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Nucleophosmin is selectively deposited on mRNA during polyadenylation

Viswanathan Palaniswamy, Karen C M Moraes, Carol J Wilusz, and Jeffrey Wilusz

Department of Microbiology, Immunology & Pathology, Colorado State University, 1619 Campus Delivery, Fort Collins, Colorado 80523, USA

Abstract

Nucleophosmin (NPM), an abundant, predominantly nucleolar protein that influences numerous cellular processes, was shown to specifically associate with the bodies of messenger RNAs as a result of the process of 3'-end formation. NPM deposition requires polyadenylation but not the 3' cleavage event to occur on the transcript. Furthermore, the protein does not associate with RNAs bearing a preformed poly(A) tail or with mRNAs that have undergone cleavage but not polyadenylation. A region within 10 bases upstream of the AAUAAA element is required for NPM association, but deposition of the protein seems to be sequence independent. NPM association with poly(A)⁺ mRNAs was also demonstrated *in vivo*. NPM, therefore, represents a mark left on transcripts as a result of 3'-end processing and may have a role in one or more of a variety of post-transcriptional processes influenced by the polyadenylation event.

Over the last few years, it has become clear that the individual steps of gene expression, from transcription through mRNA processing and export to localization, translation and mRNA decay, are intimately connected with each other to allow efficient regulation as well as surveillance of transcript quality¹. In both yeast and higher eukaryotes, processing of the nascent mRNA occurs cotranscriptionally and the factors required for each of the three steps—capping, splicing and polyadenylation—are recruited by the C-terminal domain of the large subunit of RNA polymerase II². In addition to being dependent on transcription, the capping, splicing and polyadenylation reactions can also influence one another^{3,4} as well as aspects of the transcription process itself⁵. Finally, processing of the mRNA is necessary for it to be functional in downstream processes, specifically export to the cytoplasm and translation^{6,7}. Thus, cotranscriptional RNA processing and messenger ribonucleoprotein particle (mRNP) biogenesis are essential steps in gene expression.

The mechanisms by which splicing influences gene expression in mammalian cells are relatively well understood. During the splicing process, a large complex of proteins, the exon junction complex (EJC), is deposited on the mRNA 20–25 nucleotides (nt) upstream of each exon-exon junction. The EJC is anchored by eIF4AIII and contains factors that mediate mRNA export (Ref (also called Aly), Y14 and Magoh) as well as splicing factors (RNPS1, SRM160 and UAP56) and factors involved in nonsense-mediated mRNA decay and translation (Upf1, Upf2 and Upf3)^{8,9}. The presence of the EJC seems to enhance 3'-end formation, as tethering

Correspondence should be addressed to J.W. (jeffrey.wilusz@colostate.edu).

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of the RNPS1 component results in increased efficiency of this process¹⁰. The EJC is exported along with the spliced mRNA and has been shown to influence both translation efficiency and nonsense-mediated decay in the cytoplasm^{10–12}. In contrast, although it is clear that the poly(A) tail enhances translation initiation¹³ and there is evidence that 3'-end formation is important for mRNA export¹⁴, understanding of the coupling between 3'-end formation and downstream processes is less mature. A simple hypothesis is that the poly(A) tail itself acts in a similar way to the EJC to mark an mRNA as competent for export and translation. However, observations using a reporter mRNA whose 3' end is generated by ribozyme cleavage rather than by normal cleavage and polyadenylation suggest that the situation is more complex¹⁵. This unadenylated mRNA is, as may have been predicted, very poorly exported from the nucleus. Notably, when a template-encoded poly(A) tail is inserted upstream of the site of cleavage, the mRNA is still retained in the nucleus. This result indicates that the mere presence of a poly(A) tail is not sufficient for mRNA maturation. Instead, the mRNA has to actually experience the polyadenylation process to acquire the correct mRNP structure for export. This led us to investigate whether 3'-end formation, like splicing, deposits a protein mark on the mRNA that can influence downstream processes.

