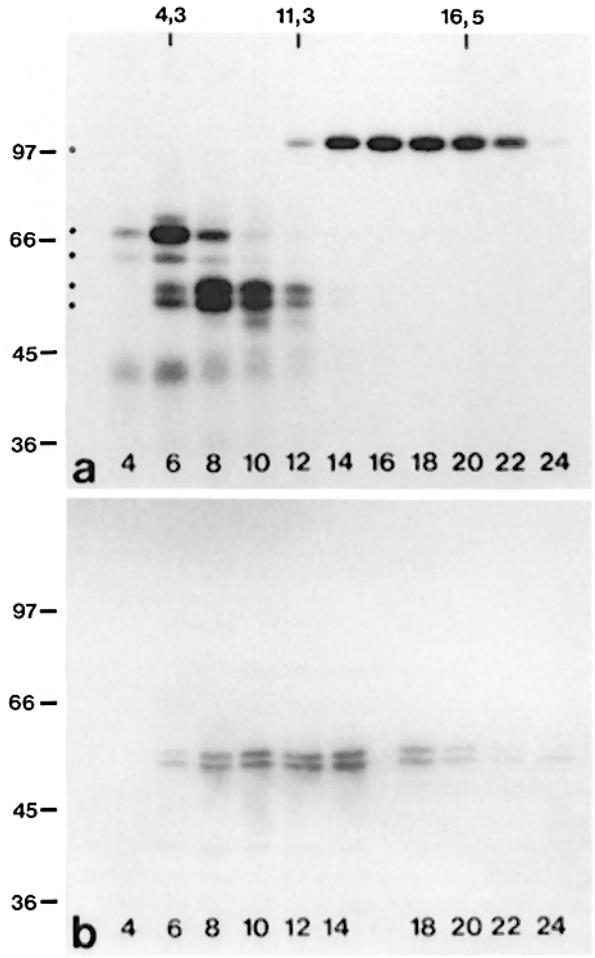

Figure 5. Sedimentation analysis of particles containing mRNA binding proteins. Ovary S100 extract was fractionated (5-30% glycerol gradient; 400-μl fractions) and 5 μl of every second fraction assayed (*a*) for mRNA binding using polyadenylated mRNA or (*b*) by immunoblot with antibodies against p54 and p56, as in Fig. 4 (no protein was loaded from fraction 16). The dots to the left in *a* indicate p100, p70, p60, p56, and p54 (10% SDS-PAGE). The ~4.5S mRNA binding particle peak is centered at fraction 6, ~6S particle peak at fraction 8, and ~15S particle peak at fraction 18. The position of reference proteins of known S value were determined in a parallel gradient (from left to right, at top; 4.3S, 11.3S, and 16.5S).

potential, i.e., p54 and p56, were purified from ovary S100 supernatant fractions in a three step scheme originally developed for the purification of the 14.5S ATPase homooligomeric ring shaped particle (Peters et al., 1990). After anion exchange chromatography, fractions containing the 97-kD polypeptide of the 14.5S particle were concentrated by ammonium sulfate precipitation, and the ~6S particle containing p54 and p56 was separated by velocity gradient centrifugation. During all stages of purification, p54 and p56 were observed in approximately equimolar amounts as estimated by SDS-PAGE and Coomassie blue staining. Because p54 and p56 appear as subunits of a discrete particle, antibodies were raised against both p54 and p56 (Fig. 4). The antibodies obtained were specific for p54 and p56 (Fig. 4 *a*, lane *3*; Fig. 5 *b*; Fig. 8, *b* and *c*), recognizing all their major isoforms (Fig. 4 *c* and Fig. 8, *d* and *e*).

