PIPPin Is a Brain-specific Protein That Contains a Cold-shock Domain and Binds Specifically to H1° and H3.3 mRNAs*

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During maturation of mammalian brain, variants of both linker (i.e. H1°) and core (i.e. H3.3) histone proteins accumulate in nerve cells. As the concentration of the corresponding transcripts decreases, in postmitotic cells, even if the genes are actively transcribed, it is likely that regulation of variant histone expression has relevant post-transcriptional components and that cellular factors affect histone mRNA stability and/or translation. Here we report that PIPPin, a protein that is highly enriched in the rat brain and contains a cold-shock domain, binds with high specificity to the transcripts that encode H1° and H3.3 histone variants. Both mRNAs are bound through the very end of their 3′-untranslated region that encompasses the polyadenylation signal. Although PIPPin is present both in the cytoplasm and the nucleus of nerve cells, PIPPin-RNA complexes can be obtained only from nuclear extracts. The results of two-dimensional electrophoretic analysis suggest that a relevant proportion of nuclear PIPPin is more acidic than expected, thus suggesting that its RNA binding activity might be modulated by post-translational modifications, such as phosphorylation.

During development of an organism and tissue differentiation, chromatin must be remodeled to permit entrance of transcription factors and hence expression of genes at the right places and times. Although a critical moment for setting new patterns of chromatin organization is the S phase of the cell cycle, it is now clear that chromatin can be remodeled also in the absence of DNA replication, by energy consuming complexes (1–4). The possibility that remodeling also allows entrance, at topologically defined regions of the nucleus, of specific histone isoforms, which might locally modify chromatin organization even more, is provocative and deserves of attention.

We previously demonstrated that, in the developing rat brain, the concentration of H1° and H3.3 mRNAs decreases between the embryonal day 18 (E18) and the postnatal day 10 (P10), whereas the corresponding genes are transcribed at the same rate at any stage studied, suggesting that the two genes are regulated mainly at post-transcriptional level (5, 6). As post-transcriptional control processes, including regulation of splicing (7), vectorial transport of mature mRNAs (8–10), regulation of mRNA stability (11–13), and availability for translation (14, 15), are mediated by several classes of RNA-binding proteins (for review, see Refs. 16–18), it is likely that developing rat brain contains mRNA-binding factors involved in the binding and regulation of mRNAs encoding histone variants. We reported in a previous paper (19) cloning and analysis of a cDNA encoding a putative RNA-binding protein, specifically expressed in the rat brain and conserved from Drosophila melanogaster to man. The protein, that contains two regions with chemical homology to double-stranded RNA-binding motifs (16) was called PIPPin after the first four amino acids of the second of these motifs (PIPP, in one-letter code).

Here we report that PIPPin contains also a potential cold-shock domain (CSD1; for review, see Refs. 20 and 21). Within the latter, two short sequences are particularly interesting as they correspond to the so called ribonucleoprotein motifs 1 and 2 (RNP1 and RNP2), respectively, and are conserved among PIPPin and several other prokaryotic as well as eukaryotic nucleic acid-binding proteins.

The presence, in PIPPin, of a CSD flanked on both sides by putative double-stranded RNA-binding motifs strongly suggested that the protein could really be an RNA-binding factor. The results reported in the present paper clearly demonstrate that this is in fact the case. Moreover, we found that PIPPin binds preferentially to RNAs encoding H3.3 and H1° histone variants.

Using an in vitro culture system, we further demonstrate that PIPPin is present both in the cytoplasm and nucleus of nerve cells; however, its ability to bind RNA seems to be confined to the nucleus. We report that about one-half of nuclear PIPPin is more acidic (pI ≈ 6.0) than expected (pI = 7.7), thus suggesting that the protein would be post-translationally modified (perhaps by phosphorylation), in order to bind RNA.

EXPERIMENTAL PROCEDURES

Animals—Harlan Sprague-Dawley rats and New Zealand rabbits (Stefano Morini, San Polo d’Enza, Italy) were housed and handled according to European Community Council Directive 86/609, OJL 358 1, 12 December 1987 (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985).

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were prepared from embryonic day 16 rat cerebral cortices, and cultured for 5–8 days in a selective, serum-free medium, on Primaria tissue culture dishes (Falcon), precoated with laminin (Roche Molecular Biochemicals), as described in detail elsewhere (22, 23).

To obtain total post-nuclear, mitochondrial, microsomal, and post-mitochondrial (P100) cell subfractions, fresh tissues from developing and adult rats or cultured neurons were processed as described previously (24). Nuclear extracts were prepared according to Dignam et al. (25).

