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Purification of two thermostable components of messenger ribonucleoprotein particles (mRNPs) from *Xenopus laevis* oocytes, belonging to a novel class of RNA-binding proteins

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We have purified and partially sequenced two proteins from *Xenopus laevis* previtellogenic oocytes, belonging to messenger ribonucleoprotein particles (mRNPs). The purification procedure rests on the thermostability of these proteins, which remain soluble after heating the cell extracts at 80°C. The thermostable proteins can be identified with two of the most abundant components (mRNP3 and mRNP4) of the mRNPs, described by Darnbrough and Ford (1981) [Eur. J. Biochem. 118, 415–424]. mRNP3 and mRNP4 are homologous to each other, but to no other protein of known sequence. The abundance and semi-periodic distribution of proline residues in mRNP3 and mRNP4 sequences suggest that these RNA-binding proteins adopt an unusual type of conformation

mRNA-containing particle; mRNP; Amino acid sequence; Sequence homology; *Xenopus laevis*; Oogenesis

1. INTRODUCTION

Protein synthesis in early embryonic development of *Xenopus laevis* is supported by mRNA, tRNA and ribosomes of maternal origin [1]. All these components are stored in oocytes for long periods of time. The best known storage system is that of 5 S RNA and tRNA, which involves integration of these RNAs in nucleoprotein particles, known as thesaurisomes [2–5]. A large fraction of the oocyte's mRNA is also stored in nucleoprotein particles, called mRNPs [6,7]. The mRNPs are less well characterized than the thesaurisomes. They sediment in a broad region of the sucrose or glycerol gradients, ranging from 40 S to 100 S [7]. Besides mRNA, the mRNPs contain a set of four major (mRNP 1–4) and four minor proteins (mRNP5–8) [7]. A fraction of the mRNA-binding proteins sediments more slowly (6–15 S), and forms various kinds of mRNA-free heteropolymers [8,9].

The function of the mRNPs is poorly understood. These particles protect mRNA against degradation [7], and presumably regulate its translation [8,10]. A subset of the mRNP proteins is phosphorylated by one or several kinases associated with the particles [8,9,11]. The phosphorylation state of the mRNP proteins strongly influences their ability to bind mRNA [9]. However, the involvement of phosphorylation in the postulated functions of the mRNPs is far from proven.

In this paper we describe an easy and efficient method to purify two of the most abundant mRNP proteins. These proteins remain soluble when purified mRNPs or whole cell extracts are heated at 80°C, and are recovered as a complex with mRNA or 5 S RNA. We identify the thermostable proteins with mRNP3 and mRNP4 [7], and confirm that these proteins can be phosphorylated by simply incubating the mRNPs with [γ-^{32}P]ATP [8,9,11]. Amino acid sequencing of several peptides derived from mRNP3 and mRNP4 revealed that these proteins are homologous to each other, but to no other protein listed in current data banks. We conclude that mRNP3 and mRNP4 belong to a novel class of RNA-binding proteins.

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2. EXPERIMENTAL

Post-mitochondrial extracts from immature ovaries were fractionated by centrifugation through 15–30% sucrose density gradients containing 5 mM $MgCl_2$ [3]. This separates the major nucleoprotein particles, which sediment at 7 S [4], 42 S [2,3] and 80 S. mRNPs were isolated by the same procedure, except that a cushion of 66% sucrose was placed on the bottom of the centrifuge tubes [12]. Under these conditions the mRNPs concentrate on top of the sucrose cushion, and can be recovered in a semi-purified form [12].

The mRNP proteins were phosphorylated in vitro [11] by incubating fractionated or non-fractionated cell homogenates with [γ-^{32}P]ATP (Amersham; spec. radioact. 5000 Ci/mmol).

Non-fractionated extracts of immature ovaries [3] and selected fractions from the sucrose gradients were heated at 80°C for 10 min in 50 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$. This treat-

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ment precipitates nearly all soluble proteins. The extracts were cooled in ice and centrifuged at 10 000 rpm for 10 min. This clarifies the extract and leaves the thermostable proteins in the supernatant (80 S).