In higher eukaryotes, the process of 3'-end formation is well characterized and has been reconstituted *in vitro*¹⁶. Mammalian 3'-end formation requires poly(A) polymerase as well as four complexes of proteins: cleavage polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF) and cleavage factors I and II. CPSF specifically binds the canonical AAUAAA signal upstream of the cleavage site¹⁷, whereas CstF interacts with U-rich sequences downstream¹⁸. Together, these factors recruit the cleavage factors and specify the site of cleavage¹⁹. After the cleavage event, CPSF promotes poly(A) addition by poly(A) polymerase. The poly(A) tail is generally around 200 nt in length and is complexed with nuclear poly(A)-binding protein in the nucleus or cytoplasmic poly(A)-binding protein in the cytoplasm²⁰. None of these factors, however, seems a likely candidate for a polyadenylation mark, as they either do not remain bound to the mRNA after the polyadenylation process is complete or do not specifically require the process of 3'-end formation to occur in order to associate with the mRNA.

We therefore developed a strategy using HeLa cell nuclear extracts to visualize proteins that bind RNA substrates only when they have undergone 3'-end formation. In this manner, we identified human NPM, an abundant nucleolar shuttling protein, as a candidate polyadenylation mark. Our results suggest that NPM binds the body of the mRNA upstream of the AAUAAA poly(A) signal and that binding requires the process of poly(A) tail addition but not cleavage. We find that NPM is associated with poly(A)⁺ mRNAs in living cells, consistent with our *in vitro* observations. To our knowledge, NPM is the first protein shown to be specifically deposited on the mRNA body during the process of poly(A) tail addition.

Results

A protein mark left by the process of 3'-end formation

To observe proteins binding the mRNA body in a polyadenylation-dependent manner, we took advantage of the well-characterized *in vitro* assay that has been routinely used in the study of 3'-end formation^{16,21}. The SVL RNA substrate, derived from the SV40 late poly(A) signal, was radiolabeled at U residues and incubated in HeLa nuclear extracts. At designated time points, samples were analyzed both for polyadenylation progress and for proteins that were associated with the body of the transcript. As expected, during the course of the experiment the SVL RNA was cleaved and polyadenylated (Fig. 1, SVL lanes, top gel). At each time point, a sample of the reaction was exposed to UV light and the cross-linked proteins were separated by SDS-PAGE. Several proteins were cross-linked to the RNA body (Fig. 1, SVL lanes, bottom

gel), and of these, two (at ~32 kDa and ~40 kDa) appeared to have increased cross-linking at later time points. These two proteins, therefore, could be candidate polyadenylation marks.

We next wanted to determine whether binding of these proteins was specific for the SV40 late poly(A) signal or reflected a more general event that resulted from 3'-end processing. We therefore used two additional independent polyadenylation substrate RNAs derived from adenovirus type 5 precursor mRNAs (pre-mRNAs), E1B and IVA2. Both of these RNA substrates underwent efficient cleavage and polyadenylation in the HeLa nuclear extracts (Fig. 1, lanes E1B and IVA2, top gels). UV cross-linking analysis of the same reaction (Fig. 1, bottom gels) revealed that a 32-kDa protein was also associated with E1B and IVA2 substrates at later time points, when appreciable polyadenylation had occurred. The 40-kDa protein did not bind the E1B or IVA2 substrates and thus seemed to be specific for the SVL RNA. The 32-kDa protein, therefore, has the key property that we would predict for a polyadenylation mark: it binds the body of multiple RNA substrates apparently only after they have undergone proper 3'-end processing.

To identify the 32-kDa protein, we performed large-scale polyadenylation reactions similar to those described above but using a biotinylated SVL RNA substrate in an affinity-purification approach. After incubation with HeLa nuclear extract, the biotinylated RNA was recovered, along with any associated proteins, by binding to streptavidin agarose. The proteins were then separated by SDS-PAGE and visualized by silver-staining. We performed the procedure using either extract alone, untreated extract containing the RNA substrate or extract containing substrate that had been incubated for 60 min to allow polyadenylation to occur. Several proteins were purified by this procedure (Fig. 2a), and a 32-kDa protein appeared to bind specifically to the RNA substrate that had undergone polyadenylation. This band was excised and subjected to MS. Eleven peptides were identified that all matched NPM (also called B23) (Fig. 2b), an abundant nucleolar shuttling protein with a molecular weight of 32 kDa (ref. 22).