Characterization of the Native State of mRNA Binding Proteins

When S100 extract proteins were separated by sucrose and glycerol gradient centrifugations and assayed for mRNA binding (Fig. 5 *a*), p60, p70, and p42 were detected in slow sedimenting (~4.5S) particles, indicative of monomers. In contrast, both p54 and p56 displayed the same monodisperse distribution with a peak at ~6S. A third, much larger particle, which contained protein p100, appeared in a somewhat broad distribution with a mean of ~15S. Thus, three different particles can be defined on the basis of their mRNA binding. However, when the fractionated S100 extract was examined by immunoblotting (Fig. 5 *b*), p54 and p56 were present over an extended range of particles, from ~6S to ~15S particle fractions, albeit at different intensities. The absence of mRNA binding by p54 and p56 in the ~15S particles (compare lanes *8* and *18* of Fig. 5, *a* and *b*) may be due to modifications or to interference by additional components in the 15S particles.

Dependence of mRNA Binding Ability on Kinase Activity and Protein Phosphorylation

When aliquots of fractions containing the 4.5S, 6S, and 15S

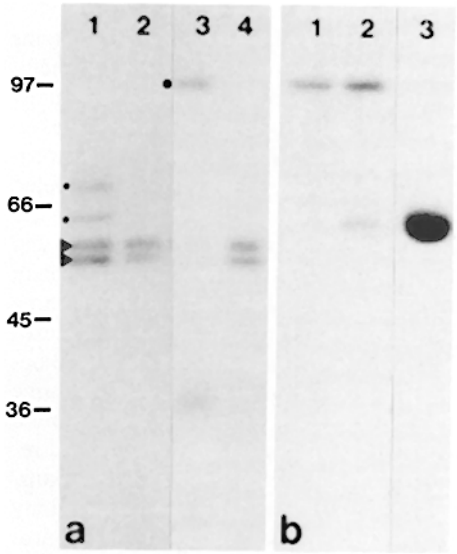

Figure 6. Influence of protein phosphorylation on mRNA binding. The influence of protein phosphorylation on mRNA binding was probed by assaying fractionated particles after phosphatase treatment. (*a*) 4.5S and 6S (lanes *1* and *2*) and 15S particles (lanes *3* and *4*) of fractionated ovary S100 extract were treated with alkaline phosphatase and tested for mRNA binding with polyadenylated Ll mRNA. 7.5 μl of fractions 6 or 16 (see Fig. 5) were incubated without (lanes *1* and *3*) or with (lanes *2* and *4*) alkaline phosphatase (20 U) in the absence of ATP for 30 min at 37°C, and then brought to the standard mRNA-binding conditions (on ice); polyadenylated radiolabeled mRNA was added and the standard assay was continued. In both *a* and *b*, the *dots* indicate p100, p70, and p60; and the *arrowheads*, p56 and p54 (10% SDS-PAGE). (*b*) 15S particles were treated with varying amounts of alkaline phosphatase at 20°C and then tested for mRNA binding. 4.5 μl of fraction 18 was treated with 0, 2, or 10 U of alkaline phosphatase (lanes *1–3*) in the presence of 1 mM ATP at 20°C for 30 min, and then tested for mRNA binding as in *a*.