Protein concentration of all fractions was determined by the method described by Lowry et al. (26), using bovine serum albumin as a standard.

**Results and Sequences**—Some of the plasmids used for *in vitro* transcription of both radiolabeled and cold RNA probes were already described elsewhere (24). The other plasmids were constructed by subcloning different portions of the original inserts into the BlueScript KS+ plasmid (Strategene). Briefly, to synthesize transcripts corresponding to most of the H1° mRNA, we used as template the original pH11+ (EMBL accession number X70685; Ref. 27), that contains an insert of 1711 nucleotides (nt). To obtain smaller H1° transcripts, we used the subclones described previously (24) and reported in Fig. 3B (H1°, a to d). To synthesize H3.3 mRNA, we used as template pH3H, obtained by ligation of the 5′ region of PDH 33-2 and the 3′ region of PDH 33-1 inserts (EMBL accession number X76383; Ref. 5). To obtain smaller transcripts, corresponding to different portions of the untranslated region of the coding portion of 3′-UTR (B) of H1° RNA, we subcloned the following two regions of the H3.3 insert: 1) from nt 537 to nt 1107 (pF4; corresponding to the whole 3′-UTR, indicated as R4, in Fig. 3B); 2) from nt 909 to 1107 (pM4; corresponding to the last 198 nt of 3′-UTR, indicated as M4, in Fig. 3B). To synthesize the c-erbA-α2 transcript, we used the pAL13 plasmid, that contains an insert of about 1280 nt, corresponding to the full-length c-erbA α2 mRNA (26).

Finally, to synthesize a maltose-binding protein (MBP)-PIPPin fusion protein, the coding region of the PIPPin insert, from the Cx1 plasmid (accession number X89962; Ref. 19) was amplified, by polymerase chain reaction, using the following primers: 5′-dAGCGATTCT-GACATCAGACATCAGAC-3′; 5′-dCCCTGCAGCATGATCCACACACCTGGCCAGA-3′. The 5′- and 3′-primers included EcoRI and Psd1 sites (underlined), respectively, to allow oriented cloning of the amplified fragment into the pMAL-ct plasmid (New England BioLabs). All the subclones mentioned were sequenced, from both, by the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech).

Sequences of CDS-containing proteins were retrieved by Blast search (29) at the National Center for Biotechnology Information Website and aligned using MacMolly Tetra Program by GenoSoft. The corresponding accession numbers for SwissProt (SP), GeneBank (PB) and PIR data bases, are: Escherichia coli CspA, SP-P15277; E. coli CSPG, SP-P36996; E. coli CSP, SP-Q47130; Bacillus cereus CspA, SP-Q45096; B. cereus CSPB, SP-Q45097; B. cereus CSPC, SP-Q45098; Bacillus subtilis CSPB, SP-Q36201; Xenopus laevis FRGY12, GP-M9454; X. laevis FRGY11, GP-M5949; Caenorhabditis elegans LIN29, GP-U75915; D. melanogaster c-erbA, SP-Q47130; X. laevis FRGY1, GP-P54434; mouse MSY1, PIR-I58915; human Y1, SP-P16991; mouse Y1, SP-P43482; chicken Y1, SP-Q06066; X. laevis Y1, SP-P21573; rabbit P50, PIR-A55971.

For secondary structure predictions, the amino acid sequence of PIPPin was submitted to The PredictProtein server at EMBL (30, 31) and a Blast search against ProDom data base was run (26). MacMolly Tetra Program by GeneSoft was also used to compare the nucleotide sequences of H1° and H3.3 mRNAs.

**Purification of Fusion Protein, Antibody Production, Preparation of Serum, and Purification of PIPPin-specific IgG**—The MBP/PIPPin fusion protein was expressed in *E. coli* and purified from crude bacterial extract by affinity chromatography on amylose resin (Amersham Pharmacia Biochem) and eluted from the column with 100 mM glycine-HCl (pH 2.5). In the second step, anti-MBP antibodies were removed from the IgG fraction, obtained in the first step, by chromatography on MBP-Sepharose. The fraction not bound by the column is enriched in anti-PIPPin antibodies, while bound anti-MBP IgGs were eluted with 100 mM glycine-HCl (pH 2.5). All the purified fractions were dia lyzed against PBS and stored at −20 °C.