To confirm that NPM was indeed the 32-kDa protein of interest, we performed the UV cross-linking analysis with radiolabeled SVL RNA, as in Figure 1, and then used an antibody to NPM to immunoprecipitate cross-linked NPM protein from the extract. The NPM antibody specifically precipitated a 32-kDa cross-linked band from the sample that had undergone polyadenylation but not from the sample taken before incubation at 30 °C (Fig. 2c). Control serum from normal mice did not precipitate any cross-linked material (data not shown). We therefore conclude that the 32-kDa band that associates with the RNA body as a result of 3'-end processing is NPM.

An artificial poly(A) tail cannot induce NPM binding

To investigate in more detail the requirements for NPM binding, we performed several experiments with other substrate RNAs and under different conditions. First, we determined whether NPM bound an RNA substrate with a point mutation in the AAUAAA polyadenylation signal²³. The AAUACA mutant was a poor substrate for polyadenylation and, as predicted, was unable to bind NPM (Fig. 3a); cross-linked NPM was immunoprecipitated using the wild-type substrate but not the AAUACA mutant RNA.

We next wished to determine whether the actual process of 3'-end formation was required for NPM binding or whether simply the presence of a poly(A) tail on the RNA substrate was sufficient. To distinguish between these possibilities, we compared the UV cross-linking profiles in HeLa nuclear extracts of the SVL RNA substrate and an SVL derivative to which a ~150- to 200-nt poly(A) tail had been added artificially before the assay (SV-A₂₀₀). As before, upon incubation in the HeLa nuclear extract, the SVL RNA substrate became polyadenylated and concomitantly bound NPM (Fig. 3b, SVL lanes). In contrast, the pre-polyadenylated substrate RNA, SVL-A₂₀₀, did not change its polyadenylation status upon incubation in HeLa

nuclear extract and also did not bind NPM (Fig. 3b, SV-A₂₀₀ lanes). This result demonstrates that the presence of a poly(A) tail is not sufficient to recruit NPM to the RNA body, indicating that the cleavage or polyadenylation step of 3'-end processing, or both, are essential for NPM binding to the RNA.

We tested whether the cleavage reaction was sufficient for NPM binding by replacing the ATP and phosphocreatine in the reaction with $\alpha\beta$ -methylene ATP (a nonhydrolyzable ATP analog) and EDTA. Under these conditions, the substrate RNA undergoes cleavage but polyadenylation does not occur²⁴. Although NPM bound efficiently to the RNA substrate in a standard polyadenylation reaction, there was no NPM associated with the RNA when poly(A) addition was blocked (Fig. 3c).

We next wished to determine whether both aspects of 3'-end processing—cleavage and poly (A) addition—were necessary for NPM binding to the body of the polyadenylated product. To examine whether NPM binding requires the RNA to undergo cleavage, we incubated a precleaved SVL RNA substrate in HeLa nuclear extract. Precleaved RNA substrates undergo efficient polyadenylation in HeLa nuclear extracts¹⁶. The pre-cleaved SVL RNA was polyadenylated efficiently and also bound NPM after polyadenylation (Fig. 4).

We tested whether NPM was also bound to the unadenylated population of the precleaved substrate RNA after incubation, using oligo(dT) selection after UV cross-linking. The cross-linked NPM was seen at 60 min only in the bound fraction and not in the unbound population of proteins associated with poly(A)⁻ RNA (Fig. 4b). Together, the above experiments show that the process of polyadenylation, but not 3'-end cleavage, is required for NPM deposition on RNA substrates.

