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Binding of *Xenopus* oocyte masking proteins to mRNA sequences

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ABSTRACT

It has been shown previously that maternal mRNA, synthesized and stored in growing oocytes, is stabilized and blocked from translation through various mechanisms including restricted polyadenylation and the binding of proteins to 3′ regulatory elements. In addition to binding sequence-specific proteins, the bulk of stored mRNA is packaged with a set of 'masking' proteins, the most abundant of which are the phosphoproteins pp56 and pp60. In this report these proteins are shown to be bound to heterogeneous mRNA sequences and not to the 3′ poly(A) tract. Crosslinking studies demonstrate that all of the pp56/60 present makes direct contact with the RNA. *In vitro* binding studies confirm that pp56/60 interact with single-stranded RNA of heterogeneous sequence, such as occurring in the maternal mRNA encoding cyclin B1. However, binding is equally effective to capped and polyadenylated cyclin mRNA, to truncated mRNA lacking 5′ and 3′ non-coding regions and even to the antisense sequence. Lengths of 70 – 80 nucleotides are protected from ribonuclease digestion after protein binding. Although no extended binding motif could be detected, binding does appear to have some specificity in that it is not competed out by 100-fold excess of double-stranded RNA, transfer RNA, poly(A) and various other homopolymers and heteropolymers. The sequence which competes most efficiently is the mixed polypyrimidine, poly(C,U). Crosslinking of RNA-protein complexes, followed by ribonuclease digestion, suggests that the arrangement of proteins on RNA is as dimers. Dimerization appears to be stabilized by phosphorylation of pp56/60. These results are discussed in terms of the known structures of pp56/60.

INTRODUCTION

Amphibian oocytes accumulate large amounts of stored mRNA which are maintained in a stable and untranslated state over periods of months until eventually mobilized into polysomes at oocyte maturation and after fertilization (reviewed, 1). Expression of this maternal mRNA fulfils the requirements of the early embryo for new proteins: the rapid cell cycles taking the embryo to mid-blastula preclude transcription until after this stage (2). A prominent feature of maternal mRNA is its association with a specific set of 'masking' proteins (plus other, less abundant proteins) to form messenger ribonucleoprotein (mRNP) particles (reviewed, 3). In *Xenopus* oocytes, the major masking proteins have apparent molecular masses of 56 and 60 kD (4–6) and are maintained in a phosphorylated state through the activity of an mRNP-bound protein kinase (7).

Sequencing of cDNA clones (8) and amino acid sequencing of isolated proteins (8, 9) have revealed the pp56/60 masking proteins to be closely-related members of the family of 'Y-box' proteins (10). These proteins interact specifically with a Y-box DNA sequence (CTGATTGGCCAA) which contains a reverse CCAAT box and can stimulate transcription from promoters containing this element (10–12). The mRNAs encoding pp56/60 are detected only in germ cells (8, 10) where the proteins may act on known germ-cell-specific Y-box promoters (13). Thus pp56/60 may have a dual function in oocytes: to regulate transcription of oocyte-specific genes, and to stabilize the mRNA sequences transcribed from those genes. (To what extent the resulting general packaging of mRNA contributes to the blocking of its translation *in vivo* is not known). Such a dual function is not without precedent: transcription factor TFIIIA both positively regulates 5S rRNA genes and binds to and stabilizes 5S rRNA in *Xenopus* oocytes (14).

Although the DNA binding specificity of pp60 (also known as FRG Y2) has been studied (10, 13), little is known of the RNA binding specificities of pp60 and its partner masking protein pp56. Here we examine the occurrence and location of pp56/60 in native mRNP particles and study their preference of binding *in vitro* to sequences contained in synthetic mRNAs.

MATERIALS AND METHODS

Isolation of mRNP particles

Previtellogenic ovary was dissected from *Xenopus laevis* at 4–8 weeks post-metamorphosis. To label RNA *in vivo*, the ovarian tissue was divided into equal amounts (approximately 100 μg wet weight) and incubated at 20°C for 18h in the presence of [2-^{3}H]adenosine (21 Ci/mmol), or [5-^{3}H]cytidine (27 Ci/mmol)

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or [5-^{3}H]uridine (26 Ci/mmol, all from Amersham International), each at a final concentration of 0.5 mCi/ml in Barths' solution (15). Ovary was sonicated in 4 volumes of homogenization buffer (HB: 8% glycerol, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 mM Tris-HC1, pH 7.5). After centrifugation at 10,000×g for 10 min at 2°C, the clarified supernatant was adjusted to 0.25M NaCl and applied to 1 ml column of oligo(dT)-cellulose (Pharmacia) equilibrated with binding buffer (BB: 0.25M NaCl, 2 mM Mg Cl_2, 10 mM Tris-HC1, pH 7.5). The unbound material was reapplied to the column three times and eventually washed through with BB. Poly(A)$^+$RNP was eluted with 2.5 ml of 2 mM Tris-HC1, pH 7.5 at 40°C and immediately frozen and stored at −70°C. To phospholabel the poly(A$^+$)RNP, 0.5 ml of the thawed column eluate, containing approximately 0.25 mg of RNP, was adjusted to 2 mM $MgCl_2$, 2 mM $MnCl_2$, 2 mM 2-mercaptoethanol, 10 mM Tris-HC1, pH 7.5, and incubated at 20°C for 20 min in the presence of 50 μCi [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham International). The endogenous, mRNP-bound, protein kinase (7) is activated under these conditions to phosphorylate the pp56/60 RNA-masking proteins.

