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Isolation of proteins comprising native gene-specific messenger ribonucleoprotein particles using paramagnetic beads

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Abstract

A method for the rapid isolation of proteins comprising native messenger ribonucleoprotein particles (mRNPs), that utilises streptavidin-coated paramagnetic beads, coupled with biotinylated oligodeoxyribonucleotides, to pull down general and transcript-specific mRNP complexes from crude cell-free extracts, is described. Two biotinylated oligo probes were tested for their efficacy in isolating mRNPs from tobacco pollen extracts; a 25-mer oligo(dT) probe for the isolation of total mRNPs, and a transcript-specific oligo complementary to the pollen-specific *ntp303* mRNA sequence. Following annealing to the target mRNAs, the oligo probes, with associated ribonucleoprotein particles, were captured by streptavidin-coated paramagnetic particles and pelleted in a magnetic field. After several washing steps, the bound RNPs were released and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot immunodetection. This one-step mRNP enrichment method could be used either to enhance the detection of low-abundance mRNA-binding proteins in subsequent northwestern and RNA UV cross-linking experiments or the direct identification and biochemical characterisation of RNA-binding proteins. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Magnetic isolation; Protein purification; RNA-binding proteins

1. Introduction

Ribonucleoprotein particles (mRNPs) are macromolecular complexes formed by the non-covalent association of RNA-binding proteins with either heterogeneous nuclear RNA in the nucleus (hnRNPs) or fully processed mRNA in the cytoplasm (mRNPs) ([1,2], see [3]). Proteins forming mRNPs participate in every stage of mRNA biogenesis and mRNA translation, including pre-mRNA splicing, polyadenylation, nuclear-cytoplasmic transport, translation initiation and mRNA turnover. In addition, a number of specialised post-transcriptional functions have been ascribed to mRNPs, such as the storage of mRNAs in quiescent cells and the establishment of polarity in dividing cells by the asymmetric subcellular distribution of specific mRNAs [4].

Therefore a critical technical hurdle in the biochemical characterisation of any post-trancriptional process is the isolation and identification of the relevant RNPs. mRNPs were first isolated as proteins that co-sedimented in CsCl or sucrose gradients with non-polysomal mRNAs [3]. Related group of approaches is represented by the size fractionation of post-mitochondrial supernatant by centrifugation through sucrose cushion [5,6] and/or in linear sucrose or glycerol gradients [7] followed by oligo(dT) affinity chromatography [8]. Amongst the first mRNPs to be identified, poly (A)-binding protein and p50, a member of *Y*-box family of transcription factors proteins, were isolated by above poly(T) or poly(U) sepharose chromatography (see [8,9]). Other general RNPs have been co-purified with mRNA following in vivo or in vitro photochemical cross-linking (reviewed by [10]). However, gene specific RNPs have only been identified by reconstituting RNA–protein interactions in vitro on syntheic mRNAs. These synthetic mRNAs are either fixed to a solid support [11] or in someway tagged to facilitate their rapid removal from solution [12,13]. An example of the latter was the iron responsive element (IRE) binding protein, which was purified from a human liver cell

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lysates with a biotinylated IRE RNA, using free avidin and biotin agarose beads [13]. Recently, a more efficient RNP purification scheme has been developed which uses a hybrid synthetic RNA containing the target RNA sequence and a streptomycin-binding aptamer sequence [12]. Proteins complexed with this hybrid RNA are purified from cell-free extracts using streptomycin-conjugated sepharose beads. In addition to affinity purification, sequence-specific RNA-binding proteins have been identified from expression library screens using in vitro or in vivo RNA–protein interaction assays, such as north-western blotting [14] or the yeast three hybrid system [15].

This paper describes an alternative method for the isolation of proteins comprising native non-cross-linked mRNP particles from crude plant cell extracts, using biotinylated oligodeoxyribonucleotide probes and streptavidin-coated paramagnetic beads. The biotinylated oligonucleotide is first annealed to the target mRNP, and the resulting complex is purified from solution with streptavidin-coated paramagnetic beads. General mRNP particles were isolated from tobacco pollen cell extracts using an oligo(dT) probe, whereas the possibility of purification of transcript-specific mRNPs was tested with the probe complementary to *ntp303* transcript. *ntp303* is a pollen-specific gene encoding for pollen tube wall-specific glycoprotein with apparent M_w 69 kDa [16]. This gene is transcribed but not translated during pollen maturation. Highly abundant *ntp303* mRNA is stored in immature pollen and it is used for massive translation after germination [17].