NPM deposition requires sequences upstream of AAUAAA

Thus far, the data demonstrate that the process of poly(A) addition results in deposition of NPM on the mRNA body. As three unrelated RNA substrates, SVL, E1B and IVA2, all bind NPM upon polyadenylation, the NPM-binding site could be the conserved canonical poly(A) signal (AAUAAA). Alternatively, binding of NPM may be sequence independent. To investigate the binding requirements of NPM, we tested two shortened versions of the SVL substrate for their ability to bind. The first substrate, SVL-AAUAAA, essentially represents a minimal polyadenylation substrate and consists of the AAUAAA plus 6 bases downstream, terminating at the normal cleavage site (Fig. 5a)²⁵. This substrate was polyadenylated efficiently *in vitro* but did not associate with NPM (Fig. 5b). This result suggests that NPM interacts not with the AAUAAA element but more probably with sequences upstream. We therefore used a slightly longer substrate, SVL+10, identical to SVL-AAUAAA RNA but containing an extra 10 nt upstream of the AAUAAA (Fig. 5a). This substrate was also polyadenylated efficiently, but in contrast to the SVL-AAUAAA substrate, SVL+10 RNA bound NPM with a similar efficiency to the full-length SVL substrate (Fig. 5b). We therefore conclude that NPM probably binds the SVL RNA substrate within the 10-nt region upstream of the AAUAAA. This region is not particularly conserved between SVL and the other two polyadenylation substrate RNAs (E1B and IVA2) tested. We therefore made analogous short RNA substrates from IVA2 and E1B substrates (E1B+10 and IVA2+10) as well as an SVL substrate with 10 nt of randomized sequence upstream (SVL+10_{rand}). All four of these short substrates were polyadenylated efficiently and also bound NPM (Fig. 5b). It is noteworthy that the only uridine residue in the IVA2 substrate lies just 2 bases upstream of the AAUAAA signal. As the substrate RNAs were labeled with [α -³²P]UTP and UV cross-linking detects only short-range interactions, it seems likely that NPM is deposited very close to the AAUAAA, at least in this instance. Overall, our results suggest that, similar to the binding of the EJC to splicing substrates⁸, binding of NPM is not sequence specific but rather may depend on distance from the site of cleavage or from the AAUAAA element.

NPM associates with poly(A)⁺ mRNAs *in vivo*

Finally, we wished to assess whether NPM could interact with polyadenylated mRNAs in living cells. To approach this question, we used an oligo(dT) pull-down assay. SVL RNA was incubated in HeLa nuclear extract for 60 min and polyadenylated products were isolated using an oligo(dT) column. Proteins associated with the RNA were then separated by SDS-PAGE and analyzed by western blotting. NPM association with the oligo(dT) column increased over 50-fold after polyadenylation of the SVL RNA *in vitro* (Fig. 6a).

Notably, however, even at time zero, before incubation, some NPM was associated with the oligo(dT) column. This suggests that NPM may be associated with endogenous poly(A)⁺ RNAs in the extract. To confirm this, HeLa nuclear extract was passed over an oligo(dT) column in the presence or absence of RNase. RNase treatment abolished retention of NPM on the column (Fig. 6b). These results strongly suggest that NPM associates with endogenous poly(A)⁺ RNAs *in vivo*. To confirm this, *in vivo* UV cross-linking was performed, followed by oligo(dT) chromatography under denaturing conditions. NPM was clearly cross-linked to poly(A)⁺ mRNAs in living cells (Fig. 6c). Although nuclear poly(A)⁺ RNAs did interact with NPM *in vivo*, we did not detect a large amount of cytoplasmic NPM associated with poly(A)⁺ mRNAs (Fig. 6c). Therefore, we conclude that NPM specifically associates with nuclear poly(A)⁺ mRNA as a result of the process of polyadenylation.

Discussion

In this study, we have identified NPM as a candidate polyadenylation mark. NPM meets several criteria that might be expected for such a mark: it becomes associated with the body of the RNA substrate as a result of the process of polyadenylation, its binding seems to be sequence independent, it shuttles between the nucleus and cytoplasm and it is found associated with poly(A)⁺ RNAs in living cells.

