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RNA-binding ability of PIPPin requires the entire protein

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Abstract

Post-transcriptional fate of eukaryotic mRNAs depends on association with different classes of RNA-binding proteins (RBPs). Among these proteins, the cold-shock domain (CSD)-containing proteins, also called Y-box proteins, play a key role in controlling the recruitment of mRNA to the translational machinery, in response to environmental cues, both in development and in differentiated cells. We recently cloned a rat cDNA encoding a new CSD-protein that we called PIPPin. This protein also contains two putative double-stranded RNA-binding motifs (PIP_1 and PIP_2) flanking the central CSD, and is able to bind mRNAs encoding H1° and H3.3 histone variants. In order to clarify the role of each domain in the RNA-binding activity of PIPPin, we constructed a number of different recombinant vectors, encoding different regions of the protein. Here we report that only recombinant proteins that contain all the putative PIPPin domains show RNA-binding ability.

Keywords: RNA-binding factors - CSD-containing proteins - PIPPin - histone variants - H1° histone - H3.3 histone - PIPPin domains

Introduction

Immediately after or even during transcription, eukaryotic mRNAs associate with different families of RNA-binding proteins (RBPs), with formation of ribo-nucleoprotein particles (mRNPs) [for review, see Refs. 1-3]. Cold Shock Domain (CSD)-containing-proteins, also called Y-box-proteins, form a highly conserved family of RBPs present in all organisms [4-7] that show both single stranded (ss)-DNA- and/or ss-RNA-binding activities and have been suggested to couple nuclear history of a transcript to its translational fate [4, 8-11].

One possible mechanism to alter chromatin structure and transcriptional activity of specific genes, during embryonic development and differentiation, involves entrance into chromatin of specific histone variant proteins [12-13].

We previously demonstrated that the genes encoding H1° and H3.3 histone protein variants, in the developing rat brain, are regulated mainly at post-transcriptional level [14-15]. In searching for

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factors able to bind histone variant mRNAs, we cloned a cDNA encoding a novel protein that we called PIPPin [16] and that is selectively expressed in some nerve cells of the rat brain and cerebellum [17]. PIPPin binds specifically both H1° and H3.3 histone mRNAs at the very end of their 3'-untranslated regions (3'-UTR), around the putative polyadenylation signals [18], and contains two putative double-stranded RNA-binding motifs (PIP1 and PIP2), each on one side of a central CSD [18].

The present study was undertaken to clarify the role of each of the PIPPin putative domains in RNA-binding ability. We found that only the entire PIPPin, but none of the single domains, is able to bind H1° and H3.3 mRNAs.

Materials and methods

Preparation of recombinant MBP-PIPPin proteins containing different combinations of PIPPin domains

Preparation of maltose-binding protein (MBP)/PIPPin fusion protein was described elsewhere [18]. In order to obtain fusion proteins containing only one of the putative PIPPin domains, the corresponding nucleotide sequences from the Cx1 plasmid (accession number X89962; Ref.16), were amplified by polymerase chain reaction (PCR), using the primers shown in Table 1a.

The 5'- and 3'-primers included *EcoRI* and *PstI* sites (underlined), respectively, to allow oriented cloning of the amplified fragments into the pMAL c2 plasmid (New England BioLabs).

Preparation of recombinant proteins containing different combinations of PIPPin domains and bearing an N-terminal tag of six histidines

In order to synthesize recombinant PIPPin, the entire putative coding region of the PIPPin insert, from the Cx1 plasmid (accession number X89962; Ref.16), was amplified, by polymerase chain reaction, using the attB-modified custom primers (Invitrogen) (Table 1b).

The 5'- and 3'-primers included attB1 and attB2 recombination sequences (underlined) for cloning the amplification products into the pDONR201 plasmid vector (BP reaction: Gateway™ Technology, Invitrogen) and then into the pDEST™ 17 plasmid vector (LR reaction: Gateway™ Technology, Invitrogen). The expression clone pDEST™ 17 was used for transformation of E. coli BL21-SI competent cells and expression of N-terminal histidine fusion with PIPPin (6His-tagged PIPPin), according to the protocols recommended by the manufacturers (Gateway™ Technology, Invitrogen).

In order to obtain proteins containing different combinations of the putative PIPPin domains, the corresponding nucleotide sequences were amplified by polymerase chain reaction (PCR), using the primers shown in Table 1c.