2. Material and methods

2.1. Plant material

Flower buds were collected from *Nicotiana tabacum* L. cv. Samsun plants grown under greenhouse conditions. Immature pollen grains at late bicellular stage of development (stage 5, flower bud lengths 48–51 mm) were isolated from anthers according to Ref. [18].

2.2. Isolation of mRNPs

Tobacco pollen grains (100 mg) were ground in liquid nitrogen with 1 ml of incubation buffer (100 mM Tris–HCl, pH 7.8, 400 mM NaCl, 30 mM Mg–acetate, 2 mM DTT, 0.5 mM PMSF, 1 mM cycloheximide, 250 mM sucrose) in a sterile mortar with a pestle. The homogenate was transferred into 1.5 ml eppendorf tube and incubated on ice for 15 min. The post-mitochondrial supernatant was obtained by two consecutive centrifugations at 20 000 × g, 4 °C for 12 min.

Two custom synthesised (Integrated DNA Technologies, Coralville, I A) 5′-biotinylated oligodeoxyribonucleotide probes were used for the isolation of mRNP complexes: oligo(*ntp303*), 5′-ATAGTGAGCTTGCTTGGGTCGGCCG-3′ and oligo$(dT)_{25}$. The biotinylated probe (50 pmol) was mixed with the post-mitochondrial supernatant and incubated on ice for 5 min to hybridise with the target mRNPs. Streptavidin-MagneSphere particles (0.6 ml) (SA-PMP; Promega Corp., Madison, WI) resuspended in incubation buffer were used to pull down the biotinylated probes with associated mRNP particles according to the manufacturer's instructions. The bound RNPs were washed with washing buffer 1 (WB1; 10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 20 mM Mg–acetate) and washing buffer 2 (WB2; 10 mM Tris–HCl, pH 7.8, 500 mM NaCl, 20 mM Mg–acetate). The RNA-binding proteins were released by boiling the streptavidin particles in SDS sample buffer (2% SDS, 50 mM Tris–HCl, pH 6.8, 5% β-mercaptoethanol, 10% glycerol) for 2 min, and stored at − 80 °C. Alternatively, mRNPs were released under non-denaturing conditions by incubating the mRNP–streptavidin complexes in protein elution buffer (PEB; 20 mM Tris–HCl, pH 7.8, 2 M LiCl, 10 mM EDTA) at either room temperature or 37 °C for 5 min. Eluted proteins were mixed with an equal volume of 2 × STM buffer (4% SDS, 100 mM Tris–HCl, pH 6.8, 10% β-mercaptoethanol, 20% glycerol), denatured by boiling for 2 min and stored at − 80 °C.

2.3. In vitro translation

Two micrograms of mRNA was translated under in vitro conditions using the rabbit reticulocyte lysate system (Promega corp.) in the presence of $[^{35}S]$methionine according to the manufacturer's instructions. mRNAs isolated with both oligo(dT) and oligo(*ntp303*) probes and mRNA purified from pulled ribonucleoproteins were used as a template. Control *ntp303* mRNA was transcribed in vitro on XhoI-cleaved pBluescript vector with sub-cloned *ntp303* cDNA.

2.4. Protein analysis

Proteins were separated by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) through 12.5% gels (12.5% T, 2.7% C for the resolving gel and 3% T, 2.7% C for the stacking gel) according to Ref. [19]. Proteins (10–30 μg) were loaded per lane and visualised by silver staining [20]. For the immunodetection of eIF4E and nuclear-encoded mitochondrial core protein 2 of the cytochrome c reductase complex, protein samples were fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Immobilon P, Millipore) by semi-dry blotting [21]. Membranes were blocked 1 h in TBS-Tween containing

5% skimmed milk and probed with the polyclonal antibody against eIF4E (Combe and Twell unpublished; 1/2000) and the monoclonal antibody against core protein 2 (1/500) for 2 h at room temperature. The blots were washed three times with TBS-Tween and subsequently incubated with 1/8000 dilution of anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Sigma) for 1 hour at room temperature. After three washes in TBS-Tween, the staining pattern of the secondary antibodies was visualised according to the manufacturer's instructions. The anti-eIF4E antibody was kindly given by Dr J. Combe from University of Leicester, UK. The antibody against core protein 2 was kindly provided by Dr H.-P. Braun from University of Hannover, Germany.