The 80 S supernatants were analyzed by polyacrylamide gel electrophoresis in the presence of SDS [13]. The proteins were revealed by autoradiography or Coomassie blue staining. The 80 S supernatants were also fractionated by size exclusion HPLC [14] in the absence of SDS. The HPLC fractions were analyzed for RNA and protein content by polyacrylamide gel electrophoresis followed by silver staining [15].

The major proteins present in the 80 S supernatants were purified by preparative electrophoresis [14]. The gels were stained with 300 mM copper sulfate for 5–10 min [16]. The protein bands were cut off from the gels and washed first with 250 mM Tris-HCl, pH 9, 250 mM EDTA (3 × 10 min), and second with 20 mM Tris-HCl, pH 6.8, 150 mM glycine, 0.1% SDS (10 min). Each protein was electroeluted from the gel as described [14], dialyzed against V8 buffer (125 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS), concentrated in Centricon 10 (Amicon), and digested for 1 h at 37°C with staphylococcal V8 protease (Miles) at a protein/enzyme ratio of 10:1 [17]. Proteolysis was stopped [17] and the resulting peptides were separated by gel electrophoresis as described previously [14]. The peptides were revealed by autoradiography or Coomassie blue staining. Each stained peptide was again purified by electrophoresis and transferred onto a polyvinylidene difluoride membrane [18]. A strip carrying the peptide band was cut off from the membrane and submitted to Edman degradation in an Applied Biosystems 470-A gas-phase sequenator coupled to a PTH analyzer.

3. RESULTS AND DISCUSSION

We heated total and purified extracts of previtellogenic oocytes and analyzed the thermostable products (Fig. 1). Only two major proteins remain soluble (Fig. 1; lanes 2 and 3). These proteins correspond to the larger two components of a typical tetrad of bands (mRNP 1–4) which can easily be recognized in electrophoregrams of mRNPs (Fig. 1, lanes 4 and 5; and [7]). The tetrad is present in a broad region of the sucrose gradients, ranging from 40 S to 100 S (Fig. 1, lanes 4–7). This distribution corresponds to that of the mRNPs [7]. The larger two components of the tetrad can also be detected in the 7 S fraction (Fig. 1, lane 8). This most probably represents the RNA-free form of the mRNP proteins [8,9].

The apparent molecular masses of the thermostable proteins (57 and 59 kDa; Fig. 1) closely agree with the reported molecular masses of mRNP3 and mRNP4 (56 kDa and 59 kDa; [7]). We therefore identify the major thermostable proteins seen in Fig. 1 with mRNP3 and mRNP4.

