Association of Purα with RNAs Homologous to 7 SL Determines Its Binding Ability to the Myelin Basic Protein Promoter DNA Sequence

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Cell type and developmental stage expression of the myelin basic protein (MBP) gene in mouse brain is regulated at the transcriptional level. Earlier studies from our laboratory have led to the identification of a DNA binding protein from mouse brain, named Purα, which interacts with the MB1 regulatory motif of the MBP and stimulates its transcription in glial cells. In this report, we demonstrate that a cellular RNA, with significant homology to 7 SL RNA is associated with Purα. Results from band shift competition studies indicate that Purα-associated RNA (PU-RNA), inhibits the interaction of immunopurified Purα with the MB1 DNA sequence. Results from Northern blot studies indicated that PU-RNA is expressed during various stages of brain development. Of interest, this RNA was found in association with Purα that was produced in the mouse brain at the early stage of brain development. Results from Northwestern analysis using a PU-RNA probe identified the regions within Purα that are important for Purα/PU-RNA association. Production of Purα at the early stage of brain development and its association with PU-RNA at this stage, when Purα exhibits poor binding ability to the MB1 DNA sequence, suggests that PU-RNA may function as a co-factor that negatively regulates Purα interaction with the MBP promoter sequence.

Cell type-specific gene activation is controlled by tissue-specific transcription factors that usually recognize and interact with DNA sequences located within the promoter or enhancer. In some instances, binding of these regulatory proteins to the DNA sequence is facilitated upon self-association of the proteins or their interaction with other members of transcription factors. Such a protein-protein interaction may, in turn, alter the protein conformation and facilitate its binding to the DNA molecules and/or its communication with other transcription factors.

The focus of our investigations during the past 10 years has been to decipher the regulatory pathways that participate in cell type-specific gene transcription in the brain. We have focused our attention on the myelin basic protein (MBP) gene, whose product is a major component of the myelin sheath in the central nervous system (for a review see Ref. 1), and its expression is controlled in manners both cell- and stage-specific during brain development (2–4). In mouse brain, expression of the MBP occurs postnatally such that it is first detected at the end of the first postnatal week, increases dramatically to peak at 18–21 days, and decreases to about 20% of peak levels in mature animals (5). Earlier studies have indicated that programmed expression of MBP is regulated at the level of transcription (6–8). Functional dissection of the MBP regulatory sequence led to the identification of a regulatory motif named MB1, which spans from nucleotide −14 to −50 with respect to the transcription start site. Analysis of nuclear proteins derived from mouse brain identified a 39-kDa protein that forms a nucleoprotein complex with a DNA fragment containing the MB1 sequence (9). Subsequent studies indicated that the 39-kDa protein, which is named Purα, recognized the GGC/GGA-rich sequences within MB1 DNA in a single-stranded configuration and has the ability to increase transcription of the MBP promoter both in vitro and in vivo (10, 11). Of interest, Purα binding activity to the MB1 sequence occurs in a developmental stage-specific manner that coincides with the pattern of MBP transcription (10). Evidently, at the early stage of brain development (days 3–7, postnatally), the level of Purα association with MB1 is extremely low, whereas during the phase of myelination (18–20 days) and in adults (day 30), its binding activity to MB1 drastically increases. Preliminary examination of Purα production indicated that although its levels increase between the first and second week of brain development, a significant amount of Purα is detected at the early stage of mouse brain development, which may not account for its extremely low level of association with the MB1 DNA sequence at this stage. These observations imply the participation of a co-factor(s) that may determine Purα binding activity to the DNA molecule. The ability of Purα to interact with a GGN repeat in a single-stranded form prompted us and others (12) to examine its binding ability to RNA molecules. Here, we describe our results demonstrating that Purα obtained from brain extract is in association with RNA molecules that have the ability to inhibit its binding activity to the MB1 DNA. Of interest, this RNA, which we have named PU-RNA, has significant homology to the 7 SL RNA, is expressed during various stages of brain development, and is found in association with Purα in 5-day-old mouse brain. Thus, our data suggest that PU-RNA functions as a co-factor by determining the binding activity of Purα to the MBP promoter sequence and indirectly participates in the developmental activation of the MBP promoter in mouse brain.
EXPERIMENTAL PROCEDURES

Nuclear Extract Preparation—Nuclear extracts from mouse brains at different stages of development were prepared according to the method of Dignam et al. (13) except that the second wash with buffer A was eliminated. The concentration of protein extract was measured using the Bradford assay (Bio-Rad), and the extracts were stored at −70 °C.