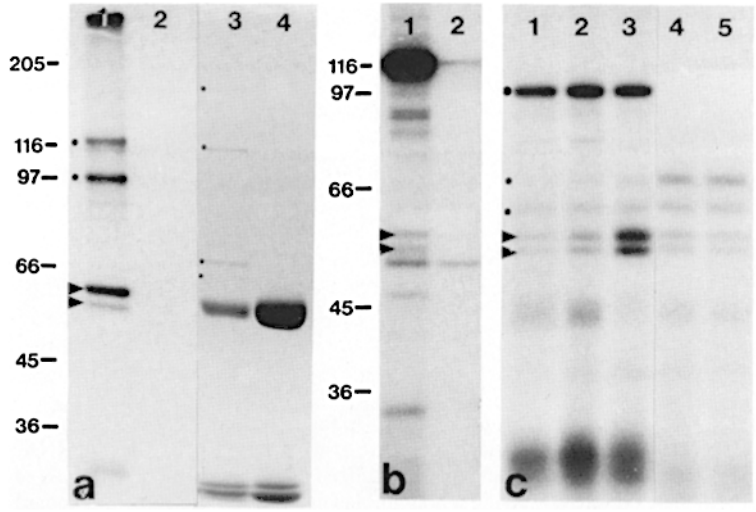

Figure 7. Involvement of protein kinase in mRNA binding. (*a*) 15S particles (4.5 μl of fraction 18; shown in Fig. 5) were incubated with 2 μCi γ^{32}P-ATP in the presence of 10 mM $MgCl_2$ for 30 min at 20°C, and immunoprecipitated with p54/p56 (lane *1* and *3*), or control antibodies (lane *2* and *4*). Autoradiographic exposure (lanes *1* and *2*) and Coomassie blue staining (lane *3* and *4*) detect the polypeptides co-immunoprecipitated with p54 and p56. Aliquots of 15S particle kinase reaction were incubated with p54/p56 antibodies covalently bound to Sepharose or control serum adsorded to protein A Sepharose in buffer B. In lane *1*, the dots indicate phosphorylated polypeptides of ~120 and ~100 kD, and arrowheads indicate p54 and p56; in lane *3*, the dots indicate polypeptides of ~160, ~120, ~70, and ~60 kD. The intensely stained polypeptides in lanes *3* and *4* are immunoglobin heavy and light chains. (*b*) Protein kinase activity sedimenting with the 15S mRNA binding particles (lane *1*) was inhibited by the addition of heparin to 0.1 mg/ml (lane *2*). 5 μl of fraction 18 (shown in Fig. 5) was incubated with 2 μCi^{32}P-ATP in the presence of 10 mM $MgCl_2$ for 30 min at 20°C and the TCA-precipitable proteins separated by SDS-PAGE (10%). (*c*) The influence of protein kinase activity on mRNA binding of 6S and 15S particles was tested after incubation under conditions which were either permissive (lanes *2* and *3*) or inhibitory for protein kinase activity (lanes *4* and *5*). Aliquots (7.5 μl) of fraction 13 containing both 6S and 15S particles (see Fig. 5) were tested for mRNA binding under standard assay conditions (lane *1*), or after incubation at 20°C for 30 min under suboptimal (lane 2; 1 mM ATP, 2 mM $MgCl_2$) or optimal protein kinase conditions (lanes 3–5; 10 mM ATP, 10 mM $MgCl_2$). Heparin (0.1 mg/ml) was present throughout the 30-min incubation at 20°C (lane *4*), or added immediately before the mRNA binding incubation period (lane *5*). The arrowheads indicate p56 and p54; the dots, p100, p70, and p60 (10% SDS-PAGE). Autoradiographs are shown in *b* and *c*.

particles (Fig. 5) were treated with phosphatase and then tested for mRNA binding, the binding of p60, p70, and p100 was no longer observed (Fig. 6 *a*, lanes *2* and *4*). Surprisingly, the loss of p100 mRNA binding in the 15S particles was accompanied by the appearance of mRNA binding by p54 and p56. This was further investigated by limited phosphatase treatment of the 15S particles (Fig. 6 *b*). Loss of mRNA binding by p100 was accompanied the appearance of mRNA binding of p60 (lane *2*), which was greatly enhanced upon total loss of p100 binding activity (lane *3*). Since the native particle only exhibits mRNA binding by p100 (Fig. 5), this indicates that the organization of the 15S particles is dependent on phosphorylation, and that the mRNA binding of p54, p56, and p60 is regulated by their association within the 15S particles.

The 15S particle was tested for protein kinase activity and the endogenous substrates analyzed after SDS-PAGE separation of particles immunoprecipitated with the p54/p56 antibodies (Fig. 7 *a*). Both p54 and p56 were detected as substrates of the endogenous kinase (Fig. 7 *a*, lane *1*). The results also showed co-immunoprecipitation of phosphorylated polypeptides of ~100 and ~120 kD (lane *1*), and polypeptides of ~60, ~70, ~120, and ~160 kD by Coomassie blue staining (lane *3*). The phosphorylated ~120-kD polypeptide migrated slightly slower than the Coomassie blue-stained ~120-kD polypeptide. While the exact composition of the p54/p56 15S particles is under further investigation, these results establish both their heterotypic complex nature and that they are substrates of an endogenous kinase.