Preparation of bacterial extracts and purification of recombinant PIPPin

Bacteria were harvested by centrifuging at 5500 x g for 15 min, at 4°C, resuspended in Lysis Buffer (LB: 50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole) and stored for at least one night at –20°C. After thawing, the bacterial suspension was treated with lysozyme (1.0 mg/ml), sonicated for 2 min with a microtip sonicator (Vibracell VR6000), using a power setting of 60 W and finally incubated with RNase A (10 μg/ml) and DNase I (5 μg/ml) for 30 min on ice. The sonicated suspension was centrifuged at 5500 x g for 20 min, at 4°C to remove cell debris, and the supernatant used for purification of recombinant 6His-tagged PIPPin. Briefly, recombinant PIPPin was purified by metal-affinity chromatography on a selective nickel-nitriloacetic (Ni-NTA)-conjugated agarose matrix (QIA*express* System, QIAGEN) that shows high selectivity for molecules tagged with 6 consecutive histidine residues. Bound PIPPin was eluted with LB containing 250 mM imidazole. The entire procedure was performed according to the manufacture's instructions. Purified PIPPin was dialyzed against distilled water and stored at –20°C.

Recovery of insoluble proteins

Different proportions of the recombinant proteins produced by bacteria were found to be insoluble and were

Table 1. Primers used for PCR amplification of sequences encoding different combinations of PIPPin putative domains: a, primers used for preparation of MBP-PIPPin fusion proteins; restriction sites used for cloning are underlined; b,c, primers used for preparation of recombinant proteins containing N-terminal 6 histidine tags and corresponding to either entire PIPPin (b) or to different combinations of PIPPin domains (c); sequences required for recombination are underlined.

a

1 (PIP1) 5′-dAGCGAATTCATGACATCAGAGTCTACATGACCC-3′
2 (PIP1) 5′-dCCCCTGCAGCTAAGGGCCAGCCGAGGCTCGAGCTGT-3′
3 (PIP2) 5′-dAGCGAATTCATGTGTCCCATCCCAACCCAAGAAC-3′
4 (PIP2) 5′-dCCCCTGCAGCTAGGATCCCACAACCTGGCCAGA-3′
5 (CSD) 5′-dAGCGAATTCAAGGGCGTCTGTAAAGCAGTTCTCA-3′
6 (CSD) 5′-dCCCCTGCAGCTACTTGTAGGTCACCTCATCTCCTTC-3′

b

5′-dGGGG-ACAAGTTTGTACAAAAAAGCAGGCTTATCCAGACGTGACCCTTGCCAGCCA -3′;
5′-dGGGGACCACTTTGTACAAGAAAGCTGGGTCGGATCCCACAACCTGGCCAGACCA-3′.

c

1 (PIP1) 5′-dGGGG-ACAAGTTTGTACAAAAAAGCAGGCTTAATGACATCAGAGTCTACATCACCC-3′
2 (PIP1) 5′-dGGGGACCACTTTGTACAAGAAAGCTGGGTCAGGGCCAGCCGAGGCTCGAGCTGT-3′
3 (CSD) 5′-dGGGG-ACAAGTTTGTACAAAAAAGCAGGCTTAAAGGGCGTCTGTAAGCAGTTCTCA-3′
4 (CSD) 5′-dGGGGACCACTTTGTACAAGAAAGCTGGGTCCTTGTAGGTCACCTCATCTCCTTC-3′
5 (PIP2) 5′-dGGGG-ACAAGTTTGTACAAAAAAGCAGGCTTAATGTGTCCCATCCCACCCAAGAAC-3′
6 (PIP2) 5′-dGGGGACCACTTTGTACAAGAAAGCTGGGTCGGATCCCACAACCTGGCCAGACCA-3′

not released into the supernatant of sonicated cells. To recover from the pellet the otherwise insoluble proteins, a modification of the protocol described above was used. After thawing on ice, the bacterial pellet was resuspended in Modified Lysis Buffer (MLB: 25 mM Tris-HCl, pH 7.7; 100 mM KCl; 0.1 mM EDTA; 12.5 mM $MgCl_2$), containing 0.1% Nonidet P-40 and 4.0 M Urea, and incubated at room temperature for 30 min. The bacterial suspension was then centrifuged at 15.000 x g at 4°C and the supernatant was dialyzed sequentially against solutions containing 2.0 M- and 1.0 M- urea, respectively. The suspension was finally dialyzed against MLB lacking $MgCl_2$, and used for the chromatographic purification of the recombinant protein, as described above.