Immunoprecipitation and RNA Extraction—For immunoprecipitation, essentially 100 µg of nuclear extract was incubated with 1 µg of either nonimmune serum or clone 9C12 anti-Pur antibody for 2 h at 4 °C in buffer containing 12 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.5), 150 mM KCl, 2.5 mM CaCl2, 2.5 mM MgCl2, 0.1% Nonidet P-40, and 0.1 mM diithiothreitol. The immunocomplex was collected with 50 µl of prewashed Pansorbin, and the pellets were washed three times in the above buffer. Subsequently, the pellets were resuspended in phosphate-buffered saline and analyzed by the Western blot technique for detection of Pur or used for extraction of RNA. For RNA extraction, total nucleic acids (DNA and RNA) were extracted from the pellets and the supernatants of the immunoprecipitate using phenol/chloroform extraction followed by ethanol precipitation. The nucleic acid pellets were redissolved in buffer containing 10 mM Tris-HCl (pH 7.0) and 30 mM NaCl and treated with RNase-free DNase for 15 min at room temperature in the presence of RNase inhibitor. The reaction mixture was subjected to phenol/chloroform extraction and ethanol precipitation. This step was repeated twice to ensure complete removal of the DNA molecules. The RNA samples were stored at −70 °C in 75% ethanol until use.

Immunoprecipitation Band Shift Assay—One hundred micrograms of mouse brain nuclear extracts were precipitated using anti-Pur antibody according to the procedure described above with the exception that CaCl2 and MgCl2 were removed from the buffer. The immunoprecipitates were washed three times, and the protein-antibody complexes were eluted in 50 µl of high salt buffer C (13) for 1 h at 4 °C. Pansorbin was removed by centrifugation at 14,000 rpm for 1 min, and the supernatants that contained the eluted protein were collected for use.

The eluate was diluted with low salt buffer B (13) to a final KCl concentration of 150 mM. For band shift studies, 5 µl of immunopurified protein and total nuclear extract were incubated for 30 min in binding buffer containing 12 mM Heps (pH 7.9), 4 mM Tris-HCl (pH 7.5), 2.5 mM CaCl2, 2.5 mM MgCl2, 50 mM KCl, 0.1 mM diithiothreitol, and 30,000 cpm of the 32P-labeled MBIA oligonucleotide DNA (5'-TCAGAGGGCGCTCTTTGAAGGTG-3'). The protein-DNA complexes were resolved on 6% native polyacrylamide gels (19:1) (Bio-Rad) in 0.5 M Tris-HCl, 1 M glycine, and 0.5% SDS, and the gels were fixed with 10% acetic acid for 30 min in 70 °C in 75% ethanol until use. The autoradiograms were dried and analyzed using the PhosphorImager (Molecular Dynamics, Inc.). The full-length GST fusion Pur protein and the various Pur complexes were visualized by the SuperSignal West Dura kit (Pierce) and then exposed to film or phosphorimager to obtain the autoradiogram.