**Northern and Western Analyses**—Total RNA from cultured neurons was isolated, according to Chomczynsky and Sacchi (33), and separated by electrophoresis on 1.5% agarose, 6% formaldehyde gels. RNA was then transferred to nylon membranes (Hybond, Amersham) and hybridized to 32P-labeled PIPPin probe, as already described (19). Proteins (30–50 μg of total or fractionated cell extracts) were separated by electrophoresis on 15% polyacrylamide slab gels (SDS-PAGE), and immunoblotted as described elsewhere (24).

Two-dimensional electrophoretic analysis was performed as described by Roccheri et al. (34), with isoelectrofocusing (IEF) in the 5–7 pH range, in the first dimension, and SDS-PAGE in the second dimension. The pH gradient profile of IEF was determined by using proteins of known pl as standards.

**Preparation of In Vitro Transcripts and T1 Nuclelease Protection Assay**—Plasmids were linearized by restriction with suitable enzymes which cut the vectors downstream to the inserts, and used as templates for *in vitro* transcription of either unlabeled or 32P-labeled H1°, H3.3, and c-erbAα2 transcripts, from the T3 (pMHI+ and pH11+ d) or T7 (pD3H, pP4, pM4, pH11 α, pH11 γ, pH11 ε, and pAL13K) RNA polymerase promoters, according to the manufacturer’s instructions. T1 protection assays were carried out as described elsewhere (24).

To identify any H3.3 RNA binding activity, recognizable as PIPPin, *in vivo* postnuclear or nuclear cell fractions (up to 1 mg of total proteins) were incubated with 5.0 × 106 cpm of labeled H3.3 RNA and subjected to T1 assay. The putative PIPPin-RNA covalent complexes were then immunoprecipitated from the mixture with anti-PIPPin antibodies.

**Immunoprecipitation Assay**—To immunoprecipitate putative PIPPin-RNA covalent complexes, obtained by T1 protection assay, the cell lysates were incubated with 10 μl of preimmune serum to remove specific binding (preclearing step). After 20 min on ice, 20 μl of Protein A-Sepharose (Sigma), in T1 buffer (TB; 15 mM Tris-HEC, pH 7.5, 10 mM KCl, 10% glycerol, 1 mM dithiothreitol), were added and incubation was carried out for 1 h with agitation. A 2-ml suspension of 80 μl of Protein A-Sepharose was then incubated for 2–4 h with the immune (total or fractionated) anti-PIPPin serum. Sepharose from both steps was washed with 100 volumes of TB and finally boiled for 5 min in SDS-PAGE buffer.

**Immunocytochemistry**—Neurons were cultured on lamin-coated glass coverslips for 5 days. Cells were fixed with 2% paraformaldehyde, in PBS, at room temperature, for 15 min and then permeabilized for 5 min with 0.05% Triton X-100, in PBS. For the contemporary staining of both microtubule-associated protein 2 (MAP-2) and PIPPin, neurons were incubated with mouse monoclonal anti-MAP2 (Roche Molecular Biochemicals) and rabbit anti-PIPPin serum (total or fractionated), in PBS, for 1 h at room temperature, in a wet chamber. The secondary antibodies were, respectively, anti-mouse IgG, conjugated to rhodamine (Promega), and goat anti-rabbit IgG, conjugated to fluorescein (Promega), diluted, for use, in PBS (1:75). Cells were examined in an Olympus BX-50 microscope equipped with Vario Cam BW camera, and elaborated by image-pro/plus Media Cybernetics.

**Results**

**PIPPin contains a CSD and Binds RNA**—We reported in a previous paper (19) that PIPPin contains two regions (PIP-1 and PIP-2, in Fig. 1) with chemical homology to double-stranded RNA-binding motifs. A more recent search in the data bases revealed that, in the central part (residues 70–116) of PIPPin sequence (Fig. 1), a putative CSD was also present. The alignment of this domain with those present in a number of eukaryotic proteins, as well as prokaryotic, nucleic acid-binding proteins shows that the most conserved portions are the RNP1 and RNP2 motifs. Interestingly, the region in between these motifs shows higher homology to the prokaryotic than to the eukaryotic proteins, on the 3′ side of amino acid identity. Moreover, according to protein structure prediction (not shown), PIPPin would show the highest structural homology with *E. coli* CspA, that seems to be a general chaperone for RNA. Like in the other eukaryotic proteins, however, the CSD of PIPPin is flanked on both sides by additional putative RNA-binding domains.
These preliminary findings suggested that PIPPin was an RNA-binding protein. That this is the case was then clearly shown by the fact that a MBP-PIPPin fusion protein (Fig. 2, lane 1), but not MBP alone (Fig. 2, lane C), protected the radioactive H3.3 RNA, transcribed in vitro, from nucleolytic degradation by T1 RNase.