After in vitro translation, [^{35}S]methionine-labeled proteins corresponding to 100 000 dpm were loaded per lane. Radioactive proteins on the gel were visualised by fluorography [22].

3. Results and discussion

This work describes a method for the one-step isolation of native gene-specific messenger ribonucleoprotein particles. Several reports have described the isolation of general mRNPs by poly-T and poly-U chromatography [8]. However, sequence-specific RNA-binding proteins have only been purified using synthetic mRNAs that are tagged to facilitate removal from solution. In particular, biotinylated mRNAs have been used to pull-down RNA-binding proteins with streptavidin-coated paramagnetic beads [23]. In this paper, streptavidin-coated paramagnetic beads have been utilised to purify native mRNPs, using a biotinylated oligodeoxyribonucleotide complemetary to the target mRNP.

This method was tested on tobacco pollen cell extracts with two biotinylated oligonucleotide probes, including a 25 mer oligo(dT) probe for the isolation of general mRNPs and an oligonucleotide complementary to the pollen-specific *ntp303* transcript. The high level expression of NTP303 in pollen (ca. 0.1% of total poly(A)$^+$ RNA) [16] enables the isolation of detectable quantities of *ntp303* mRNPs. NTP303 serves as a convenient model to study RNA–protein interactions involved in the post-transcriptional regulation, as the *ntp303* transcript is translationally repressed during pollen development but is efficiently translated after pollen tube germination [17]. The *ntp303*-specific oligonucleotide, called oligo(*ntp303*), complemented a region of the *ntp303* transcript (nucleotides 1371-1395 of the *ntp303* cDNA), that was predicted by the mFold algorithm [24,25] to be devoid of RNA secondary structure.

Oligo(dT) and oligo(*ntp303*) were first annealed to mRNPs within a post-mitochondrial supernatant of a cell extract of pollen at the late-bicellular stage of development [26], and then bound to paramagnetic particles via streptavidin (Section 2 and Fig. 1). Non-specifically bound proteins were removed by washing at two salt concentrations, and the remaining RNPs were released and analysed by SDS-PAGE (Fig. 2). The