As several protein kinases are sensitive to inhibition by heparin (e.g., Hathaway and Traugh, 1982; Erikson and Maller, 1986), the 15S gradient fractions were assayed directly for protein kinase activity (Fig. 7 *b*) and the effects of heparin examined. Heparin was found to inhibit the protein kinase acting on p54 and p56 (Fig. 7 *b*, lane *2*). When the 15S gradient fractions were assayed directly for protein kinase activity (Fig. 7 *b*), the 120-kD was detected as the primary substrate, perhaps corresponding to the protein reported by Mulner-Lorillon et al. (1988) to show increased phosphorylation upon microinjection of purified *Xenopus* ovary kinase II into fully grown oocytes. The more intense radiolabeling of the 120-kD component after the 30-min reaction, as compared with that observed after immunoprecipitation (after a 14-h incubation in the presence of 0.5 mM ATP) probably reflects a greater sensitivity of the 120-kD polypeptide to endogenous phosphatases.

We then further used the inhibition of kinase activity to probe whether the enzyme activity itself is necessary for mRNA binding. Fig. 7 *c* shows the effect of heparin inhibition of protein kinase activity on mRNA binding of a mixture of particles (fraction 13, see Fig. 5): Addition of heparin alone (Fig. 7 *c*, lane *4*) abolished mRNA binding of p100 but did not considerably affect the mRNA binding activity of the other proteins. The 15S particles, however, containing p100 as well as p54, p56, and p60 were entirely excluded from mRNA binding. In contrast, the mRNA binding proteins present in the smaller particles (p70, p60, p56, and p54) bound mRNA independent of heparin addition (Fig. 7 *c*, lanes *4* and *5*). The effect of heparin on mRNA binding of p100 was observed even when the heparin was added after the kinase incubation period (Fig. 7 *c*, lane *5*). Inhibition of p100 mRNA binding by heparin alone suggested that stable phosphorylation was not sufficient to allow the binding of this protein, and hence of the 15S particles, but that protein kinase activity was required. Binding of both subunits of the 6S particle, i.e., p54 and p56, was found to increase after in-