The radioactive RNA-protein complex shows an apparent molecular mass of about 84 kDa, that is about 24 kDa more than MBP/PIPPin fusion protein (i.e. about 60 kDa). As the exceeding mass has to be attributed probably to RNA, we can hypothesize that about 80 nucleotides of RNA are bound.

PIPPin Binds to Messages Encoding Histone Variants at the End of Their 3'-UTR—The above results demonstrated that PIPPin did bind RNA. We then asked whether it shows any preference for the message encoding H3.3 histone. Competition experiments showed that, among those tested, only unlabeled H3.3 (Fig. 2, lane 2) and H1° (lane 3) transcripts, but not the brain-specific c-erbA2 (lane 4) RNA, were able to compete with radioactive H3.3 transcripts, when present in the binding mixture. Thus PIPPin recognizes specifically some feature, common to RNAs encoding H1° and H3.3 histone variants. The alignment of H1° and H3.3 cDNAs revealed the presence, downstream to the coding regions of the two inserts, of a number of sites with moderate to high sequence homology. One region, in particular, shows high similarity (Fig. 3A); interestingly, this sequence of about 40 nucleotides covers in both messages the terminal portion of the 3'-UTR, encompassing the polyadenylation signal (underlined in the figure). Moreover, the same region is part of a sequence that was previously suggested by us to be potentially able to form a stem-loop structure, highly conserved in vertebrate H3.3 mRNAs (Ref. 5 and Fig. 3A). To investigate the possible involvement of this region in binding PIPPin, we amplified and cloned different fragments of the original H1° and H3.3 inserts (Fig. 3B) and used these new plasmids as templates to synthesize a set of unlabeled competitor RNAs. As shown in Fig. 3C, all the unlabeled RNAs that contain the very end of the 3'-UTR of both H1° (Fig. 3C, lane H1°, d) and H3.3 RNAs (Fig. 3C, all the lanes marked as H3.3) are able to abolish binding. These data confirm that PIPPin binds specifically to the 3' end of both H1° and H3.3 RNAs, as we hypothesized on the basis of H1° and H3.3 sequence alignment.

PIPPin Is Present Both in the Nucleus and Cytosol of Brain
Cells—As a first step toward identification of PIPPin in vivo, we prepared antibodies directed against the MBP/PIPPin fusion protein. We then used the total serum from immunized rabbit or PIPPin-specific purified antibodies to study the expression of PIPPin during brain development and its intracellular localization. We identified a main band (30 kDa), the concentration of which increases at birth (P0) and keeps thereafter an almost constant level (Fig. 4A), thus showing a behavior similar to the one reported for PIPPin mRNA (19). As shown in Fig. 4B, the band is enriched in the postmicrosomal cytosolic fraction, suggesting that PIPPin is not anchored to cytoskeletal or membrane structures. PIPPin is also present in brain nuclear extracts (see below).

We asked next if PIPPin is expressed in isolated neurons. Therefore, we prepared neurons from embryonic day 16 rat cerebral cortices, and cultured them in a selective, serum-free medium (22) on laminin (23). After 5 (L5) or 8 (L8) days of culture, either total RNA or nuclear and postnuclear extracts were prepared from neurons. As shown in Fig. 5A, PIPPin mRNA is highly expressed in isolated neurons. Accordingly, the 30-kDa protein was evidenced, by Western analysis, in nuclear and postnuclear extracts from both cultured neurons (Fig. 5B, lanes L5, N and Pn), and brain (Fig. 5B, lanes P10, N, and Pn). A minor immunoreactive band (about 20 kDa) was sometimes evidenced when purified anti-PIPPin antibodies were used in the Western analyses (Fig. 5B); the origin of this band, that was not visible when we used total anti-MBP/PIPPin serum (see, for example, Fig. 5B) is not yet clear.

Similar to the results shown above, staining of PIPPin by immunofluorescence localizes the protein to both nucleus and cytoplasm, in cultured neurons. The nuclear staining is especially intense, in the largest cells present in the culture. One neuron, representative of this population, is shown in Fig. 6 (Fig. 6, B and C, PIPPin immunostaining). In the same cell, the MAP-2 clearly shows (Fig. 6, A and C) the expected cytoplasmic localization (35). Moreover, the spotted appearance of immunofluorescence in the nucleus suggests possible involvement of the protein in discrete intranuclear macromolecular complexes and/or association to the nuclear pores.