Preparation of *in vitro* transcripts and T1 nuclease protection assay

The original pMH1° (EMBL accession number X70685; Ref. 19) was linearized by restriction and used as template to synthesize ^{32}P-labelled H1° mRNA, from the T3 RNA polymerase promoter, according to Promega instructions. T1 protection assays were carried out as described elsewhere [20]. Protein-RNA complexes were analysed by electrophoresis on denaturing 15% polyacrylamide and autoradiography, as described [20].

Results

Recombinant proteins containing only one or two of PIPPin putative domains do not bind RNA

We previously reported that the amino acid sequence of PIPPin contains three different regions that show homology to motifs present in other RNA-binding proteins [16,18]. In particular, it contains two putative double stranded RNA-binding domains (called PIP_1 and PIP_2) and a central cold shock domain (CSD) (Fig.1A). In order

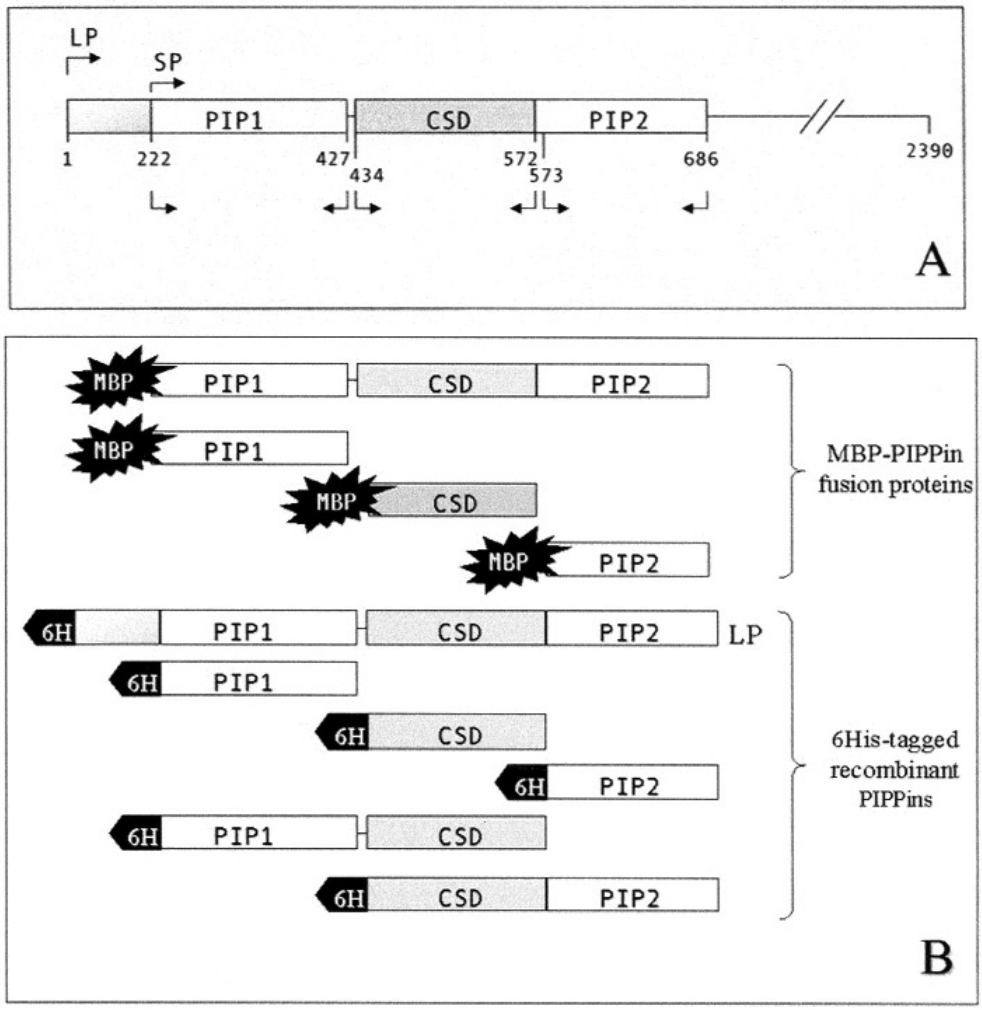

Fig. 1 Schematic drawing of recombinant proteins containing different combinations of PIPPin putative domains.