Synthesis of riboprobes

Radiolabelled RNA (riboprobe) was synthesized by *in vitro* transcription, through inserts cloned into pGEM vectors, in the presence of [α-^{32}P]CTP (400 Ci/mmol, Amersham International) as described previously (16). pXlcBl contains an insert of 1411 bp encoding a complete, translatable mRNA for *X. laevis* cyclin B1 (17). Linearization with BamH1 and transcription with T7 RNA polymerase (Pharmacia) generates full-length sense strands. pJS23 contains 324 bp of the coding region of pXlcBl (bp 128−451) which, on linearization with HindIII and transcription with SP6 RNA polymerase, generates sense strands and, on linearization with EcoR1 and transcription with T7 RNA polymerase, antisense strands. Some transcripts were capped at their 5′ ends by adding m^{7}G(5′)ppp(5′)G (Boehringer) to the synthesis reaction (18). Radiolabelled RNA was recovered from unincorporated label by spin-column chromatography using Sephadex G-50 (Pharmacia) and was further purified by extraction with phenol-chloroform (16). The RNA was further polyadenylated at its 3′ end using ATP and poly(A) polymerase (Gibco-BRL) (18). For use in crosslinking studies 5BrUTP (Sigma Chemical Co.) was substituted for UTP in the synthesis reaction, because, as described previously (19), 5BrUTP improves the efficiency of UV-stimulated crosslinking of bound protein to RNA.

RNA polymers

The ribohomopolymers: polyadenylic acid [poly(A)]; polyguanylic acid [poly(G)], polycytidylic acid [poly(C)], and polyuridylic acid [poly(U)], the heteropolymers: poly(A,G), poly(A,U), poly (A,C) and poly (A,U), and the double stranded homopolymers: poly(A). poly(U) and poly(I).poly(C), were purchased from Sigma Chemical Co and dissolved at 10 mg/ml in distilled water. Dephosphorylated 5′ ends were end-labelled using [γ-^{32}P]ATP (3,000 Ci/mmol) and polynucleotide kinase as recommended on the enzyme data sheet (Pharmacia). Radiolabelled poly(C,U) was synthesized from 1 mM CTP (containing 50 μCi of [α-^{32}P]CTP) and 1 mM 5BrUTP using poly(A) polymerase (Gibco-BRL) and yeast transfer RNA (Boehringer) as primer.

Conditions for protein-RNA binding

Proteins separated by preparative SDS-PAGE of poly (A)$^+$RNP were recovered as described previously (6). To remove SDS from the isolated proteins, urea was added to 8.5 M and the denatured proteins (5 μg in 50 μl) were dialysed extensively (over 2h) against dialysis buffer (DB: 2 mM EDTA, 20 mM Tris-HC1, pH 7.5) containing 8.5 M urea. Radiolabelled RNA (0.2 μg) and competitor RNA (0−2 mg) were added at this stage and the protein was renatured by continuously lowering the urea concentration over a period of 2h until the protein-RNA mixture was contained in DB alone. Proteins were translocated directly from native mRNP particles to added riboprobe by first destabilizing the RNP particles in 20 mM $MgCl_2$ (20) and then allowing RNP complexes to form by gradually reducing the concentration of $MgCl_2$. Mixtures containing 2.5 μg of mRNP (approximately 2 μg of protein bound to 0.5 μg of RNA), 0.1 μg if radiolabelled riboprobe and any competitor polynucleotide (up to 1 mg) were adjusted to 20 mM $MgCl_2$, 20 mM Tris-HC1, pH 7.5 in a total volume of 100 μl. The $MgCl_2$ concentration was reduced to 2 mM by stepwise dilutions with 50 μl aliquots of 20mM Tris-HC1, pH 7.5 over a period of 30 min. RNP complexes were collected by binding to filter discs (Millipore, HAWP) and unbound RNA was washed off with 20 mM Tris-HC1, pH 7.5. Bound RNA was estimated by Cerenkov counting of the filters in 5 ml of water.

Ribonuclease protection

Lengths of radiolabelled RNA, protected from digestion with ribonuclease by virtue of bound protein, were determined by electrophoresis through gels of 6% acrylamide containing 8M urea (sequencing gel mix) followed by autoradiography. The RNP complexes were digested for 30 min at 20°C with 50 μg/ml of ribonuclease A and 50 units/ml of ribonuclease T1 or 1/10 or 1/100 dilutions of this. Two volumes of urea-saturated formamide, containing 5mM EDTA, xylene cyanol and bromophenol blue, were added to the digests which were then heated to 90°C for 3 min and immediately loaded on to the gel. After electrophoresis at 260 volts for 90 min, the gels were fixed for 30 min in a solution containing 30% methanol, 10% acetic acid, vacuum dried and set up for autoradiography using Agfa X-ray film.

Ultraviolet cross-linking

Proteins in direct contact with radiolabelled RNA were covalently crosslinked to the RNA by irradiation with ultraviolet light (21, 22). Solutions containing 20−25 μg/ml of RNP complexes (liquid depth, 2−3 mm) were stirred at 0°C while being irradiated with ultraviolet light (output 600 J/m^2/sec at 254 nm). After irradiation for 40 min, samples were adjusted to 0.2% Nonidet P-40 (Sigma) and unprotected RNA was digested with 50 μg/ml of ribonuclease A and 50 units/ml of ribonuclease T1 for 30 min at 20°C. Samples for SDS-PAGE were adjusted to 8M urea, 1% SDS, 2.5% mercaptoethonol and 5% glycerol (23). Denatured proteins retaining crosslinked fragments of radiolabelled RNA are detected by autoradiography of dried gels.