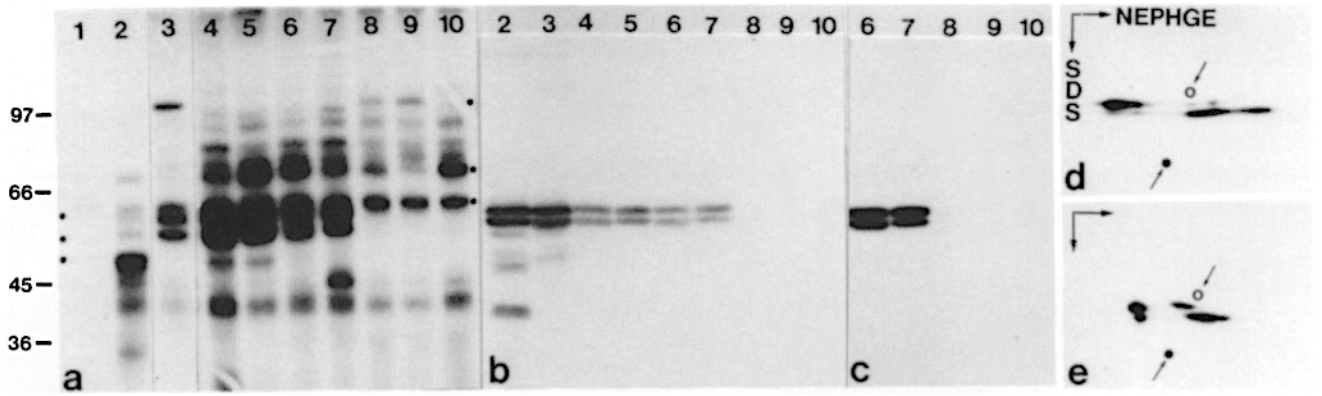

Figure 8. Profiles of mRNA binding proteins during oogenesis and early embryogenesis. (*a*) The mRNA binding of proteins in S100 extracts prepared from ovaries, unfertilized eggs or staged embryos, in comparison with somatic tissue, were tested with polyadenylated mRNA. 50 μg of S100 extract from liver (lane *1*), immature ovary (lane *2*), mature ovary (lane *3*), unfertilized eggs (lane *4*), and embryos of stage 6, 9, 11.5, 15, 22, 28 (lanes *5–10*) were incubated with radiolabeled mRNA having a nonradiolabeled poly(A) tail, under standard conditions (except for liver, where 20 mM vanadylate was also included), and the proteins separated on a 12% gel. The dots to the left indicate p56, p54, and a 48-kD polypeptide, and those to the right indicate p100, p70, and p60. The intensity of the 48-kD polypeptide in lane *2* suggests that it has maximal RNA binding early in oogenesis. (*b* and *c*) Immunoblot with p54/p56 antibodies, corresponding (lanes *2–10*) to 50 μg of the same S100 extracts tested for mRNA binding in *a*. The proteins were separated on a 10% gel, and the blot probed with a 1:20,000 antibody dilution using ^{125}I-Protein A for detection. A longer exposure of lanes *6–10* is shown in *c*. (*d* and *e*) Immunodetection of p54 and p56 isoforms in previtellogenic oocyte (*d*) and unfertilized egg (*e*). The low-speed supernatant equivalent of a single stage II oocyte and unfertilized egg were separated by NEPHGE in the first dimension with BSA (*open circles*) and actin (*filled circles*) added as references, and 10% SDS-PAGE in the second dimension. The proteins were transferred to nitrocellulose and processed as in *b*.

cubation under conditions of kinase type II phosphorylation (Fig. 7 *c*, lane *3*). Again, addition of heparin prevented this increase (Fig. 7 *c*, lanes *4* and *5*).

The heparin inhibition of mRNA binding by p100 in the 15S particles did not facilitate mRNA binding of any of the other proteins, in contrast to the phosphatase effect of rendering p60, or p54 and p56, in the 15S particles, competent for mRNA binding (Fig. 6). Together, these results indicate that in the 15S particles, the mRNA binding proteins are organized in a manner which is, at least partly, dependent on phosphorylation, and that initial mRNA binding by these particles via p100 requires an active protein kinase.

Developmental Regulation of Protein mRNA Binding Ability

S100 extracts were prepared from various stages of oogenesis and embryogenesis to determine whether the mRNA binding of the soluble proteins is developmentally regulated. Fig. 8 *a* shows a panel of mRNA binding assays with polyadenylated substrate mRNA and S100 extract from such stages, in comparison with S100 extract from *Xenopus* liver in which significant amounts of RNA binding-proteins were not detected (lane *1*). The binding by p54 and p56 increased during oogenesis, declined between embryonic stages 6, 9, and 11.5 and was lost between stages 11.5 and 15.

After the observation of reduced mRNA binding by p54 and p56 in postgastrula stages, we examined the presence of these two polypeptides in the S100 extracts by immunoblotting with antibodies to p54 and p56 (Fig. 8, *b* and *c*): both polypeptides were found at their highest level early in oogenesis and then to gradually decrease during oogenesis and early embryogenesis. The substantial reduction of p54 and p56 in the soluble pool of stage 15 and later embryos clarifies the loss of mRNA binding noted in these stages. Upon very long exposure time, some p54 and p56 are detected in these postgastrular extracts (not shown). The decline of p54 and p56 levels during oogenesis, which includes maturation to the egg, is accompanied by an increase in their observed mRNA binding (Fig. 8, *a* and *b*), indicating that the mRNA binding of these proteins is not solely determined by their protein levels. Some insight to this observation was obtained by two-dimensional analysis of p54 and p56 of previtellogenic oocytes (Fig. 8 *d*) and unfertilized eggs (Figs. 8 *e* and 4 *c*), showing a variety of isoforms, and a significant modification of p54 to more acidic isoforms in the unfertilized egg. The acidic isoforms of p54 and p56 in the unfertilized egg, are most probably due to phosphorylation upon maturation of the oocyte, and correlate with an increased mRNA binding. Two dimensional immunoblots of the immature and mature ovary S100 extracts were indistinguishable from the previtellogenic oocyte immunoblot (Fig. 8 *d*), indicating that additional factors must regulate the mRNA binding which increases between these oogenic stages.