RESULTS

Previously, we identified and molecularly cloned a gene whose product, Purα, increased the level of transcription from the MBP promoter both in vitro and in vivo (10, 11). Purα associates with the MBP regulatory motif, MB1, which is located between nucleotides −14 and −50, with respect to the transcription start site and contains the putative Purα binding site, GGNGGN. Our preliminary observations, along with the results from other laboratories, suggested that Purα has strong binding affinity to single-stranded DNA and associates with RNA molecules (11, 12). In order to further investigate the association of Purα with RNA and to determine the importance of such an interaction in binding of Purα to the MB1 DNA sequence, mouse brain nuclear extract was treated with anti-Purα antibody, and the ability of the immunopurified Purα to bind to the MB1 DNA probe was examined by band shift assay. As shown in Fig. 1A, the immunopurified Purα bound to the MB1 DNA probe and formed a complex with electrophoretic mobility similar to that seen upon binding of Purα from the unfractionated nuclear extract and MB1 probe (compare lanes 2 and 3). The protein content of the immunocomplexes obtained by the band shift assay showed no significant increase (MB1 probe lane 3, lane 4). Fig. 1A (bottom) also shows results from Western blot analysis of total brain nuclear extract (lane 1) and the immunopurified Purα protein obtained from brain nuclear extract (lane 3). The band corresponding to the 39-kDa Purα was detected in total nuclear extract, and the immunocomplex was obtained from the extract treated with anti-Purα antibody. To gain some evidence regarding the association of RNA mol-
ecules with the immunopurified Purα and to evaluate the effect of such an association on the Purα-MB1 assembly, total ribonucleic acid was extracted from the Purα immunocomplex and was used as a competitor in an MB1-directed band shift assay. As shown in Fig. 1B, inclusion of 1 ng of PU-RNA in the DNA binding reaction decreased the interaction of immunopurified Purα with the MB1 DNA probe (compare lanes 2 and 3). At a higher concentration (3 ng) of PU-RNA, no signal corresponding to the Purα-MB1 complex was detected (Fig. 1B, lane 4). The addition of RNase to the binding reaction containing 3 ng of PU-RNA restored the assembly of the Purα-MB1 complex (Fig. 1B, lane 5), suggesting that the observed inhibitory effect is dispensable and mediated by the RNA molecules. The addition of PU-RNA and RNase to the MB1 DNA alone had no effect on the electrophoretic mobility of the MB1 probe (Fig. 1B, lane 6). To further examine the specificity of this observation, the competition experiment was repeated with 3 ng of PU-RNA (PU) or an equal amount of RNA obtained from the supernatant of the immunoprecipitate (S). As shown in Fig. 1C, whereas the PU-RNA effectively blocked association of Purα to the MB1 probe, the supernatant RNA did not abrogate the formation of the Purα-MB1 complex. These observations indicate that RNA species are associated with the immunopurified Purα and that the participant RNA molecules have the ability to control DNA binding activity of Purα.

The computer-assisted search for RNAs with the Purα binding site led us to suspect that Purα may be associated with the RNAs encompassing Alu core sequences, the GGAGGC repeat (15). All 7 S RNAs represent the only known abundant RNA type that contains the Alu-like core repeat sequence (16–18). Of interest, in neural cells brain-specific RNAs, named BC1 and BC200, represent the transcripts of transcriptionally active Alu genes and contain a high degree of homology to 7 S RNA (19–22). In order to examine whether immunopurified Purα is associated with 7 S or BC200 RNAs, we designed oligonucleotides derived from the conserved regions of these transcripts and used them as primers for cDNA priming and PCR amplification in a reaction containing Purα-associated RNA molecules. Under optimal conditions, no DNA fragment corresponding to the expected 95-base pair species indicative of 7 S or BC200 sequences was amplified. However, under similar conditions, an approximately 290-base pair fragment was obtained, indicating that the PCR primers were able to hybridize to the regions further upstream from the designated region and amplify a larger DNA fragment. Fig. 2 illustrates the primary sequence of the central region of the amplified fragment, which exhibits significant homology to 7 SL RNA. Despite the unexpected size of the amplified DNA fragment, an advanced gapped BLASTN search confirmed that the amplified cDNA corresponding to the PU-RNA sequence may belong to the 7 SL family of cellular RNA and has the highest homology score of 613 and a probability event of 4.2e−78. Fig. 2 illustrates the alignment of PU-RNA to the neuron-specific BC200 (BC) and left arm of 7 SL RNAs (7S), where the primers for RT-PCR were generated and to the 7 SL (SL) RNA with the highest homology score. PU-RNA differs significantly from the BC200 and left arm of 7 SL, which were used to generate primers, as examined by an advanced BLASTN search, which was optimized to find only the nearly identical sequences. The @ symbol depicts the nucleotides that are conserved. As shown in Fig. 2, only two single base substitutions and one single base deletion distinguish PU-RNA from the highest homology human 7 SL sequence. In order to identify putative Purα binding sites positioned in the single-stranded areas of PU-RNA, secondary prediction was determined. PU-RNA shows extensive stem-loop structure with the stability of −69.7 kcal/mol at 25 °C. Through this analysis, we were able to identify at least two putative Purα binding sites (GGN) that are located within the loops of the PU-RNA (data not shown).