Density gradient centrifugation

Samples of 2.5 μg of RNA-protein complexes, with or without prior cross linking and with or without ribonuclease digestion (see above) were layered directly on to 5 ml preformed gradients of 6−48% CsC1 in 0.2% Nonidet P-40, 50 mM sodium

phosphate, pH 7.0. After centrifugation at 40,000 rpm for 16h at 18°C in an SW65 rotor of the Beckman L-7 ultracentrifuge, 200 μl samples were collected by careful pipetting from the top of the gradients. Density points were measured by refractometry and radioactivity was estimated by liquid scintillation counting of samples precipitated with 10% trichloroacetic acid.

RESULTS

Identity of proteins bound to native mRNA sequences

The native mRNP particles of oocytes are selectively bound to columns of oligo(dT)−cellulose by virtue of their 3′ poly(A) tail (3, 7, 15). On increasing the salt concentration of eluent to 2M NaCl the only proteins remaining bound to mRNA are the pp60/56 masking proteins. These proteins can then be eluted by washing the column with nucleic acid denaturants, for example 60% formamide (Figure 1).

In contrast, pp56/60 bind relatively weakly to columns of poly(A)-Sepharose, the mRNP protein binding most strongly having an apparent mass on SDS-PAGE of 70kD (p70). p70 remains bound in 1M NaCl and is eluted with 2M guanidine hydrochloride (Figure 1A). These binding characteristics are identical to those of the poly(A)-binding protein (PABP) isolated from yeast (24) and mammalian cells (25). It has been confirmed recently from cDNA sequencing, that the *Xenopus* PABP has a mass of 68.5 kD (26), which is very close to the value recorded here. Furthermore, salt stability of poly(A) binding to a PABP/β-Gal fusion protein (27) is similar to the salt stability of binding of the native 70 kD protein to poly(A)-Sepharose.

As previously demonstrated (22), oocyte masking proteins are efficiently crosslinked to mRNA molecules on exposure of mRNP particles to ultraviolet light. Figure 1B shows that the phospholabelled forms of pp56/60 are entirely crosslinked to poly(A)$^+$ RNA, remaining bound to oligo(dT)-cellulose in the presence of 0.5% SDS and being eluted with 60% formamide or after incubation with ribonuclease A. The putative PABP is not released from oligo(dT)-cellulose with ribonuclease A, but is released in conditions of nucleic acid denaturation (60% formamide, Figure 1C). That pp56/60 are co-eluted with heterogeneous RNA sequences whereas p70 is co-eluted with poly(A) is demonstrated by labelling mRNP particles *in vivo*. More than 90% of the C and U residues, are released from oligo(dT)-cellulose after treatment with ribonuclease A, but about 35% of A residues presumably consisting of poly(A) tracts, are released only after subsequent addition of 60% formamide. (Table 1). Taken together, these results are consistent with the interpretation that in native mRNP particles, the pp56/60 masking proteins are bound to heterogeneous mRNA sequences, whereas p70 is bound to the poly(A) tail. On the basis of protein staining, the mass ratio of each of pp56 and pp60 to the 70kD PABP is greater than 20:1 (Figure 1A) which would correspond to the relative lengths of heterogeneous sequence to poly(A) in the mRNA population and might indicate similar binding densities of the two classes of protein to RNA.

Binding of pp60/56 to synthetic mRNA

The mRNA encoding the cell-cycle regulatory protein cyclin B1 is expressed as a maternal message in *Xenopus* oocytes (17) and synthetic copies are used in this study as RNA templates for protein binding. Templates were transcribed from linearized plasmid vectors using bacteriophage RNA polymerases (16) to produce either a full-length translatable mRNA (from pX1cB1) or a truncated transcript corresponding to only part of the coding region (from pJS23). RNA from pJS23 was synthesized also in an antisense orientation.

Radiolabelled RNA transcripts (riboprobes) do not stick to HAWP filters (Millipore) in the solutions used, but mRNP proteins are efficiently bound. A simple assay for binding of proteins to a riboprobe is to measure the percentage of radiolabelled RNA retained on filters. However, a technical problem in working with pp56/60 is that the isolated proteins readily aggregate in the absence of ionic detergents or chaotropic agents. To avoid this problem the proteins were translocated from native mRNA molecules to riboprobes first by destabilizing the