Discussion

Nucleocytoplasmic transport of mRNA is accompanied by a change in its associated proteins, from hnRNP to mRNP (e.g., Lindberg and Sunquist, 1974; Beyer et al., 1977; Greenberg, 1981; Mayrand and Pederson, 1981), with the cytoplasmic mRNA-associated proteins probably serving roles in stabilization, translation, and intracellular localization of mRNAs (for review see Dreyfuss, 1986). To gain insight into the regulatory principles involved in the formation and stabilization of mRNPs we systematically searched for soluble mRNA binding-proteins, using the amphibian oocyte as a particularly suitable cell because of its high production rate and storage capacity of maternal mRNPs.

Using a sensitive in vitro binding assay to identify possible soluble mRNA-binding proteins, we have detected in the cytoplasm of *Xenopus* oocytes, eggs, and embryos, three discrete and compositionally different forms of particles containing proteins that readily and stably associate with defined mRNA molecules resulting in the formation of mRNPs. Obviously these free, i.e., unoccupied proteins and particles are excellent candidates for a role in the translational regulation of the stored maternal mRNAs characteristic of these stages. Our demonstration of a cytoplasmic pool of mRNA-binding proteins also raises the general question of the regulation of their activity in relation to mRNP and polyribosome formation.

Several observations suggest that the mRNP particles formed in vitro are specific and biologically relevant: (*a*) they are selective for mRNA and can be competed by competent, i.e., ovarian RNA. (*b*) The mRNPs formed are similar in size to native mRNPs from the same cell and also seem to have several polypeptides in common, including p54 and p56 (as shown by immunoblotting; data not shown), although a direct correlation is not possible (c.f. Darnbourgh and Ford, 1981; Richter and Smith, 1983; Dearsly et al., 1985; Cummings and Sommerville, 1988; Swiderski and Richter, 1988). (*c*) The complexes formed exclude other nucleic acid–binding molecules such as ribosomal proteins, specific hnRNA-binding proteins (Dreyfuss, 1986), the small polypeptides binding to AUUUA motifs (see Malter, 1989), and histones which are known to be abundant in oocyte S100 extracts and to avidly bind to RNA in vitro (see Laskey et al., 1978; Kleinschmidt et al., 1985). (*d*) The observed changes in two of the polypeptides (p54 and p56) correlate with the developmentally regulated translational activation of stored maternal mRNAs.

Three Forms of Soluble mRNA Binding Particles

The smallest class of "free" proteins observed are the ~4.5S particles containing p42, p60, and p70, probably in the form of individual molecules. Of these, p60 occurs in both the nucleus and the cytoplasm, suggesting that it is an early assembly protein in mRNP formation. It is probably identical to the poly(A)-binding protein identified in amphibian oocyte hnRNPs with which it immunologically cross-reacts (this study; see Kloetzel et al., 1982; Dearsly et al., 1985; Kick et al., 1987; Cummmings and Sommerville, 1988). However, our present findings also show that p60 can bind, in addition to the poly(A) tail, to upstream sites of mRNA and to nonpolyadenylated mRNA as well. The polypeptide p70, which is very basic and found among the soluble cytoplasmic proteins, may be related to the ~70-kD poly(A)-binding mRNP component (Blobel, 1973; Sachs et al., 1986; Ullrich et al., 1988) identified in *Xenopus* oocytes and embryos by Swiderski and Richter (1988) and Zelus et al. (1989), although the latter authors were unable to detect it in any preneurula stage by immunoblots.