In a different series of studies, we assessed the fraction of Purα-associated RNA that encompasses sequences with homology to 7 SL RNA. Results from sequencing of 12 clones obtained by RT-PCR of Purα-associated RNAs utilizing random primers revealed that five clones have homology to the Alu repeat,
which is common in 7 SL RNAs (data not shown).

In the next series of studies, we examined the level of Purα and the associated PU-RNA at various stages of brain development. As shown in Fig. 3A, the 39-kDa Purα protein was detected by Western blot analysis of the brain protein extracts from mouse at 5, 10, 12, and 45 days after birth (lanes 1–4, respectively) with the notion that its levels increased at day 10 and remained virtually constant thereafter. In parallel, we performed immunoprecipitation of the extract from mouse brain at various ages utilizing anti-Purα antibody, and the RNA molecules were extracted from Purα immunocomplexes and were used in an RT-PCR assay for detection of the 295-bp PU-RNA. As shown in Fig. 3B, the 295-bp PU-RNA was detected in the Purα immunocomplex derived from 5-day-old mouse brain. In order to examine the level of PU-RNA total RNAs were obtained from mouse brain at various stages of development and examined by Northern blot technique. As shown in Fig. 3C, strong signals indicative of a high level of PU-RNAs were detected at the early, middle, and late stages of mouse brain development (lane 1). The integrity of the RNAs is demonstrated in Fig. 3D, where the levels of 28 and 18 S RNAs remain unchanged. Our observation on the association of PU-RNA with Purα at the early stage of brain development is interesting in light of our earlier studies (10) demonstrating that association of Purα with MB1 DNA is developmentally regulated, with extremely low levels at the early stages of brain development (2–5 days) and maximizing in adults. The observed discrepancy in the abundance of Purα in newborn mice (shown in Fig. 3A) and its extremely low binding ability to MB1 suggested that binding of Purα to MB1 DNA at the early stage of brain development may be regulated by a distinct mechanism. The inhibitory action of PU-RNA on the DNA (MB1) binding activity of Purα and its association with Purα produced in 3–5-day-old mouse brain suggested that PU-RNA may play a role in down-modulating binding of Purα to MB1 DNA at the early stage of brain development. Thus, one may envision a model whereby PU-RNA, by inhibiting DNA binding activity of Purα may indirectly affect MBP gene transcription at early, but not late, stages of brain development. In accord with this concept, results from band shift studies indicated that RNase treatment of the nuclear extract from 5-day-old mouse brain induces binding of nuclear proteins to the MB1 DNA probe. In this study, nuclear extracts from 5-day-old, 10-day-old, and adult (60-day-old) mouse brain were mixed with the oligonucleotide probe containing the Purα binding site as described previously (10). The arrow indicates the position of the Purα-DNA complex. B, DNA binding reaction mixture derived from 5-day-old brain extract (lanes 1 and 2) and adult brain (lanes 3 and 4) were incubated with and without RNase prior to gel electrophoresis.
Fig. 4. Production of Pur in brain during development and its association with PU-RNA. A, Western blot analysis of nuclear extracts derived from mouse brain at various stages of development. The arrow depicts the position of the 39-kDa Pur protein. B, RT-PCR analysis of PU-RNA in immunopurified Pur derived from mouse brain at various stages of development. The arrow points to the position of 290-nucleotide cDNA from PU-RNA. C, Northern blot analysis of RNA from mouse brain at various stages of brain development, as indicated above, utilizing PU-RNA probe. D, ethidium bromide staining of RNA preparations from mouse brain at various stages of development. The positions of 28S and 18S RNAs are depicted.

our previous data (10), a significant increase in Pur binding activity was detected in the mature brain extract (Fig. 4A, compare lanes 2 and 4). Treatment of the extract with RNase enhanced association of Pur (from 5-day-old mouse brain extract) to the DNA probe and exhibited an insignificant effect on binding activity of Pur from adult brain extract. These observations, along with the data presented in Fig. 1, strongly suggest that the association of Pur with the RNA molecule modulates DNA binding activity of Pur.