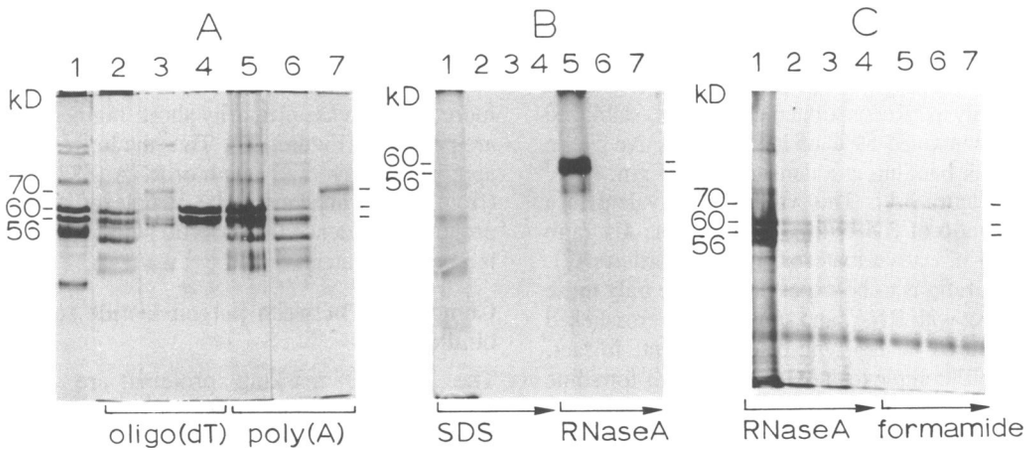

Figure 1. Characterization by SDS-PAGE of proteins bound to native mRNA. **(A)** Binding of mRNP proteins to affinity columns. Shown are: the total protein complement of mRNP particles (track 1); the fractions bound to oligo(dT)-cellulose and eluted with 0.5M NaCl (track 2), 1.0M NaCl (track 3) and 60% formamide (track 4); the fractions bound to poly (A)-Sepharose and eluted with 0.5M NaCl (track 5), 1.0M NaCl (track 6) and 2M guanidine hydrochloride (track 7). Stained gel. **(B)** Binding of phospholabelled proteins derived from UV-irradiated mRNP particles to oligo(dT)-cellulose and fractions eluted with 0.5% SDS (tracks 1−4) and 60% formamide (tracks 5−7), Autoradiograph. **(C)** Binding of mRNP proteins to oligo (dT)-cellulose and fractions eluted after incubation for 30 min at 20°C with 50 μg/ml of ribonuclease A (tracks 1−4) and 60% formamide (tracks 5−7). Stained gel. Band densities were estimated from photographs and autoradiographs using a Shimadzu CS-9000 Scanning Densitometer. Apparent molecular masses are indicated (kD).

Table 1. Release of nucleotide residues from mRNP particles bound to oligo(dT)-cellulose after digestion with ribonuclease A (50 μg/ml) then by denaturation with 60% formamide. Previtellogenic ovary was labelled *in vivo* and mRNP particles were isolated and UV-crosslinked before applying to the column.

^{3}H-labelled nucleoside	Percentage radioactivity eluted by:	
	RNase A	Formamide
C	92.9	7.1
U	94.5	5.5
A	64.5	35.5

RNP complexes in 20 mM $MgCl_2$ (20), then by allowing new complexes to form by diluting to 2mM $MgCl_2$. On including the full-length, sense riboprobe in the filter-binding assay, 60−80% of input radiolabel is bound at near saturating amounts of mRNP-derived protein (Figure 2). However the truncated sense riboprobe was equally effective in binding protein, as was the truncated antisense riboprobe (Figure 2). Therefore, 5′ and 3′ non-coding sequences appear not to be important for protein binding, as does the presence of a poly(A) tail. Extension of the poly(A) tail to 150−300 nucleotides using poly(A) polymerase (18) and addition of a $m^7G(5')ppp(5')G$ cap to the 5′ end (18) had no noticeable effect on protein binding to the full-length riboprobe (not shown). The observation that antisense RNA sequences are as effective as sense RNA sequences in protein binding indicates that an extended, defined RNA sequence is not required for recognition by the proteins.

The extent of incorporation of riboprobe into RNP complexes is proportional to the input ratio of protein to total RNA (Figure 2), indicating that the synthetic RNA sequences perform as effectively as the naturally-occurring mRNA population in the binding of protein. This parity continues towards the maximum input mass ratio (protein: RNA) of 4:1 which is observed for native mRNP particles (7). The retention of riboprobes on filters was not achieved by using non-RNP proteins. For instance, substitution of bovine serum albumin for mRNP proteins gave less than 2% binding at a protein : RNA mass ratio of 36:1 (Figure 2)

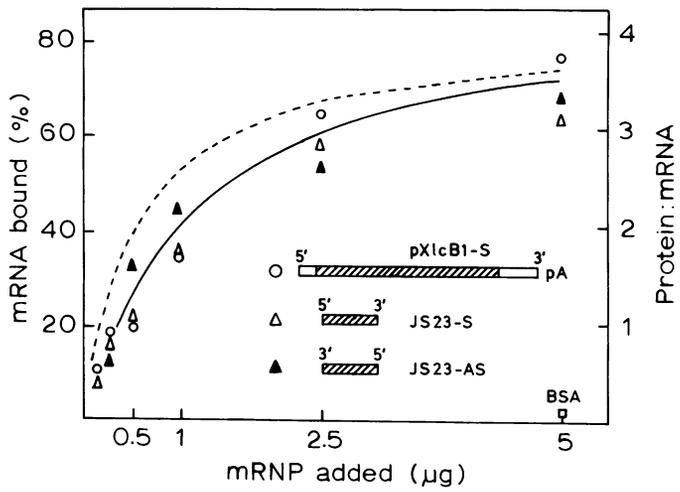

Figure 2. Translocation of proteins from mRNP particles to radiolabelled riboprobes representing full-length cyclin B1 (○), a short segment of the coding region (△) and the antisense copy of this same segment (▲). The percentage of radiolabel bound to nitrocellulose filters is plotted against input mass of mRNP. The solid line represents the mean of the experimental points, the broken line represents the input mass ratio of protein: RNA. Binding of full-length probe to filters on substituting bovine serum albumin for mRNP is also shown (□). For all further experiments the binding reaction contained 2.5μg of mRNP for every 0.1μg of full-length riboprobe.