Part A shows the map of the original cDNA insert of 2390 nucleotides that encodes PIPPin. Numbers refer to the cDNA nucleotide sequence and indicate positions of sequences encoding the two putative double-stranded RNA-binding domains (PIP1 and PIP2) and the central cold-shock domain (CSD). Positions and directions of the primers used to amplify regions encoding each domain are indicated by converging arrows.

LP (long PIPPin), complete open reading frame present in the insert; SP (short PIPPin), open reading frame starting at the first ATG codon present in the insert.

Part B shows schematically the two groups of recombinant proteins used in the present study: i) proteins expressed as C-terminal fusions with the bacterial maltose-binding protein (MBP) and ii) proteins with an N-terminal tag of six histidines (6H).

to clarify the role of each of the putative domains in RNA-binding ability of PIPPin, we produced first a collection of MBP-PIPPin fusions proteins (Fig.1B), each containing only one of the putative RNA-binding domains (PIP1, PIP2 or CSD), and analyzed their ability to protect *in vitro* transcribed H1° and H3.3 RNAs from digestion by T1 RNase, as described elsewhere [20]. As shown in Fig. 2, only a fusion protein containing all the three putative domains binds both H1°- (Fig.2A, lane b) and H3.3- (Fig.2B, lane a) RNA, while fusion proteins containing only one out of the three domains do not bind RNA at all (Fig. 2A, lanes c-e; Fig.2B, lanes b-d). These preliminary findings suggested

that, when isolated, single portions of PIPPin are either unable to bind RNA stably or even to fold correctly. The latter possibility might be overcharged by fusion of a small domain to a large protein (MBP). Therefore we prepared a new set of recombinant proteins containing only a 6-histidine tag at the N-terminus. As we found that most of these 6His-tagged proteins are largely insoluble, we extracted them from the bacterial pellet with urea-containing buffer. After solubilization, recovered proteins were refolded according to the protocol described under "Materials and Methods", based on a series of dialyses against buffers of decreasing urea concentration. Interestingly, this procedure was apparently unnecessary for the protein corresponding to the entire long PIPPin (6H-LP), as it was normally recovered in sufficient amount from the supernatant of sonicated bacteria. As shown in Fig.3, only the proteins that contain all the three putative domains (Fig. 3A, lane d; Fig.3B, lane a) are able to bind RNA.

RNA-binding capacity of recombinant PIPPin increases after protein refolding

While producing recombinant 6His-proteins, we noticed that the 6H-LP protein was more soluble than the deleted PIPPin forms. However, a significant proportion of even 6H-LP tended to remain in the bacterial pellet. In order to recover also this fraction of protein we used again the denatura-