To directly investigate the effect of PU-RNA on the association of Pur to the MB1 DNA sequence, various amounts of in vitro transcribed PU-RNA were used as competitors in DNA binding studies. As shown in Fig. 5A, inclusion of PU-RNA in the binding reaction decreased the intensity of the band corresponding to Pur association with the MB1 DNA probe. In a separate series of studies, we compared the relative binding affinity of Pur to DNA and RNA molecules with similar nucleotide compositions. Toward this end, synthetic MB1 ribonucleic acid was used as a probe in the binding reaction in the absence and presence of unlabeled MB1 RNA, MB1 DNA, and nonspecific RNA competitors. As illustrated in Fig. 5B, both MB1 RNA and MB1 DNA were able to inhibit PU-RNA-Pur complex formation. From the intensity of the ribonucleocomplex, however, it is evident that under the identical conditions, MB1 DNA is more effective than the MB1 RNA in dissociating the PU-RNA-Pur complex.

The primary amino acid sequence of Pur demonstrates the well-defined modular nature of the protein, which has no strong homologies to the known proteins (23, 24). The structural domains within Pur include (i) the stretch of 18 glycines, interrupted by one serine, in the N-terminal region of the protein, (ii) three basic-aromatic (class I) and two acidic-leucine (class II) repeats in the middle portion of the protein, and (iii) the region of limited homology to T-antigen, which includes the putative phosphorylation site for casein kinase II and is followed by glutamine- and glutamate-rich domains (23). In an attempt to determine the region within Pur that is responsible for its association with PU-RNA, we performed Northwestern experiments utilizing PU-RNA as a probe. To carry out these experiments, we created and utilized recombinant cDNA clones expressing different regions of Pur in the prokaryotic system. Fig. 6A illustrates the structural organization of wild type and the various mutants of Pur that were used in this experiment. The glutathione S-transferase (GST) fusion proteins containing the various regions of Pur as illustrated in Fig. 6A were produced in bacteria and resolved by SDS-polyacrylamide gel electrophoresis. The fractionated proteins were transferred to nitrocellulose, and the transblot was reacted with the riboprobe containing PU-RNA. The equal loading of proteins was verified by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining of the proteins (data not shown). As shown in Fig. 6B, PU-RNA bound to the full-length Pur. Deletion of the first 85 N-terminal amino acids resulted in a severe reduction of PU-RNA binding to the N-85 mutant. None of the subsequent N-terminal Pur deletion mutants were able to bind to PU-RNA. The first C-terminal mutant (mutant 1–215), which lacks amino acids 216–322, exhibited binding activity to PU-RNA. Further C-terminal deletion mutant (mutant 1–174), which deletes amino acids 174–322, completely lost its ability to bind to PU-RNA. Neither the internal deletion mutant nor the 85/230 mutant, which consists of the sequence deleted in mutant A72–231, was able to bind to PU-RNA. These results suggest that at least two distinct regions of Pur, located at the N terminus and between amino acids 174 and 215, are required for binding of Pur to PU-RNA; however, neither of these regions alone is sufficient for binding. Cooperation between these two regions of the protein is probably responsible for the binding of Pur to PU-RNA. Also, our data indicate that the DNA binding site of Pur, which is located between amino acid residues 66 and 246, overlaps with the region responsible for its binding to RNA.