The population of ~6S particles includes complexes of the avidity mRNA-binding polypeptides p54 and p56, in near-equimolar stoichiometry, suggestive of a heterodimer, and these polypeptides have also been identified as constituents of oocyte mRNP particles of a wide range of sizes. This correlates with results described in previous reports. A polypeptide of ~56 kD has been reported in *Xenopus* oocytes as a constituent of 40–60S RNP particles by Richter and Smith (1983, 1984), and two polypeptides of ~54 and ~56 kD have been shown to occur in 40–120S RNPs by Sommerville and colleagues (Kloetzel et al., 1982; Dearsly et al., 1985; Cummings and Sommerville, 1988). Neither of these groups has identified these two polypeptides among the soluble proteins of mature oocytes, and the 6–18S particles described by Cummings and Sommerville (1988) which do contain a 56-kD polypeptide, were only found in previtellogenic oocytes.

The results of our present study show that the complex of p54 and p56, i.e., the 6S particle, is cytoplasmic and that both subunits bind mRNA independent of its polyadenylation, in a very stable way, as demonstrated by the resistance to elevated ionic strength. The same fraction of 6S particles also contains protein kinase activity, but we have not yet resolved whether p54, p56, or both carry the enzyme activity, or whether there is an additional minor protein responsible for the phosphorylation of p54 and p56, which has as yet escaped our detection.

Using the p54/p56 antibodies described here, we have isolated cDNAs from λgt11 expression libraries containing the major portion of the coding sequence (>40 kD) of p56. The partial amino acid sequence determined therefrom reveals a remarkable richness in glutamine (Q) residues, including five QQ dipeptides, in hydroxyamino acids, (S,T), proline (P), and arginine (R) residues, with a conspicuous QQRPPP-RRFQQRF motif. We did not detect any of the known RNA-binding consensus motifs (Sachs et al., 1986; for review see Bandziulis et al., 1989). Extensive searches of several protein and nucleic acid sequence data banks (e.g., Swiss Prot Release 14.0, Protein Identification Resource Release 25, EMBL release 24, and Genbank release 64) have not shown significant homology of this sequence to any of the known RNA-binding proteins. Therefore, we conclude that p56, and probably also p54, represent new kinds of RNA binding proteins.

The larger mRNA binding particles, with a broad distribution centered at ~15S, are the only moieties containing p100 and exhibit exclusive mRNA binding by p100 in the native particles. Cummings and Sommerville (1988) described, in previtellogenic oocytes, a fraction of 6–18S particles with phosphopolypeptides at 16, 28, and 56 kD, which also contained kinase activity, but these authors did not test for mRNA binding. Our immunoprecipitation of an ~100-kD phosphopolypeptide with p54/p56 antibodies, together with the increased mRNA binding activity of p54, p56, and p60 after phosphatase treatment of 15S particles, indicates that this set of mRNA binding polypeptides is present in the same particles. It also suggests that the mRNA binding of p54, p56, and p60 is regulated by their association in the 15S particles.

Dependence of mRNA Binding on Phosphorylation in All Three Particle Classes

All five major mRNA-binding proteins present in the cytosol fraction of oocytes and eggs occur in phosphorylated forms. Intense phosphorylation has also been reported for polypeptides of 56 and 60 kD present in particle fractions from previtellogenic oocytes (Cummings and Sommerville, 1988), which may be identical to the proteins discussed here. Moreover, we have found that the mRNA binding ability of these five polypeptides depends on their phosphorylation, as it is lost upon phosphatase treatment (p60, p70, and p100) or in-

creased after incubation at conditions optimal for protein kinase activity (p54 and p56). The apparent insensitivity of both polypeptides of the 6S particle to phosphatase treatment is probably explained by efficient rephosphorylation by kinase activity intrinsic to these particles. The modification of p54 and p56 to more acidic isoforms in the unfertilized egg is associated with an observed increase in their mRNA binding (Fig. 8).