As reported recently by several authors [8,9,11],

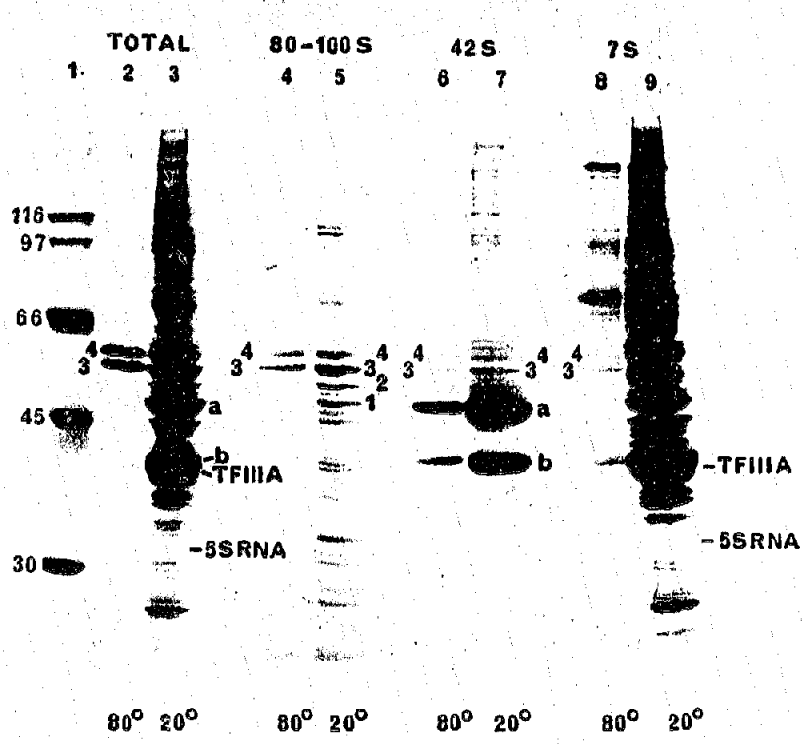

Fig. 1. Effect of heat treatment on total and purified ovary extracts. Four immature ovaries were homogenized in 500 μl of Tris-HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$. The homogenate was clarified by a low-speed centrifugation (10 000 rpm for 10 min). A 2.5-μl aliquot of the total extract was heated at 80°C for 10 min, cooled in ice and analyzed by electrophoresis in a 10.4% polyacrylamide gel in the presence of SDS. Another 2.5-μl aliquot was left at 20°C. The remainder of the homogenate was centrifuged through a 15–30% sucrose density gradient. Aliquots from 80–100 S, 42 S and 7 S fractions were analyzed with or without previous heating. Lane 1 contains protein markers of known sizes. Bands 1–4 correspond to mRNP proteins 1–4 (mRNP1–4). Band a is thesaurin a; band b is thesaurin b.

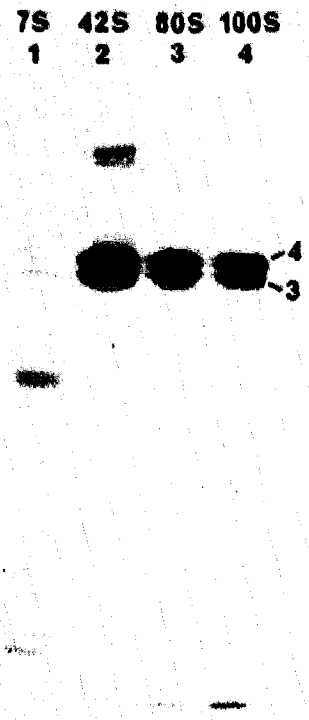

Fig. 2. In vitro phosphorylation of mRNP3 and mRNP4. Aliquots (20 μl) from 7 S, 42 S, 80 S and > 100 S fractions from a clarified ovary homogenate were incubated with [γ-^{32}P]ATP (10 μCi/ml), heated at 80°C for 10 min, cooled and analyzed by electrophoresis as in Fig. 1. The gel was dried and exposed to an X-ray film for 18 h at −70°C.

mRNP3 and mRNP4 can be phosphorylated in vitro by kinase(s) associated with the mRNPs (Fig. 2). The labeling reaction occurs in all fractions of the sucrose gradients (Fig. 2). The reaction is more efficient in the 42 S fractions than in the 80–100 S ones, although mRNP3 and mRNP4 are more concentrated in the latter fractions (Fig. 1). The low efficiency of the phosphorylation reaction in the 7 S fraction (Fig. 1) is misleading. It can be ascribed to the presence of large amounts of cold ATP in this region of the sucrose gradients. Dialyzed 7 S fractions strongly phosphorylate mRNP3 and mRNP4 when supplemented with [γ-^{32}P]ATP (data not shown).