Physical properties of reconstituted mRNP complexes

That the cyclin mRNA sequences did form distinct RNP complexes in the protein binding reaction can be demonstrated by buoyant density analysis. Reconstituted complexes, stabilized by UV-crosslinking, contained 59% of input radioactive cyclin mRNA in a single peak banding at a density of 1.45 gm, cm^{-3} in CsCl gradients (Figure 3A). This value is equivalent to a protein : RNA mass ratio of 2.5:1 (28), less than the 4:1 ratio (1.34 g cm^{-3}) typical of native maternal mRNP particles (7). A lower protein:RNA ratio is to be expected because only those proteins in direct contact with RNA, and subsequently crosslinked to the RNA are measured in this type of experiment. In fact, the reconstituted mRNP complexes are stable enough for some (16%) of the input radioactivity to peak at 1.43 g cm^{-3} (equivalent to 2.4M CsCl) in the absence of UV or chemical crosslinking (Figure 3C) The calculated mass ratio of 2.5:1 is equivalent to 83 kD of protein per 100 nucleotides. Assuming the true masses of pp60 and pp56 (predicted from cDNA clones, 8, 10) to be 36 kD and 37 kD respectively, and assuming that the masking proteins alone are crosslinked to the RNA (see Figure 5), we would expect, on average, one protein molecule to be bound per 44 nucleotides.

Treatment of the reconstituted complexes with ribonuclease A after crosslinking yielded a reduced peak of radioactivity (32% of input), this time at a density of 1.25 g cm^{-3} (Figure 3B). This value approaches that of protein alone (28) and indicates that short lengths of RNA are protected from nuclease digestion through protein binding.

The actual lengths of RNA protected by non-crosslinked masking proteins can be estimated by electrophoresis of fragments generated on digesting the RNP complexes with ribonuclease. The autoradiograph (Figure 3D) shows bands corresponding to lengths of 70−80, 45−55 and less than 20 nucleotides. After treatment with low ribonuclease concentrations the major protected fragments are 70−80 nucleotides, after treatment with ten-fold more ribonuclease the major protected fragments are 45−55 nucleotides and after treatment with one hundred-fold more ribonuclease primarily short fragments (<20 nucleotides) are produced (Figure 3D). Thus the longest protected fragments approach the average length of RNA (88 nucleotides) estimated (from density analysis) to be complexed with a pair of masking proteins. (Evidence for pp56/60 being bound to RNA as dimers is presented later).

Competition between polynucleotide sequences for protein binding

The pp56/60 masking proteins are found bound to a heterogeneous population of maternal mRNA molecules. As shown here with cyclin B1 mRNA probes, and also elsewhere with other types of maternal mRNA (19), no sequence preference for binding is apparent. In order to investigate further what structural features of mRNA might be recognized by the proteins, various polynucleotides were added, along with the cyclin riboprobe, to the mRNP reconstitution reaction. The effect of adding unlabelled polynucleotides at a 100-fold excess over radiolabelled riboprobe is shown in Figure 4A. Polypurines, poly(A), poly(G) and poly (A,G) did not compete with mRNA for protein binding. In fact they enhanced the binding of masking

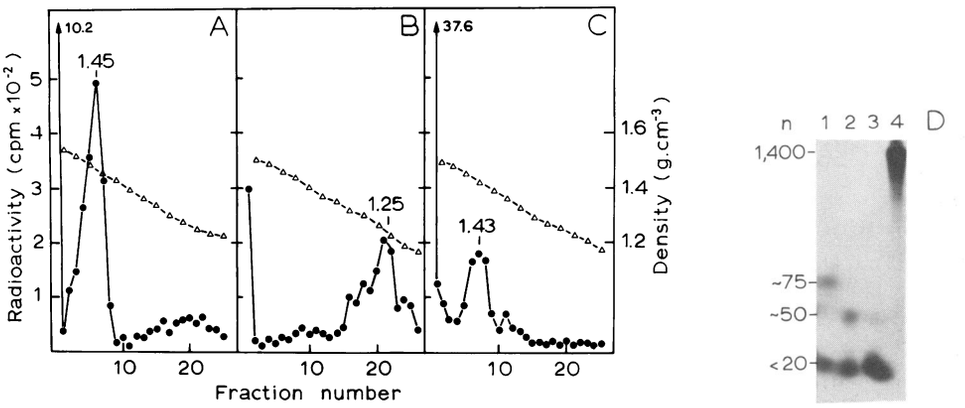

Figure 3. Physical properties of reconstituted mRNP complexes. Complexes formed from the radiolabelled cyclin B1 riboprobe and mRNP proteins, as described in Figure 2, were analysed on CsCl density gradients after UV-crosslinking **(A)**, UV-crosslinking followed by digestion with 50μg/ml ribonuclease A **(B)** and no treatment **(C)**. Distribution of radioactivity (●) and gradient profile (△) are indicated. RNA fragment sizes, after digestion of non-crosslinked complexes with ribonuclease, were estimated by electrophoresis and autoradiography **(D)**. RNA recovered from samples digested with 0.5 μg/ml RNase A/0.5 units/ml RNase T1 (track 1), 5 μg/ml RNase A/5 units/ml RNase T1 (track 2), 50 μg/ml RNase A/50 units/ml RNase T1 (track 3) and undigested (track 4). Fragment sizes were estimated using denatured 5S rRNA and tRNA and checked on DNA sequencing gels.