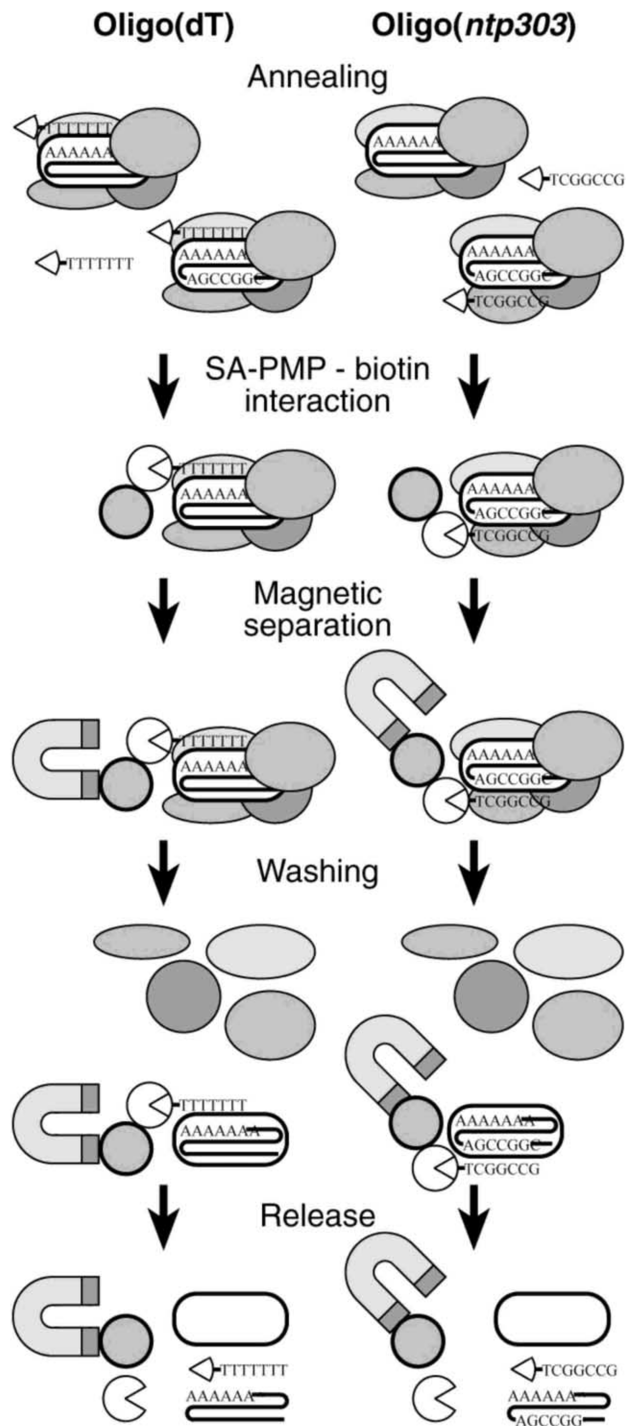

Fig. 1. Schematic representation of magnetic purification method. The isolation steps of total (Oligo(dT)) and *ntp303* (Oligo(*ntp303*)) mRNPs are explained in Section 2.

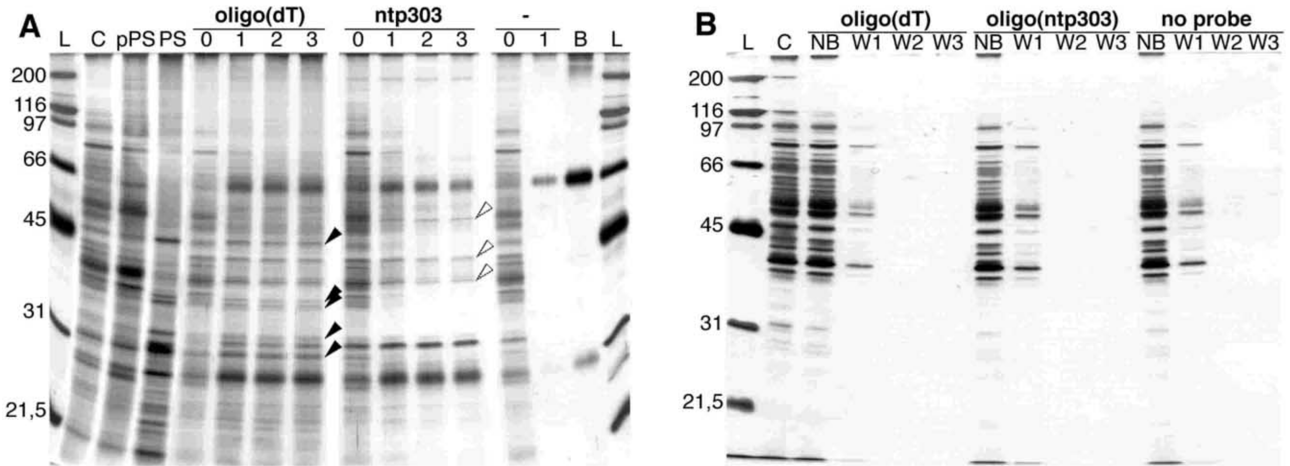

Fig. 2. Magnetic purification of total and *ntp303* mRNPs. (A) The profile of proteins present in mRNPs purified from immature tobacco pollen with oligo(dT) and oligo(*ntp303*) probes, under different wash conditions; without washing (0), washed once with WB1 (1), once with both WB1 and WB2 (2) and once with WB1 and twice with WB2 (3). The protein profiles are compared against total cytosolic proteins (C), post-polysomal (pPS) and polysomal fractions (PS) isolated by sucrose step-gradient centrifugation [23] and a mock mRNP purification carried out in the absence of a biotinylated oligonucleotide (–). The polypeptides released from the streptavidin-conjugated paramagnetic beads are also shown (B). Solid arrows denote shared polypeptide bands between the oligo(dT) and the polysomal protein fractions and open arrows, similarities between the oligo(*ntp303*) and post-polysomal protein banding patterns. (B) Analysis of the oligo(dT), oligo(*ntp303*) and the mock mRNP purification wash fractions collected under different washing conditions; one wash with WB1 (W1), one wash with WB1 and WB2 (W2), and one wash with WB1 and two washes with WB2 (W3). The wash fractions are compared against total cytosolic protein (C) and unbound protein fractions (NB).