mRNP3 and mRNP4 occur in heat-treated cell extracts as nucleoprotein complexes. These complexes can be purified by size exclusion HPLC. Heat-treated, purified mRNPs contain a large particle (M_r > 700 000), comprised of mRNA, mRNP3 and mRNP4 (data not shown). Heat-treated, non-fractionated cell extracts contain a smaller particle, in which mRNP3 and mRNP4 are associated with 5 S RNA. We interpret these observations as follows. The RNA and protein moieties of the mRNPs dissociate upon heating and reassociate upon cooling. In heated mRNPs the components able to reassociate are mRNP3, mRNP4 (Fig. 1) and mRNA. In heated, non-fractionated cell extracts, the major RNA-binding proteins are still mRNP3 and mRNP4 (Fig. 1), but the major RNA-components are 5 S RNA and tRNA released from the

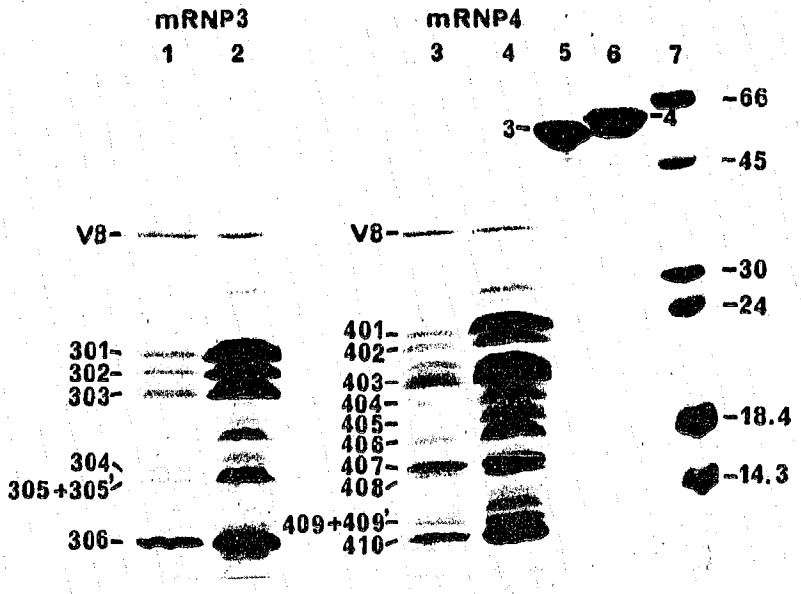

Fig. 3. Purification of peptides generated from mRNP3 and mRNP4 by partial digestion with V8 protease. mRNP3 and mRNP4 were purified by preparative electrophoresis and digested with V8 protease. Aliquots of mRNP3 and mRNP4 digests were separated by electrophoresis in a 12.5% polyacrylamide gel. Lanes 1 and 3 contain 3 μg of protein digest. Lanes 2 and 4 contain 10 μg of protein digest. Lanes 5 and 6 contain 4 and 5 μg, respectively of purified, non-digested mRNP3 and mRNP4. Lane 7 contains protein markers of known sizes. Two bands (305 + 305′ and 409 + 409′) contain two peptides of similar size.

thesaurisomes [2-5]. Under these conditions, mRNP3 and mRNP4 associate with 5 S RNA rather than with mRNA. Therefore, mRNP3 and mRNP4 bind either mRNA or 5 S according to the relative concentrations of the latter molecules in the cell extracts. However, this does not imply that 5 S RNA is a component of the native mRNPs.

The N-terminal residue of mRNP3 and mRNP4 is blocked, so that direct sequencing by Edman degradation was impossible. Internal peptides were generated by partial digestion with V8 protease. Eighteen peptides were purified (Fig. 3) and sequenced (Fig. 4). Under the conditions used, V8 protease cuts the peptide bonds between glutamate and various amino acids, except proline (Fig. 4C; see also Fig. 3 in [14]). Four stretches of sequence were elucidated, corresponding to 15–20% of the total length of both proteins. Two stretches are identical in mRNP3 and mRNP4 (Fig. 4A and B). Two other stretches are similar in sequence (Fig. 4B and C). Peptide no. 304 derived from mRNP3 contains a short sequence (QQRPP(P)X(R)(F)) which matches the repetitive motif (QQRPPPRRFQQRF) identified by Murray et al. [9] in the sequence (unpublished) of a partial mRNP4 cDNA clone. This suggests that the 13-residue motif bound in mRNP4 may also be present in mRNP3. Although fragmentary, the sequence data (Fig. 4) indicate that mRNP3 and mRNP4 are homologous.