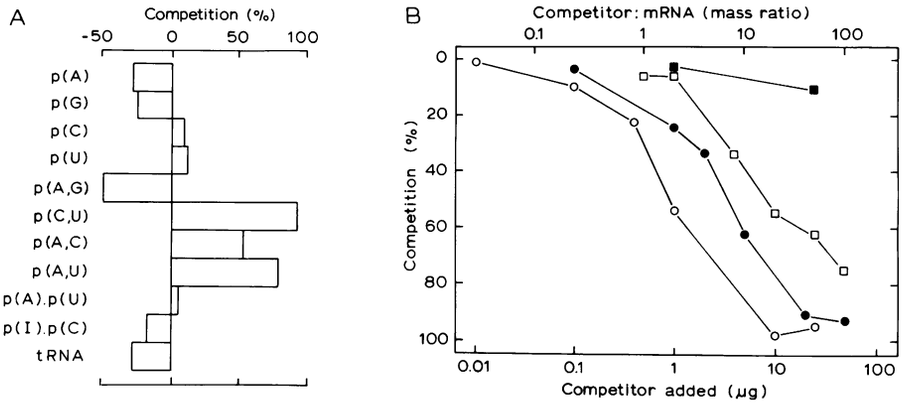

Figure 4. Effect of competing polymers added to the cyclin B1 riboprobe/mRNP protein binding assay. The procedure was as described in Figure 2 except that 10 μg of the polymers indicated were added to the reconstitution reaction. This gives a 100-fold excess of competitor to riboprobe **(A)**. The effects of increasing amounts of poly(C,U) ○, poly(A,U) ●, poly (A,C) □ and poly (A).poly(U) ■ are also shown **(B)**.

protein to the riboprobe. Whereas the polypyrimides, poly(C) and poly(U) appeared to compete slightly with the riboprobes, the mixed polypyrimide poly(C,U) competed well. The mixed polymers, poly(A,C) and poly(A,U) were slightly less effective competitors. Double-stranded RNA sequences [poly(A).poly(U), and poly(I).poly(C)] and structures (tRNA) did not compete with mRNA for protein binding (Figure 4).

These results indicate that the binding of masking proteins to RNA is specific only in the sense that the target sequence or RNA structure is simple. Titration of competing polymers (Figure 4B) shows that poly(C,U) is almost equally effective with cyclinB1 mRNA for protein binding. An 8-fold excess of poly(A,U) and a 20-fold excess of poly(A,C) are required for comparable binding.

Identification of proteins in reconstituted mRNP complexes

Complexes formed *in vitro* between mRNP proteins and radiolabelled cyclin B1 mRNA were crosslinked and extensively digested with ribonucleases. After complete denaturation in SDS and 2-mercaptoethanol, the proteins were analysed by SDS-PAGE. Autoradiographs of the gels show that the denatured proteins retain enough crosslinked radiolabelled RNA to detect their apparent molecular mass. Proteins labelled in this way migrate as two bands corresponding to pp56 and pp60 (Figure 5A). Generally most of the radioactivity resolved on the gels occurs in these two bands with variable amounts occurring in additional bands migrating at about 100 kD and 200 kD (see below). As found in filter binding, interaction of pp56/60 with mRNA is not competed out with a 100-fold excess of unlabelled poly(A), but is competed out with a 100-fold excess of unlabelled poly(C,U) (Figure 5A, tracks 3 and 4). Thus the competition effects seen in the filter binding assays relate primarily, if not exclusively, to pp56/60.

Protein dimers and multimers

If the protein/RNA binding reaction is carried out in the presence of 1mM ATP, and this is followed by UV crosslinking and ribonuclease digestion, there results on SDS-PAGE a noticeable reduction in labelling of pp56/60 but a corresponding increase in labelling of a band migrating at about 100 kD (Figure 5A,