profile of proteins isolated with oligo(dT) and oligo(*ntp303*) probes were compared against polysomal and post-polysomal fractions [27]. Proteins released from the streptavidin beads in the absence of a biotinylated oligonucleotide, with and without pollen cell extract pre-incubation, were used as negative controls.

Set of proteins associated with SA-PMP before the first wash was very similar to that of cytosolic proteins, regardless of oligodeoxynucleotide probe used (Fig. 2A). Non-specifically bound proteins were completely removed after two washes with washing buffer 1 (WB1), leaving quantitative and qualitative differences in the proteins retained by the oligo(dT) and oligo(*ntp303*). These data are well documented on Fig. 2B, where at all cases only the first wash contained a detectable amount of proteins.

Consistent with the isolation of actively translating mRNPs, oligo(dT) retained five polypeptides (solid arrows) that co-migrated with the major polypeptides in the polysomal fraction. In contrast, these major bands were absent in the oligo(*ntp303*)-bound fraction. The *ntp303* oligonucleotide retained a 50 kDa polypetide that co-migrated with a major non-polysomal mRNP (open arrow) and was absent from the oligo(dT) and polysomal fractions. A 33 kDa polypeptide was enriched by oligo(*ntp303*) compared to oligo(dT). Two most abundant polypeptides (ca. 66 kDa and 30 kDa) eluted from oligo(dT) and oligo(*ntp303*) were also present in beads-only negative control and probably represented intact streptavidin and a proteolytic fragment of streptavidin [28]. The qualitative differences in the oligo(dT) and oligo(*ntp303*) protein profiles is consistent with general mRNPs being purified with oligo(dT) and translationally repressed ntp303-specific mRNPs being purified with the oligo(*ntp303*).

The RNA content of isolated mRNPs was analysed by in vitro translation in order to confirm the specifity of the purification protocol used (Fig. 3). There was no significant difference between populations of mRNA isolated with oligo(dT) probe and mRNA purified from general mRNPs. Compared to control *ntp303* mRNA, samples containing mRNA isolated with oligo(*ntp303*) probe and mRNA from pulled *ntp303* mRNPs were slightly contaminated with other cytoplasmic mRNAs. However, both samples were highly enriched with *ntp303* mRNA. Also in the sample containing mRNA obtained from the first wash the band corresponding to translational product of *ntp303* gene was reduced significantly documenting transcript-specific association of *ntp303* mRNPs with streptavidin particles via oligo(*ntp303*) probes. The presence of *ntp303* transcript within mRNP population isolated with oligo(*ntp303*) probe was confirmed also by northern hybridisation (data not shown).

To confirm that mRNPs are free of general cytosolic proteins, they were pulled down by streptavidin particles, protein fractions bound to both oligonucleotides were separated by SDS-PAGE and electroblotted onto PVDF membrane and decorated with antibodies specific for eIF4E, a general mRNA-binding protein that binds the 5′-terminal m^7GTP cap structure of all cellular mRNAs [29]. A duplicate blot probed with nuclear-