NPM is an essential protein that has been implicated in a wide range of cellular processes. Whereas it is highly abundant in transformed cells, it is present at much lower levels in normal tissue and shows a regulated pattern of expression²⁶. The observation that NPM is deposited on mRNAs as a result of polyadenylation may help explain some of the documented effects of NPM on a multitude of cellular processes. Although NPM was first implicated in ribosome biogenesis^{22,27}, it has since been associated with numerous other functions, including regulation of cell proliferation²⁸, gene expression²⁹, organogenesis and embryonic development³⁰. Proposed NPM functions include acting as a molecular chaperone³¹, nuclear import³², responses to UV damage³³ and regulation of apoptosis³⁴. It has been implicated in tumorigenesis by a variety of evidence, including translocations and mutations in hematological disorders³⁵, overexpression in multiple cancers²⁶ and modulation of the activity of tumor suppressors such as p53 (ref. 36). Notably, both NPM and the 50-kDa subunit of CstF are candidate substrates of the BRCA1–BARD1 ubiquitin ligase^{37,38}. Furthermore, BARD1 is associated with CstF-50 (ref. 38), and data exist that link 3'-end processing to responses to DNA damage^{39,40}. Therefore, our finding that NPM, a protein whose expression changes in response to DNA damage, is specifically deposited on mRNAs as a result of polyadenylation further strengthens the concept of networking between responses of the cell to damage and the 3'-end formation process.

NPM is a predominantly nucleolar protein; thus, our findings may also give credence to recent speculation that the nucleolus is involved in mRNA metabolism as well as being a center for ribosomal RNA processing and ribosome assembly. Studies of the nucleolar proteome have revealed that components of the RNA-processing machinery, including CstF50K and several splicing factors, are localized there^{41,42}. Moreover, components of the EJC and nonsense-mediated mRNA decay machinery have been isolated from the *Arabidopsis thaliana*

nucleolus⁴³. Together with our data suggesting that NPM is a mark for polyadenylation, these findings are consistent with a role for the nucleolus in mRNP assembly or transport. Indeed, some *Schizosaccharomyces pombe* mRNAs transiently associate with the nucleolus, and this may be important for either their export or quality control⁴⁴.

It is interesting to speculate as to the role of NPM binding to mRNAs. One hypothesis is that it acts to enhance the export and perhaps even early rounds of translation of mRNAs once their processing is complete. With regard to a role in mRNA export, NPM has been shown to interact specifically with the HIV-1 Rev protein^{45,46}, a factor that facilitates the export of unspliced viral RNAs. Notably, the Rev peptide, which specifically binds NPM, is cytotoxic and effectively inhibits tumor growth in nude mice⁴⁷. In addition, NPM has recently been shown to interact with the Ran–Crm1 complex⁴⁸. Collectively, these observations suggest that export-related activities could be a major part of the function of the NPM that is associated with poly(A)⁺ mRNA. We have not detected a large amount of NPM bound to poly(A)⁺ RNA in the cytoplasm, suggesting that any cytoplasmic role for the protein is likely to be transient. However, it remains formally possible that NPM association could enhance early rounds of translation. Finally, NPM is also a binding partner of nucleolin, an RNA-binding protein that binds AU-rich elements and that has been implicated in mRNA stabilization^{49,50}. Therefore, a potential role for NPM in mRNA decay should also be explored.

Another putative role for NPM could be in mediating the dissociation of polyadenylation factors from the mRNA after processing is complete. Such a function could be important both in facilitating export of mRNAs and in recycling the polyadenylation machinery. The presence of splicing factors on pre-mRNAs results in nuclear retention until the splicing process is complete and the splicing machinery dissociates^{51,52}. It is therefore possible that the presence of the polyadenylation machinery is inhibitory to export, in which case a factor such as NPM might be required to dissociate these proteins once the polyadenylation process is complete.

As NPM has been implicated as a chromatin-associated protein⁵³, it could also be involved in changes of chromatin structure to facilitate efficient transcription termination, a process that has been intimately linked to 3'-end processing⁵⁴. Perhaps NPM is recruited to (or from) the chromatin in the area of the DNA where 3'-end processing occurs. This has the potential to lead to changes in the DNA template that could contribute to creating the proper environment for termination of RNA polymerase II transcription.

Finally, as there is already substantial evidence for coupling of splicing and 3'-end formation⁵⁵, it is possible that binding of NPM may influence the splicing machinery, specifically splicing of the last exon. Association of NPM could represent a checkpoint for successful polyadenylation that permits splicing to proceed. Notably, NPM copurifies with a splicing factor, the EJC component RNPS1 (ref. 56), and a very recent study has found that a phosphorylated isoform of NPM is associated with splicing factors in nuclear speckles⁵⁷. This particular isoform of NPM can bind pre-mRNA *in vitro* and inhibit splicing. Thus, it will be interesting in the future to determine whether the NPM deposited during polyadenylation is phosphorylated and the way in which its binding influences the splicing process.