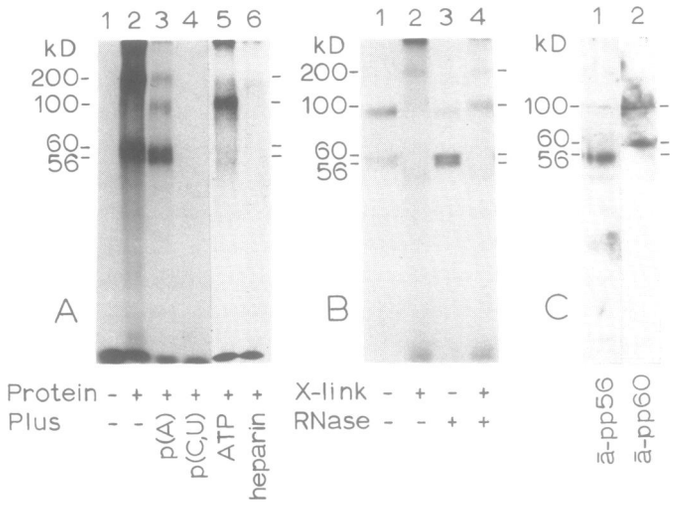

Figure 5. Identification by SDS-PAGE of proteins in reconstituted mRNP and formation of protein dimers and multimers. **(A)** Reconstitution reactions contained the radiolabelled cyclin B1 riboprobe with either no protein (track 1) or the mRNP proteins (tracks 2–6) as described in Figure 2. Additions to the reactions were 10 μg of poly(A) (track 3), 10 μg of poly(C,U) (track 4), 1mM ATP (track 5) 0.5 μg/ml of heparin (track 6). All samples were UV-crosslinked and digested with 50 μg/ml RNase A/50 units/ml RNase T1 before protein denaturation and gel analysis. (B) Phospholabelled mRNP particles with (tracks 2 and 4) or without (tracks 1 and 3) UV-crosslinking followed by no digestion (tracks 1 and 2) or digestion with ribonuclease as in (A) (tracks 3 and 4). Denatured proteins were then separated by SDS-PAGE. **(C)** Immunoblots of mRNP particles with antiserum directed against pp56 (track 1) and pp60 (track 2) detected with ^{125}I-labelled protein A (15). All pictures are from autoradiographs and apparent molecular masses are indicated (kD).

track 5). It has been shown previously (7) that continual phosphorylation by an mRNP-bound protein kinase is required to maintain optimum binding of pp56/60 to mRNA. In the presence of ATP, therefore, tighter binding may result in protein dimers being crosslinked to the same fragment of RNA. In contrast, a low concentration (0.5μg/ml) of heparin, which completely inactivates the mRNP-bound protein kinase (7), results in no observable crosslinking of protein to RNA (Figure 5A, track 6).

A possible explanation, then, for the regular occurrence of higher molecular weight bands (as seen in Figure 5A), is that they represent protein dimers and multimers covalently linked to the same fragment of radiolabelled RNA. To analyse further the arrangement of masking proteins on RNA, mRNP particles were phospholabelled *in vitro* and treated, with and without UV crosslinking and with and without ribonuclease, before analysis by SDS-PAGE. As can be seen in Figure 5B: with no crosslinking and with ribonuclease treatment, most of the phospholabel resolves at 56 and 60 kD; omitting ribonuclease results in displacement of some of this label to 100 kD; with crosslinking and no ribonuclease, most of the label fails to enter the gel; with crosslinking and ribonuclease most of the label is released to migrate to 100 kD and above. These results are consistent with the view that a stable dimer arrangement of proteins exists on the mRNA. That this dimer is a heterodimer is indicated by the immunoreactivity of protein transferred from the 100 kD band with antibodies monospecific for pp56 and pp60 (Figure 5C). It could be argued, however, that because of the close molecular masses of the two proteins, mixtures of homodimers could migrate as a single band. Nevertheless, several other lines of evidence point to heterodimerization of the masking proteins: for instance, pp56 and pp60 are always recovered in equimolar amounts from different cell fractions and from different oogenic stages (4, 7, 30); and the masking proteins are detected in an RNA-free state only as cytoplasmic complexes consisting of protein heterodimers and multimers (7, 19).

DISCUSSION

Although several abundant proteins co-isolate with oocyte polyadenylated RNA (4–7), only two of these remain bound to RNA in the presence of high salt concentrations (eg 2M NaCl) and are crosslinked to the RNA on UV irradiation (see Figure 1). These are the masking phosphoproteins pp56 and pp60 whose structure (8, 9, 13) and cellular location (7, 15, 19) have been characterized and whose expression during oogenesis correlates with inhibition of translation of the set of maternal mRNA molecules (reviewed, 3). A third protein has been identified, the poly(A)-binding protein (PABP), which is present at relatively low concentration in maternal mRNP particles (see also, 22). The molar ratio of the PABP to each of the masking proteins is about 1:20, whereas the masking proteins themselves are always present in equimolar amounts. The low concentration of the PABP probably relates to the relatively short poly(A) sequence (15–80 nucleotides) present on most maternal mRNA (29). During early development the time course for accumulation of the PABP tends to correlate with the steady state amount of poly(A) present (26). The work described here confirms that, in maternal mRNP particles, the PABP is located on the poly(A) sequence whereas the pp56/60 masking proteins are mostly, if not exclusively, located on heterogeneous sequences.

In spite of the stability of maternal mRNP particles in the presence of high salt concentrations, several conditions are known to dissociate the masking proteins from mRNA. Chaotropic agents such as 8M urea have been used in mRNP reconstitution experiments (6) and the unique resistance of pp56/60 to denaturation after heating to 80°C, being able to coalesce on any available RNA on cooling (30), provides another means of relocating the masking proteins on RNA templates. A more subtle means of regulating protein: mRNA interaction is to modulate the concentration of Mg^{2+}. It has been shown previously that a 40 kD phosphoprotein, binding to poly(A)$^+$ RNA in gastrulae of *Artemia*, is dissociated from the RNA at $MgCl_2$ concentrations above 2 mM (20). Similarly, it is found that the *Xenopus* oocyte masking proteins can be dissociated from maternal mRNA in the presence of 20 mM $MgCl_2$ and relocated on added synthetic mRNA by reducing the $MgCl_2$ concentration to 2 mM or below. This procedure was adopted for most of the binding studies described here. A possible explanation for the effect of $MgCl_2$ is that water molecules coordinated to Mg^{2+} produce hydrogen-bond donors which might interfere with the interaction of arginine side chains with the phosphate backbone of RNA molecules (31). The involvement of arginine side chains in RNA recognition is proposed for the binding of the HIV-1 Tat protein to phosphates in the RNA stem-loop structure, TAR (31). Although Tat recognizes double-stranded RNA structures and the masking proteins appear to prefer single-stranded structures, arginine residues contained within the RNA-binding sites may be a common feature of these and other proteins. It is interesting to note that both pp56 and pp60 have high contents (11–12%) of arginine residues (8, 9).