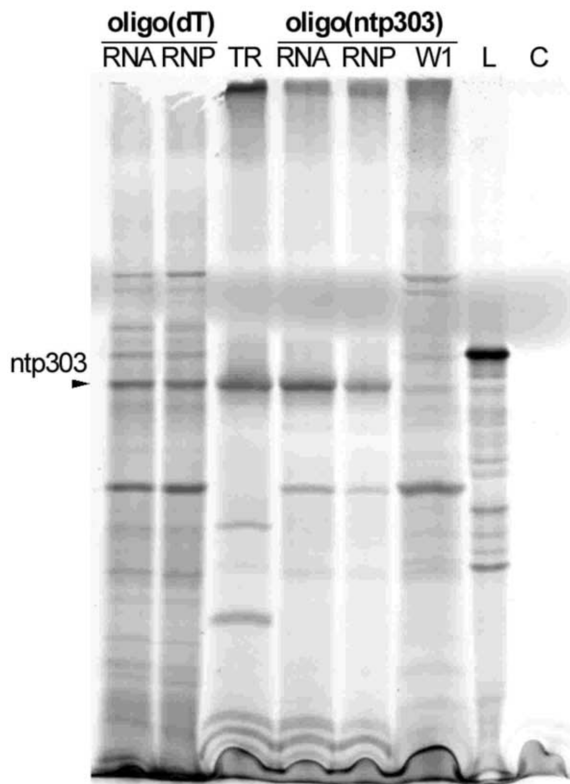

Fig. 3. SDS-PAGE of [^{35}S]methionine-labeled in vitro translation products synthesized on mRNAs isolated with oligo(dT), oligo(*ntp303*) oligodeoxynucleotide probes. mRNAs isolated with both oligoprobes (RNA) and mRNA purified from pulled ribonucleoproteins (RNP) were used as a template and they were compared to in vitro transcribed *ntp303* mRNA (TR), mRNAs washed out from *ntp303* mRNPs during the first wash (W1), control luciferase mRNA (L) and negative control containing no exogenous RNA (C). The position of 58-kDa native non-glycosylated form of p69, the translational product of *ntp303* gene is marked by solid arrowhead.

encoded mitochondrial cytochrome c reductase core protein 2 was used as a negative control (Fig. 4). eIF4E but not core protein 2 was detected in both the oligo(dT) and oligo(*ntp303*)-bound protein fractions, confirming that both oligonucleotides were specifically pulling down mRNPs..

To optimise oligonuclotide-mediated mRNP purification, the effects of pH, cation concentration and oligonucleotide-mRNP hybridisation time were investigated. The profile of proteins bound to oligo(dT) and oligo(*ntp303*) were not affected by substituting sodium ions for potassium in incubation buffer (Fig. 5A). A reduction of both sodium and potassium ion concentration in the incubation buffer, from 400 to 100 mM, did not significantly affect the profile of mRNPs bound to the two oligonucleotides (Fig. 5A). However, an increase in incubation buffer pH from 8.5 to 9.0 selectively reduced the binding efficiency of the 33 kDa polypeptide to both oligo(dT) and oligo(*ntp303*) (Fig. 5B). A hybridisation time of 5 min was sufficient to capture all major mRNPs pulled down by the two oligonucleotides. Its extension up to 30 min did not increase neither the quantity nor the quality of the isolation (data not shown).

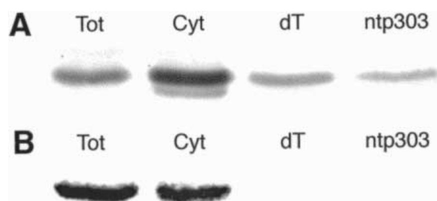

Fig. 4. Immunodetection of cap-binding protein eIF4E in oligo(dT) and oligo(*ntp303*) mRNP fractions. The distribution of eIF4E (A) and nuclear-encoded mitochondrial cytochrome c reductase core protein 2 (B) in total protein (Tot), cytoplasmic protein (Cyt), and oligo(dT) and oligo(*ntp303*) protein fractions isolated from stage 5 of immature pollen.

The protein components of captured mRNPs were released under denaturing conditions by boiling of SA-PMP with attached ribonucleoproteins in SDS sample buffer. This approach resulted in the best resolution on polyacrylamide gels. Proteins released this way are to be used for any analysis starting with SDS-PAGE, as western blot immunodetection or northwestern blot immunodetection with RNA motif of interest. Alternative non-denaturing elution buffers were tested in order to isolate native mRNPs with biological activity. Incubating the mRNP–streptavidin complexes in 10 mM Tris–HCl, pH 7.6, 2 mM EDTA at 37 °C for 5 min [8] did not release mRNPs from the oligonucleotides (data not shown). It has showed the limitation of this magnetic isolation method in small reaction volumes in bringing to ballance requirements of high salt concentrations for stabilisation of RNA–protein interactions and low salt concentration and elevated temperature for disruption of RNA/DNA hybrid. However, the major mRNPs were released from both oligo(dT) and oligo(*ntp303*) with 20 mM Tris–HCl, pH 7.8, 2 M LiCl, 10 mM EDTA (protein-elution buffer, PEB) at either room temperature or 37 °C (Fig. 6).