RNA-binding PIPPin Is Enriched in the Nucleus—To investigate whether histone RNA-PIPPin complexes could be actually formed in cell extracts, we first performed T1 RNase protection assay and then to immunoprecipitate specifically the putative PIPPin-RNA complexes. As shown in Fig. 7, a
number of radioactive bands were evidenced when H3.3 RNA was incubated with either postnuclear (Fig. 7A, lane 1) or nuclear (Fig. 7A, lane 2) brain extracts; among these bands, however, only a major one, with apparent mass of about 54 kDa, was immunoprecipitated by anti-PIPPin antibodies (Fig. 7B). The apparent mass of the observed band is about 24 kDa larger than the 30-kDa PIPPin band. The difference in size between PIPPin and the putative PIPPin-RNA covalent complex is thus exactly the same observed in the case of the complex formed by the MBP/PIPPin fusion protein (shown for internal reference in Fig. 7A, lane 3).

Interestingly, the 54-kDa complex is more concentrated in the nuclear (Fig. 7B, lane 2) than in the postnuclear extract (Fig. 7B, lane 1). Since PIPPin is present in both compartments, in order to explain this difference in its RNA binding activity, we hypothesized that this latter might be modulated by post-translational modification. With the aim of exploring this possibility, we performed two-dimensional electrophoresis and Western analysis, on both postnuclear and nuclear cell extracts. Fig. 8 shows typical results of these experiments. As expected, both fractions contain PIPPin: immunoreactive 30-kDa species were indeed clearly evidenced after the second-dimension, SDS-PAGE. However, in the first-dimension, IEF separation, cytoplasmic (Fig. 8A) and nuclear (Fig. 8B) PIPPin show different behaviors; cytoplasmic PIPPin is essentially...
FIG. 7. Identification of immunoreactive H3.3 RNA-PIPPin covalent complexes in brain homogenates. Postnuclear or nuclear cell fractions (about 1 mg of total proteins, each) were incubated with 5.0 × 10⁶ cpm of labeled H3.3 RNA and subjected to T1 assay. Putative PIPPin-RNA covalent complexes were incubated with 10 μl of preimmune serum. After 20 min on ice, 20 μl of Protein A-Sepharose (Sigma), in TB, were added and incubation was prolonged for 2 h more, under moderate shaking, at room temperature. After removing the Sepharose, precleared lysates were further incubated for 2–4 h with the immune serum. Sepharose from both steps was washed with 100 volumes of TB and boiled for 5 min in SDS-PAGE buffer. A, total radioactive covalent complexes obtained by T1 RNase protection assay, from either postnuclear (lane 1) or nuclear (lane 2) brain extracts. B, proteins immunoprecipitated, by anti-MBP/PIPPin serum, from postnuclear (lane 1) and nuclear (lane 2) H3.3-protein complexes. The radioactive MBP/PIPPin fusion protein/H3.3 RNA complex is shown in A as an internal reference (lane 3).

FIG. 8. Identification of immunoreactive PIPPin isoforms that differ in charge. Postnuclear (A) or nuclear (B) cell fractions (about 200 μg of total proteins, each) were analyzed by two-dimensional electrophoresis. First-dimension separation was IEF, in the 5–7 pH range. Second-dimension separation was 15% SDS-PAGE. After the second-dimension electrophoresis, the gel was blotted and immunostained with total anti-MBP/PIPPin serum (1:1000). Marker sizes (in kDa) are reported on the left margin, for reference. Both the acidic (30a) and basic (30b) immunoreactive isoforms of PIPPin are indicated.

present as a main form with a pI ≥ 7 (i.e. the expected pI for unmodified protein). On the other hand, a high proportion (one-half or more) of nuclear PIPPin accumulates in a region of the gel that roughly corresponds to pH 6.0. This finding suggests that a high proportion of PIPPin might be post-translationally modified in the nucleus (presumably by phosphorylation) in order to bind RNA.

DISCUSSION

Among the proteins synthesized in the maturing brain, differentiation-associated variants of both linker (such as H1+) and core histones (such as H3.3) are of great interest, as their entering chromatin may induce further modifications of the transcriptional potential of the genome, in the absence of DNA replication and cell division. It is thus most important to understand how the synthesis of these proteins is regulated in the developing brain and especially in postmitotic neurons.