The ability of the mRNA masking proteins to bind a variety of RNA templates *in vitro* is perhaps not surprising in view of

the large number of different kinds of maternal mRNA which exists in oocytes. In another report (19) these same proteins have been shown to bind *in vitro* to the mRNA encoding lamin L1 and other maternal messages. It is not, as yet, possible to say what common recognition feature of these RNA molecules imparts binding specificity. The observations made here, that truncated mRNA molecules (lacking both 5′ and 3′ non-coding regions) and antisense transcripts bind masking proteins as efficiently as does the full length cyclin B1 mRNA, indicates a loosely defined sequence or structural feature. Sufficient requirements for efficient *in vitro* binding are that the template be single stranded and contain a sequence of mixed pyrimidines (see Figure 4). Sequences peculiar to maternal mRNA, such as the UUUUUAU cytoplasmic polyadenylation motif (reviewed 1, 33), do not occur in all of the RNA templates which show positive binding of the masking proteins.

At present, the masking proteins pp56/60 are best viewed as having a general mRNA-packaging function. Specific repression of translation through protein binding has been studied previously for particular mRNA species. For instance, antisense oligonucleotides have been used to form duplexed regions on maternal mRNAs of *Spisula*, some of these structures preventing binding of translation repressor proteins (32). Sequences contained within a U-rich stretch of the 3′ untranslated region of mRNAs encoding ribonucleotide reductase and cyclin B have been identified as being responsible for unmasking and subsequent translation *in vitro* (32). Translation of several other maternal mRNAs in *Spisula, Xenopus* and mouse is influenced by extension of the 3′ poly(A) tail, which leads to recruitment into polysomes at oocyte maturation or after fertilization (reviewed 1, 33). However, not all regulation of translation occurs through 3′ sequences: the 5′ untranslated region of a set of mRNAs encoding *Xenopus* ribosomal proteins is identified as being the target for translation regulation (34). It is interesting to note that a conserved feature of the 5′ control region is a run of 8–12 pyrimidines which has protein binding activity.

Not enough is known about the identity of proteins which bind the specific regulatory motifs to say how they relate to the more generally binding masking proteins pp56/60: it is quite likely that there are different levels of selectivity in the masking/unmasking processes. Nor is enough known about the level in mRNA production at which binding of masking proteins occurs. It is significant that pp60 has been isolated from oocyte nuclei (15) and that both pp56 and pp60 are detected on nascent transcripts of lampbrush chromosomes (J.S., unpublished). In the cell, mRNA sequences may have to pass through a particular transcription/processing/transport pathway to become properly masked. If, as shown here, masking proteins can bind to sequences likely to occur in any mRNA molecule, how do some messages avoid masking to meet the translational needs of growing oocytes? From what has been mentioned above, two sorts of mechanism are possible: binding of sequence-specific proteins might exclude loading of masking proteins; masking may be determined at the level of transcription through the presence of masking proteins only in transcription units producing maternal mRNAs. This second mechanism could operate through specific interaction of masking proteins, and/or related Y-box proteins, with cognate promoter elements (13, see below) which in turn facilitates loading of masking proteins on to transcripts.

The results reported here on binding of pp56/60 to mRNA sequences *in vitro* are supported by previous observations on native materials: UV irradiation of previtellogenic oocytes results in crosslinking of pp56/60 to poly(A)$^+$RNA *in vivo* (22, J.LR. and J.S., unpublished); antibodies specific for pp56 immunoprecipitate cyclin B1 mRNA sequences contained within native mRNP particles (J. S., unpublished).

In both native and *in vitro* constructed mRNP particles, the masking proteins are bound throughout most of the length of the RNA, with the likely exception of the poly(A) tracts. Buoyant density and RNA protection studies (see Figure 3) indicate the binding of one pp56/60 pair per 90 nucleotides. It might be argued however that binding recognition does occur at a single loading site and that the proteins redistribute along the RNA or polymerize through protein-protein interaction. This latter mechanism has been suggested for the coating of HIV-1 transcripts with the retroviral encoded Rev protein (35). In fact it has been shown recently that pp60 (FRGY2) and the closely related protein FRGY1, in their capacity as DNA-binding proteins, both form multimers originating from specific recognition of the Y-box promoter element (13). Protein-protein interaction affords protection from DNase I digestion to an extended segment of DNA (>100 bp) beyond the proximal Y-box element of the *Xenopus* hsp 70 promoter (13). Thus similar mechanisms may operate in the polymerization of pp60 on both DNA and RNA to give extended coverage of the nucleic acid. The form of protein-protein interaction that could occur is suggested by the unique structural features of pp56/60 and the other members of the Y-box family of proteins (reviewed, 36). Whereas the amino terminal region of these proteins is highly conserved in amino acid sequence forming the DNA-binding domain (13), the carboxyl end consists of alternating acidic and basic regions which could interact between adjacent proteins giving a 'charge zipper' effect (12). It is easy to imagine the advantages of polymerization of masking proteins along mRNA molecules which have to be stored and protected from degradation through long periods of development. It remains to be seen how this process is regulated.

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