Described experimental approach revealed several proteins comprising tobacco pollen both general and *ntp303* mRNPs (Fig. 2), characterisation of which represents next important step but it lies behind the scope of this methodical paper. To date, many articles describing individual proteins present in messenger ribonucleoprotein particles have been published. Most data have been obtained from animal and yeast systems. Proteins identified belong among RNA-binding proteins and they are involved for example in translational repression (mRNP3 + 4) [30], cytoplasmic polyadenylation (CPEB, ElrA) [31], mRNA transport (mrnp41) [32] or mRNP linkage to microtubules (TB-RNP) [33] or actin microfilaments (p50) [8]. However, positive results concerning the protein composition and structure of mRNPs are missing. In plants, the only

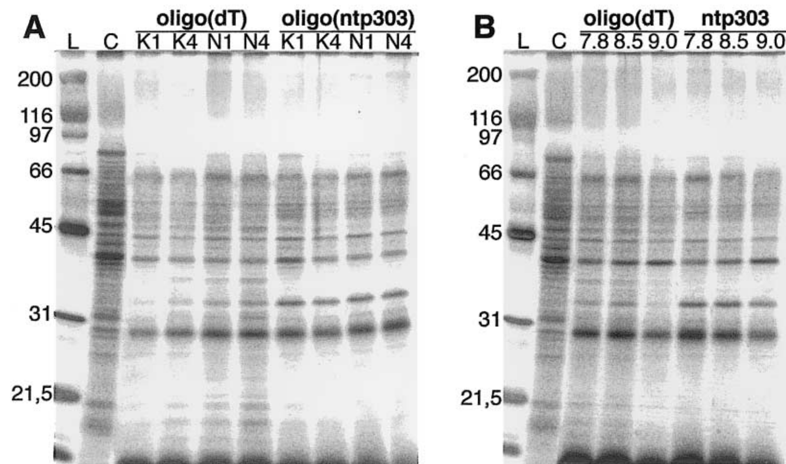

Fig. 5. The influence of incubation buffer pH and cation concentration on mRNP purification efficiency. (A) The effect of incubation buffer cation concentration on oligo(dT) and oligo(*ntp303*) mRNP isolation. mRNPs were purified with oligo(dT) and oligo(*ntp303*) with either 400 mM NaCl (N4), 100 mM NaCl (N1), 400 mM KCl (K4) or 100 mM KCl (K1). (B) The effect of incubation buffer pH on oligo(dT) and oligo(*ntp303*) mRNP isolation. mRNPs were hybridised to oligo(dT) and oligo(*ntp303*) at a pH of 7.8, 8.5 or 9.0.

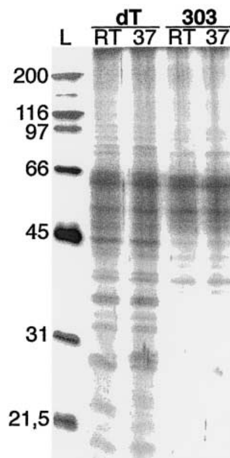

Fig. 6. Elution of mRNPs under non-denaturing conditions. Proteins bound to mRNA were released under non-denaturing conditions by incubating the paramagnetic beads in PEB (20 mM Tris–HCl, pH 7.8, 2 M LiCl, 10 mM EDTA) at room temperature (RT) or at 37 °C.

reliably characterised cytoplasmic RNP proteins are *Chlamydomonas reinhardtii* chloroplast 47 kDa RB47 interacting with poly(A) tail [34] and the multifunctional 72 kDa poly(A) binding protein, PABP (see [35]), pollen-specific homologue of which has been also cloned in *Arabidopsis* [36].

In conclusion, the method described in this article represents a novel and versatile approach for the isolation of specific messenger ribonucleoprotein complexes. In contrast to existing in vitro methods, which involved reconstituting RNA–protein complexes on synthetic mRNAs, this method retrieves native gene-specific mRNPs. The isolation of proteins present in native mRNPs by this method provides a complementary approach for the identification of RNA–protein interactions.

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