Growing evidence demonstrates the importance of regulating mRNA localization, stability, and translation, in control of gene expression, both in development and differentiated cells (for reviews, see Refs. 10, 36, and 37). The signals responsible for specific regulation of mRNA metabolism reside in the RNA message itself (38). All transcripts contain variable lengths of untranslated sequences where there are binding sites for a number of RNA-binding proteins. Many RNA-binding proteins probably assemble on the message at the moment of transcription (16, 17) and are determinant for the future fate of the transcript itself (7). Among the several classes of RNA-binding proteins identified to date, CSD-containing proteins (also called Y-box proteins: see Refs. 20 and 21) form an increasingly large family, the members of which interact with both DNA and RNA to control transcription and/or translation of specific genes (21). In the eubacterial domain, CSD proteins function mainly as RNA chaperones, a role that is gaining increasing attention, as it is now clear that many RNAs misfold or even unfold, in the absence of protein collaborators, which seem to be required also for the formation of large functional RNA-protein complexes (32). Like “protein chaperones,” most “RNA chaperones” are, however, nonspecific general factors. An interesting feature of PIPPin is, on the contrary, its specificity for the messengers encoding histones H1° and H3.3. Now, although PIPPin would show the highest structural homology with E. coli CspA, that seems in fact to be a general chaperone for RNA, its CSD, similar to other Y-box eukaryotic proteins, is flanked on both sides by other putative RNA-binding domains (PIPin and Pip2). We do not know yet if selectivity of PIPPin depends on CSD, on the two flanking domains, or on the particular combination of all these domains, but the present finding is important for at least two reasons. First, it offers an example of a tissue-specific RNA-binding factor that is also specific for one single class of messages. Second, it is quite clear that, in maturing brain, accumulation of the histone variants H1° and H3.3 is regulated mainly at the post-transcriptional level (6). However, it remains unresolved how this control is effected. Our previous studies (24) have established that factors, specific for the H1° message, are actually present in the developing brain. We show here that, in addition to those factors, H1° mRNA can also bind PIPPin, that is able to recognize H3.3 mRNA too. Taken together, the previous and present results suggest that post-transcriptional regulation of histone variants may rely on a pool of proteins, some of which are highly specific for a single message, whereas others (like PIPPin) might recognize features shared by different members of the same target group, ensuring their coordinated utilization. In this sense, an interesting finding is that PIPPin binds both H1° and H3.3 RNAs at the very end of the 3’-UTRs, which comprise the putative polyadenylation signals, suggesting that polyadenylation might be affected, in vivo. Although we do not yet have evidence of such a role, the presence of PIPPin in both the cytoplasm and the nucleus of nerve cells suggests that the protein might accompany histone messages from transcription to translation, participating possibly, as suggested by the spotted appearance of the nuclear staining, in the formation of till unknown, histone RNA-specific, macromolecular nuclear complexes.

Finally, with the goal of identifying RNA-binding PIPPin in vivo, we used the anti-PIPPin antibodies in an attempt to immunoprecipitate putative covalent H3.3 RNA-PIPPin complexes from nuclear and postnuclear brain extracts.
ple, this approach might be quite hard, as it has been suggested (18) that when RNA molecules form specific complexes with proteins, both partners (RNAs as well as proteins) undergo structural modifications (induced fit) that may hamper recognition of the protein component by antibodies; moreover, the presence of other protein- and/or RNA-binding factors might have a further masking effect. Despite these considerations, we did immunoprecipitate radioactive, covalent complexes, even if with apparently low efficiency.

Interestingly, although immunoreactive PIPPin is present in both the cytoplasm and nucleus, the concentration of PIPPin-RNA complexes is much higher in the nuclear extracts. In order to explain this finding we are forced to suppose that the protein undergoes some activating modification in the nucleus. As there are six protein kinase C and three casein kinase II potential phosphorylation sites in its amino acid sequence, we hypothesized that nuclear RNA binding activity might depend on PIPPin phosphorylation. This hypothesis was supported by the finding that a significant proportion of nuclear PIPPin is phosphorylated in order to bind RNA with high affinity.

Although not definitively proved, the suggestion that PIPPin activity might be modulated by post-translational modification is stimulating, because it implies that histone mRNA-PIPPin binding (and the consequent, till unknown, effects of this binding on histone mRNA metabolism) might be regulated, in neurons, by extracellular stimuli, such as hormones and neurotransmitters. Modifications in the rate of histone variant synthesis and perhaps of their incorporation into chromatin might then cause modifications of the structural organization and the transcription potential of neuronal chromatin.

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