DISCUSSION

Small cellular RNAs may exert a variety of regulatory activities, such as post-transcriptional mRNA processing, regulation of protein translation, and mRNA transcription. For a long
time, the 7 S RNA has been recognized as a component of the signal recognition particle, which is involved in transport of the nascent peptides into the endoplasmic reticulum (25, 26). 7 SL RNA is highly conserved between species, and from an evolutionary perspective, it is thought to serve as an RNA intermediate in the retrotransposition of the middle repetitive genomic Alu sequences (16, 17). Here we report that the transcription factor Purα, which regulates expression of the differentiation-specific MBP gene in oligodendrocytes (10, 11) engages in a complex with RNAs containing the 7 SL sequence, called PU-RNA, at the early stages of brain development. We speculate that in addition to its lower level, association of Purα with the PU-RNA, overproduced in the premyelinating brain, may account for the functional inactivation of this transcription factor before the onset of myelination. Accordingly, a decrease in the level of PU-RNA at the later stages of development and its dissociation from Purα, which permits interaction of Purα with MB1, may represent a mechanism for the regulation of transcription of MBP expression at the later stages of development and its dissociation from Purα, which permits interaction of Purα with MB1, may represent a mechanism for the maximum transcription of MBP expression during the peak of myelination. Thus, one may hypothesize that the 7 SL RNA sequence, by associating with or dissociating from a regulatory protein, may function as a co-factor for control of gene transcription. Note that while the reported protein and Purα both target a GC-rich single-stranded DNA, the former has a molecular mass (29 kDa) different from that of Purα.

As mentioned earlier, Purα has a complex structure with two series of interspersed repeats, a glutamine-rich domain (a potential candidate for an activation domain), a region of amphipathic helix, and a glycine-rich domain. Earlier studies indicated that a region positioned between amino acids 66 and 246 is critical for its DNA binding activity (29). Our observation of the requirement for the glycine rich motif in order to bind to the PU-RNA is consistent with the presence of similar glycine-rich motifs within the known RNA binding proteins (23). Class I repeats were previously implicated in the DNA binding ability of Purα (23). These regions, however, do not seem to be important for binding to RNA, since the deletion of class I repeats did not abolish binding to RNA. The class II repeats were shown to be involved in Purα interaction with human immunodeficiency virus-1 regulatory protein, Tat (30). Surprisingly, one of the class II repeats appeared to be required for the binding of Purα to RNA. These results raise the possibility that Purα-Tat interaction may be mediated by RNA. Perhaps it should be noted that involvement of RNA molecules in the interaction of proteins such as dimerization of estrogen and progesterone receptors has been previously demonstrated (31–33).

Repetitive RNA species are differentially expressed in cells in response to various conditions, such as the following: release by serum (34), stress (35), insulin (36), treatment with carcinogens (37), brain pathology (38), viral infections (39), during

![FIG. 6. Binding of PU-RNA to Purα deletion mutants. A, schematic representation of the 322-amino acid wild-type Purα and the various mutants. The amino-terminal glutathione S-transferase portion of the molecule has been deleted from the diagram. The three basic aromatic repeats are indicated by open horizontal bars between residues 66 and 246. The positions of two acidic regions between amino acids 102 and 131 and amino acids 188 and 220 are shown by shaded horizontal bars. The other motifs are indicated above the diagram. B, Northwestern analyses of the glutathione S-transferase wild-type Purα and various mutants are shown. The arrow shows the direction of the gel resolution, and the arrowhead depicts the position of the bands corresponding to wild-type Purα and the mutant 1–215.](image-url)
cell transformation (40–43), and in response to viral regulatory proteins (44). Interestingly, 7 SL RNA and the Alu transcripts can inhibit cell proliferation when overexpressed in HeLa cells (45). The possible mechanism of growth inhibitory activity stems from recent work where in vitro selected RNAs were shown to suppress cell growth via inhibition of binding of E2F1 to the DNA (46). It has long been assumed that promoter and enhancer binding factors recognize their cognate site on duplex DNA. Results from several laboratories have demonstrated that several transcription regulatory proteins, including homeodomain proteins, can bind to RNA and exhibit a regulatory effect on both transcription and translation (47–49). Earlier studies have demonstrated that Alu transcripts and 7 SL RNA can also stimulate transcription of several genes, including SV40, c-myc (41, 42), and the heat-stable enterotoxin receptor (50) promoters via unknown mechanisms. Based on the data presented in this paper, we propose that repetitive RNAs may also be involved in the regulation of expression of differentiation-specific MBP gene through their binding to transcription-activating protein Puro.

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