Stretch A in mRNP3 and mRNP4 may correspond to the N-terminal part of a structural and functional domain. V8 protease cuts preferentially both proteins within a very short region of stretch A (Fig. 4A). Presumably, this part of stretch A adopts an open, easily accessible conformation. Among the peptides generated from ^{32}P-labeled mRNP3 and mRNP4 (Fig. 2) by partial protease digestion (Fig. 3), only those including stretch A are labeled (data not shown). This indicates that the phosphorylation site(s) are located at some distance from stretch A, within a 16–22 kDa region (Fig. 4A).

An extensive search in the data banks (MIPS, NBRF) revealed no significant homology between mRNP3-4 and any known protein. Murray et al. [9] reached a similar conclusion based on a longer stretch of mRNP4 sequence. The primary structure of mRNP3 and mRNP4 is indeed unusual. The conformation of these proteins is also likely to be atypical. Polypeptide chains including motifs such as QQRPPPRRFQQRF and a sequence such as QEPEPVPQPESEPEIQKP cannot adopt a secondary structure of usual type (α-helix, β-sheet or type II trans-helix found in collagen). Some kind of periodicity emphasized by the underlined proline residues might exist in the three-dimensional structure of mRNP3 and mRNP4, and may be related to the RNA-binding properties of these proteins. This idea is supported by the following arguments. First, poly(A)-binding proteins have domains rich in proline, alanine

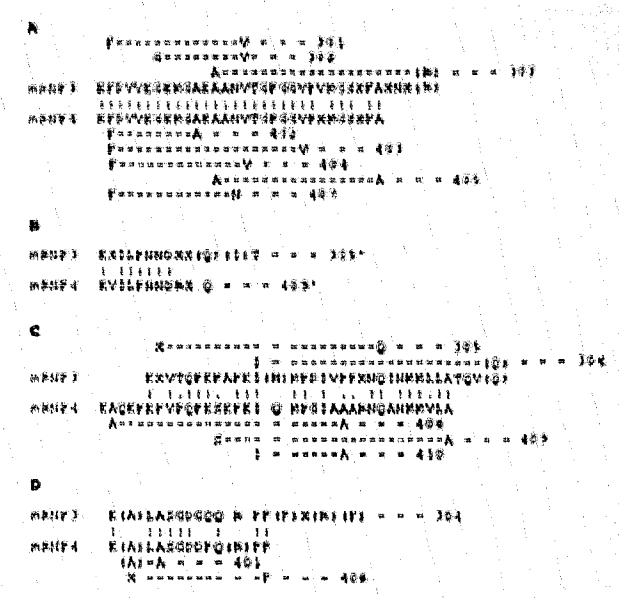

Fig. 4. Partial amino acid sequence of mRNP3 and mRNP4. The horizontal lines indicate the sequenced portion of each peptide, beginning and ending with the first and last identified residues. mRNP3 and mRNP4 contain two stretches of identical sequence (A and B) and two stretches of similar sequence (B and C). X corresponds to a non-identified residue. Residues in parentheses were not identified with certainty. Colons indicate identical residues in mRNP3 and mRNP4. Points indicate conservative substitutions [19].

and glutamine [20], with sequences such as PDPVID-PYQPAPP [21]. However, the role of these domains is presently unknown, since their removal does not appear to affect poly(A) or poly(U) binding [22]. Second, one of the tRNA-binding sites of EF-Tu is located around lysine-208 [23], and has the following sequence: PEPERAIDKP.LLP. Here again, the abundance and semi-periodic distribution of proline residues are conspicuous.

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