In conclusion, in this study we have identified NPM as a novel polyadenylation mark and characterized the requirements for its binding. The existence of this polyadenylation mark suggests a similarity and perhaps a new connection between the processes of splicing and 3'-end formation and introduces the idea that there is yet another checkpoint embedded within the mRNA-processing pathway.

Methods

Plasmids and RNAs

pSVL contains a 241-base-pair (bp) BamHI-BclI fragment containing the SV40 late polyadenylation signal inserted into the BamHI site of pSP65. Transcription of DraI-linearized templates driven by the SP6 promoter yielded a 224-base RNA (SVL). Pre-cleaved SVL RNA was generated by transcription of pSVL that had been linearized with HpaI. pE1B contains a 180-bp fragment of adenovirus type 5 (which contains the polyadenylation signal of the E1B gene) cloned into pGem4. SP6 transcription of a XbaI-linearized template yielded a 224-base RNA (E1B). pIVA2 contains the 155-bp BamHI-PvuII fragment of adenovirus pE1B (which contains the IVA2 polyadenylation signal) cloned into pGem4 at the HincII and BamHI sites. SP6 transcription of BglI-linearized template yielded a 158-base RNA (IVA2). The wild-type and mutant RNAs used in Figure 3a were transcribed with SP6 from plasmids pSVSPL:AATAAA and pSVSPL:AATACA linearized with HindIII. These plasmids were described previously²³. The template for SVL-AAUAAA was generated using the oligonucleotide 5'-CATACGATTTAGGTGACACTA TAGAATAAACAAGTT-3' and its complement. The templates for SVL+10, SVL+10_{rand}, E1B+10 and IVA2+10 were generated using the following oligonucleotides and their complements (polyadenylation signal is underlined): SVL+10, 5'-CATACGATTTAGGTGACACTATAgaaTATAAGCTGCAATAAAACAAGTT-3'; SVL+10_{rand}, 5'-CATACGATTTAGGTGACACTATAgaaACTATGTG ATAATAAAACAAGTT-3'; E1B+10, 5'-CATACGATTTAGGTGACACTATAgaaTAAAAACATAAAATAAAAAACCA-3'; IVA2+10, 5'-CATACGATTTAGGTGACACTATAgaaAAAACCCCTAAATAAAGACAGC-3'. In each construct, GAA (lowercase italic) was inserted downstream of the SP6 promoter sequence (uppercase italic) to increase transcription efficiency; these three nucleotides were retained in the transcripts tested (Fig. 5).

To generate SV-A₂₀₀ RNA, pre-cleaved SVL RNA was incubated with yeast poly(A) polymerase at 37 °C for 10 min and the reaction terminated by heating at 65 °C for 10 min. Reaction products were separated on a 6% (w/v) polyacrylamide gel containing 7 M urea. Poly(A) tail length was determined by calibration with RNA decade markers (Ambion), and RNAs with a 150- to 200-base poly(A) tail were excised and eluted.

In vitro transcription reactions were performed in the presence of [³²P]UTP and m⁷GpppG as described previously²¹. All RNAs were gel-purified before use. To prepare biotinylated UTP, bio-UTP (Roche) was substituted for [³²P]UTP.

In vitro polyadenylation reactions

Nuclear extracts from HeLa spinner cells were prepared by the method of ref. 58 and *in vitro* polyadenylation reactions were performed as previously described^{16,21}. A typical reaction contained 3% (w/v) polyvinyl alcohol, 1 mM ATP, 20 mM phosphocreatine, 12 mM HEPES (pH 7.9), 12% (v/v) glycerol, 60 mM KCl, 0.12 mM EDTA, 0.3 mM DTT and 60% (v/v) nuclear extract. Cleavage reactions were performed under the same conditions, except phosphocreatine and ATP were replaced with 1mM αβ-methylene ATP and 1 mM EDTA²⁴. Reactions were incubated at 30 °C and products were analyzed on 6% (w/v) acrylamide gels containing 7 M urea.