The observed developmental pattern of mRNA binding of p54 and p56 would be compatible with a regulatory role of the 6S particle in polyribosome formation. This may relate to the findings of dependence of translational inactivation on the phosphorylation state of mRNA-associated proteins, as observed when native oocyte mRNPs were translated in vitro after phosphatase treatment (Kick et al., 1987). These results suggest that the full set of mRNA binding phosphoproteins is necessary for mRNP stability and translational inactivation.

Developmental Regulation of mRNP Formation

The pattern of mRNA binding of p54 and p56 during oogenesis and embryogenesis seems to be correlated with mRNP formation during development: increased mRNA binding is concomitant with the increase in mRNP formation during oogenesis, including oocyte maturation and egg formation (Ballantine et al., 1979; Kandror et al., 1989), and loss of p54 and p56 from the soluble pool later in embryogenesis coincides with the increased translation of stored mRNAs (Ballentine et al., 1979; Woodland et al., 1979; Lee et al., 1984; Stick and Hausen, 1985). Since some low-level pre-mRNA transcription has been observed in stages earlier than midblastula (Shiokawa et al., 1989), the soluble pools of mRNA-binding proteins would also allow for the continuous assembly of new transcripts into mRNPs. The observed mRNA binding of p60, p70, and p100 throughout early embryogenesis (to stage 28) suggests that they may continue to serve as complex partners of newly synthesized mRNAs. This is not unexpected as it has been shown in other cells (Greenberg and Carroll, 1985) that the major mRNA-binding proteins of mRNPs are also found on polyribosome-associated mRNA.

We postulate that the mRNA binding proteins identified here, specifically the 6S particles p54 and p56, are generally involved in maternal mRNP formation in a mode largely independent of the developmentally regulated deadenylation and polyadenylation of mRNAs which occurs in the same system (e.g., Ruiz i Altaba et al., 1987; Hyman and Wormington, 1988; McGrew et al., 1989; Duval et al., 1990; Paris and Philippe, 1990). As the mRNA binding activity of these proteins depends on their phosphorylation, the observed increase in kinase II activity upon oocyte maturation (Kandror et al., 1989) and the postgastrular increases in certain phosphatase activities (Miyahara et al., 1982) may play key roles in regulating complex formation, and thus translational activity, of mRNPs.

General Conclusions

Although the amphibian oocyte builds up large stores of inactivated mRNA and therefore contains relatively high concentrations of the free, i.e., soluble forms of mRNA-binding proteins described here, the basic requirement of specific complex formation of certain proteins with certain mRNAs also has to be met in other kinds of cells, although on a smaller scale. Indeed, a poly(A) binding protein of ~70 kD has been identified in a wide variety of cell types and species (Sachs et al., 1986; Ullrich et al., 1988; Zelus et al., 1989; for review see Jackson and Standart, 1990). Thus, pools of soluble mRNA-binding proteins may be a rather general and functionally fundamental "housekeeping" feature of diverse cells whereas the build up of a large store of these proteins may be special for oocytes, and this mechanism may be necessary for early embryogenesis (see also Drawbridge et al., 1990).

We are also aware of the problems that proteins destined for binding a specific kind of nucleic acid, i.e., in this case certain mRNAs, face in their cytoplasmic environment dominated by an abundance of negatively charged molecules, including most proteins and nucleic acids other than mRNAs. Therefore, we also postulate that the mRNA-binding proteins and particles described here are themselves controlled by a category of special proteins, similar to the anionic proteins regulating storage of histones and hence chromatin formation (e.g., Laseky et al., 1978; Kleinschmidt et al., 1985; Smith and Stillman, 1989). The availability of antibodies to these proteins, and of cDNA probes, will allow us to identify such molecules and elucidate the protein–RNA and protein–protein interactions involved.

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