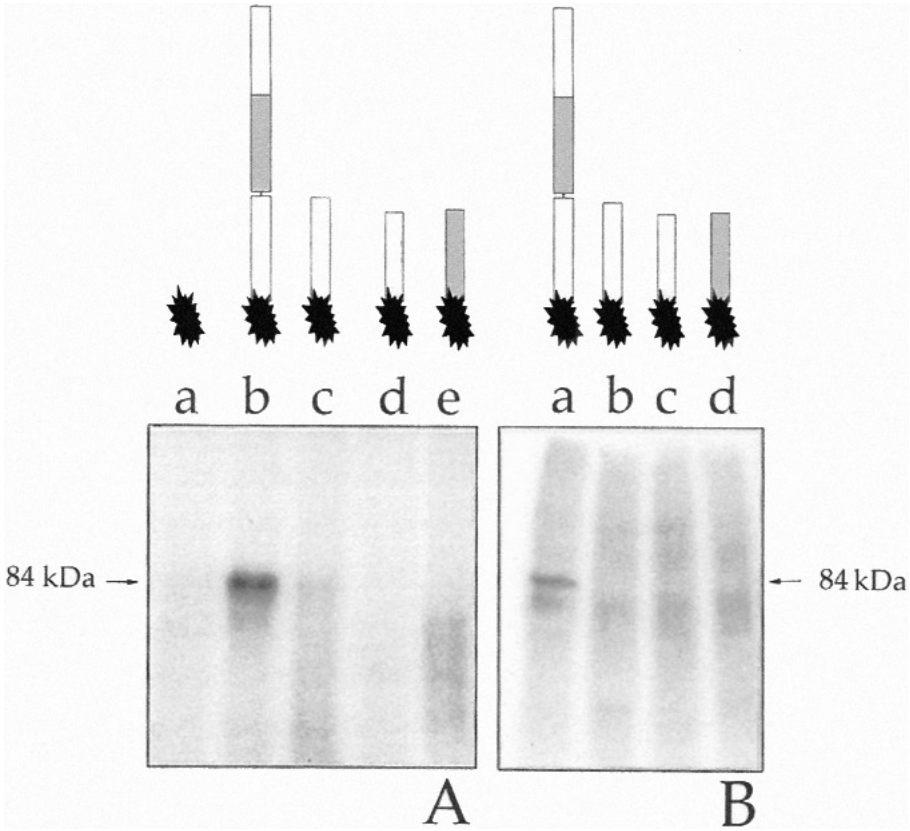

Fig. 2 T1 RNase protection assay of radiolabeled H1° and H3.3 RNAs, transcribed *in vitro* **and incubated with MBP or MBP/PIPPin fusion proteins**. 4μg of MBP (A, lane a) or MBP/PIPPin fusion proteins (A: lanes b-e; B: lanes a-d) were incubated with 0,5 x 10^6 cpm of radiolabeled H1°-(A) or H3.3- (B) RNA, for 10 min at room temperature. Samples were then incubated for 30 min with T1 RNase and exposed for 30 min to UV radiation (254 nm), on ice, as previously described [20]. Putative radioactive, covalent RNA-protein complexes were analyzed by 10% SDS-PAGE and the gel was exposed to x-ray film for 10 h, at –70°C. Recombinant proteins used (see Fig. 1) were: MBP-PIP1-CSD-PIP2 (A, lane b; B, lane a); MBP-PIP1 (A, lane c; B, lane b); MBP-PIP2 (A, lane d; B, lane c); MBP-CSD (A, lane e; B, lane d).

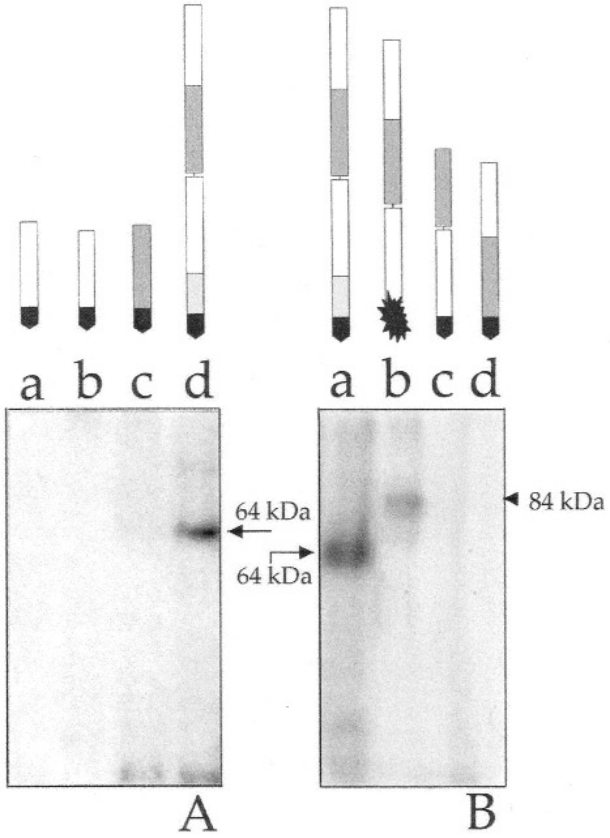

Fig. 3 T1 RNase protection assay of radiolabeled H1° RNA incubated with recombinant proteins containing N-terminal 6 histidine-tag. 2 μg of recombinant PIPPin were incubated with radioactive H1° RNA, as described in the legend to Fig.2. Recombinant proteins used (see Fig. 1) were: 6H-LP (A, lane d; B, lane a); 6H-PIP1 (A, lane a); 6H-PIP2 (A, lane b); 6H-CSD (A, lane c); 6H-PIP1-CSD (B, lane c); 6H-CSD-PIP2 (B, lane d); and MBP-PIP1-CSD-PIP2 (B, lane b).

tion/refolding protocol used for the deleted PIPPins. Surprisingly, when we analysed H1° RNA-binding ability of equal amounts of either 6H-LP directly recovered from the bacterial supernatant or 6H-LP recovered through the denaturation/refolding protocol, we found that the refolded protein binds RNA with much higher efficiency (Fig.4).