UV cross-linking and immunoprecipitation of RNA-binding proteins

In a typical reaction, 50–200 fmol of ³²P-labeled RNA was incubated in the *in vitro* polyadenylation system for the time indicated. Reaction mixtures were irradiated for 10 min at 4 °C using a Sylvania G15T8 germicidal light. RNase A was added to a final concentration of 1 mg ml⁻¹ and reaction mixtures were incubated at 37 °C for 10 min. Cross-linked proteins

were analyzed by SDS-PAGE and visualized by phosphorimaging. In cross-linking and immunoprecipitation experiments, samples were precleared after RNase treatment and incubated with antisera at 4 °C. Protein–antibody complexes were purified using protein A sepharose or *Staphylococcus aureus* protein A–positive cells.

Affinity purification and identification of nucleophosmin

Biotin-labeled SVL RNA was incubated with HeLa cell nuclear extracts for the times indicated. Reaction mixtures were then incubated with streptavidin beads for 60 min at 4 °C on a rocker platform. The beads were washed three times with wash buffer (20 mM HEPES-KCl (pH 7.5), 0.5 M NaCl and 1mM MgCl₂ with protease-inhibitor cocktail (1 mM PMSF, 1mM benzimidazole, 4 µg ml⁻¹ leupeptin, 2 µg ml⁻¹ pepstatin)). RNA-binding proteins were released by boiling the streptavidin beads in SDS sample buffer (2% (w/v) SDS, 50 mM Tris-HCl (pH 6.8), 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue) for 3 min and separated by SDS-PAGE. Proteins were visualized by silver-staining. Bands of interest were excised for in-gel trypsin digestion followed by MALDI-TOF MS at the Colorado State University Macromolecular Resource Facility.

Oligo(dT) chromatography and western blots

After *in vitro* polyadenylation, poly(A)⁺ RNA–protein complexes were purified using oligo(dT) sepharose. The beads were equilibrated with binding buffer containing 20 mM HEPES (pH 7.6), 150 mM NaCl, 1mM EDTA, 0.5% (v/v) NP40 and protease inhibitor cocktail. Protein–RNA complexes were incubated with the oligo(dT) beads for 30 min and washed extensively with binding buffer. Unbound proteins were treated with RNase A and precipitated with ethanol. The bound proteins were eluted from the oligo(dT) by boiling with protein loading sample buffer. Proteins were separated by SDS-PAGE and visualized either directly by phosphorimager or by western blotting after transfer to PVDF membrane. Monoclonal antibody to NPM (Abcam) was used according to the manufacturer's instructions. After incubating with anti-mouse horseradish peroxidase–conjugated serum (Sigma-Aldrich), the blot was developed using a chemiluminescent substrate (Pierce).

In vivo UV cross-linking

In vivo UV cross-linking of mRNA to proteins was done essentially as described in ref. 59. HeLa cells were grown to subconfluency in DMEM supplemented with 10% (v/v) FBS. Cells were washed twice with PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂. The culture plate was UV-irradiated with a 15-W germicidal lamp (Sylvania G15T8) placed 4 cm away from the cell monolayer for 3 min. The cells were released from the tissue culture plates by using trypsin and prepared nuclear and cytoplasmic extracts as described in ref. 60. Both nuclear and cytoplasmic extracts were equilibrated with binding buffer containing 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 1 mM EDTA and 0.5% (w/v) SDS with protease inhibitor cocktail. Both extracts were incubated for 1 h with oligo(dT) cellulose (Ambion), which had been equilibrated with binding buffer and agitated on a rocking platform. The slurry was washed extensively with binding buffer. The cross-linked RNA–protein complexes were eluted in 20 mM Tris-HCl (pH 7.9), 1 mM EDTA and 0.05% (w/v) SDS and precipitated with 3 volumes of ethanol. The pelleted material was digested with RNase A and separated by SDS-PAGE. NPM was visualized by western blotting as described above.

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Figure 1.