Discussion

Modulation of the structural organization of chromatin is fundamental to allow regulated gene expression both in development and in differentiated cells. Chromatin organization can be modified by covalent modification of histones [21-23] as well as by nucleosome remodelling, catalysed by DNA-dependent ATPase complexes [24-25]. Moreover, alternate histones seem to be used to modify structural organization of specific genes in response to extracellular signals, in the absence of DNA replication [13, 26].

Regulation of both linker- (i.e. H1°) and core- (i.e. H3.3) histone variant gene expression, in the developing rat brain, is mainly post-transcriptional [14-15] and possibly involves different classes of RBPs [20, 27], among which PIPPin [16,18], a Y-box protein that binds specifically both H1° and H3.3 histone mRNAs at the very end of their 3'-untranslated region, around the putative polyadenylation signal [18]. PIPPin contains a conserved central CSD, flanked on both sides by two motifs (PIP1 and PIP2) with chemical homology to dsRNA-binding proteins. The presence of additional RNA-binding domains, besides the CSD, is not unusual in Y-box proteins, where they enhance RNA-specific binding mediated by CSD [28]. In order to study the role of each of the putative RNA-binding domains present in PIPPin, we produced a collection of recombinant proteins, containing different combinations of them, and analysed their binding ability. Here we report that only the entire PIPPin is able to bind RNA, independent of the recombinant species used (i.e. either MBP-PIPPin fusions or 6His-tagged proteins). One explanation for this finding is that lack of binding is caused by failing of deleted proteins to fold into native structures: deleted proteins are indeed insoluble and were recovered from the bacterial pellet only with urea. However, after this treatment, followed by dialysis against solutions of decreasing urea concentration, recovered proteins were soluble, and presumably at least partially folded, as we used for binding assays only the proteins that remained soluble after a final centrifugation aimed at removing insoluble aggregates. Therefore, as they did not bind RNA at all, we supposed that isolated portions of PIPPin, even if well folded, are not able to bind RNA stably. Interestingly, full-length PIPPin, extracted with urea from the insoluble bacterial pellet and refolded shows much higher RNA-binding ability/μg of protein than the "soluble" fraction released into

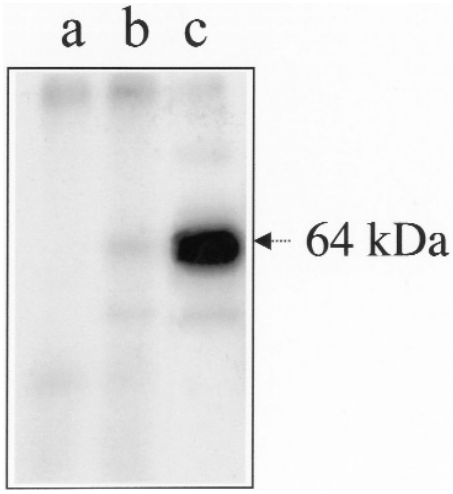

Fig. 4 T1 RNase protection assay of radiolabeled H1° RNA incubated with refolded PIPPin. 2 μg of recombinant long-PIPPin (LP) either released by sonication into the soluble bacterial fraction (lane b) or recovered by urea treatment of the bacterial pellet, and refolded as described under 'Materials and Methods' (lane c), were incubated with 0,5 x 10^6 cpm of radiolabeled H1° RNA, as described in the legend to Fig.2. An aliquot of radiolabeled H1° RNA was treated with T1 RNase and UV radiation, in parallel, without LP (lane a).

the bacterial supernatant; this finding suggests that even soluble fractions of recombinant proteins might be a mix of both uncorrectly and correctly folded species and recommends *a priori* application of refolding protocols when producing proteins to be used in functional assays as well as in medical applications.

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