A 32-kDa protein selectively associates with RNAs that have undergone polyadenylation in HeLa nuclear extracts. Radiolabeled RNAs containing the SVL, E1B or IVA2 polyadenylation signal were used in polyadenylation assays (top gels) and UV cross-linking assays (bottom gels). Top, RNA products were analyzed on 5% denaturing acrylamide gels. Positions of input and polyadenylated RNAs are indicated at right. Bottom, UV cross-linked proteins were separated on SDS polyacrylamide gels. Positions of size markers are indicated at right; arrow at left marks the position of the 32-kDa protein, whose cross-linking is associated with polyadenylation.

Figure 2.

Identification of NPM as a candidate polyadenylation mark. **(a)** Biotinylated SVL RNA (bio-SVL) was incubated with HeLa nuclear extracts for the times indicated. RNA–protein complexes were purified and separated on a 10% (w/v) acrylamide gel containing SDS and visualized by silver-staining. Arrowhead at right indicates position of the 32-kDa band. **(b)** Amino acid sequence of NPM. Sequences of peptides identified by MS analysis of the purified 32-kDa protein in **a** are underlined. **(c)** Radiolabeled SVL RNA was incubated in the *in vitro* polyadenylation extract system for the times indicated. UV cross-linking was performed and the products were either loaded directly onto an SDS polyacrylamide gel (total lanes) or first subjected to immunoprecipitation using NPM-specific antisera (IP lanes) before electrophoresis.

Figure 3.

A functional AAUAAA and the process of poly(A) addition are required for NPM association. (a) Radiolabeled wild-type (WT; AAUAAA) and mutant (Mt; AAUACA) RNA substrates derived from the SVL poly(A) signal were subjected to polyadenylation (left) and UV cross-linking (right) analysis after incubation in extracts for the indicated times. (b) Radiolabeled SVL RNA or a derivative containing a preformed ~150- to 200-base poly(A) tail (SVL-A₂₀₀) were subjected to polyadenylation (top) and UV cross-linking (bottom) analysis after incubation in extracts for the indicated times. (c) Radiolabeled SVL RNA was incubated under polyadenylation (left) or cleavage (right) conditions, and RNA products were visualized (top) and proteins cross-linked to the RNA (bottom). In addition, the cross-linked proteins were subjected to immunoprecipitation with antibody to NPM to confirm NPM binding (lower right gel).

Figure 4.

The process of polyadenylation, but not cleavage, is required for deposition of NPM on RNA substrates. (a) Radiolabeled precleaved SVL RNA was incubated in the *in vitro* polyadenylation system for the times indicated. Top, RNA products were analyzed. Positions of input and polyadenylated RNAs are indicated at left. Bottom, proteins bound to RNA were visualized by UV cross-linking. In the two lanes on the right, extracts were subjected to immunoprecipitation with antibody to NPM after cross-linking. Arrow at right marks position of cross-linked NPM. (b) Reactions were performed as in a, but after cross-linking, the poly (A)⁺ RNAs were selected on oligo(dT). Bound and unbound proteins were separated by SDS-PAGE.

Figure 5.

Sequences upstream of the AAUAAA element are required for NPM cross-linking to polyadenylated RNAs. Pre-cleaved SVL RNA and shortened versions of the SVL, IVA2 and E1B RNAs were incubated in the *in vitro* polyadenylation system. **(a)** Sequences of the shortened RNAs, with polyadenylation signal underlined. **(b)** Top, RNA products of the polyadenylation reaction were analyzed. Bottom, proteins were cross-linked to the radiolabeled RNAs and then immunoprecipitated with antibody to NPM.

Figure 6.

NPM is associated with poly(A)⁺ mRNAs *in vivo*. **(a)** SVL RNA was incubated in the *in vitro* polyadenylation system for the times indicated. Poly(A)⁺ RNA was selected by oligo(dT) chromatography. Associated NPM was detected by western blotting. **(b)** Nuclear extract alone (total lane) or extract that had been subjected to oligo(dT) chromatography in the presence or absence of RNase was separated by SDS-PAGE and NPM was detected by western blotting. **(c)** HeLa cells were irradiated with UV light to cross-link RNA-binding proteins *in vivo* and nuclear and cytoplasmic fractions were isolated. Protein-RNA complexes were isolated and subjected to oligo(dT) chromatography in the presence or absence of RNase. Products were separated by SDS-PAGE and NPM was detected by western blotting.