Molecular characterization of a novel rat protein structurally related to poly(A) binding proteins and the 70K protein of the U1 small nuclear ribonucleoprotein particle (snRNP)

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ABSTRACT

A cDNA has been isolated from a rat testis library which encodes a novel protein of 100 kDa that contains domains found in two different proteins involved in the processing of pre-mRNAs. Computer-assisted comparison reveals that one sequence motif of 30 amino-acid residues is very similar to a region conserved in the C-terminal part of eukaryotic poly(A) binding proteins (PABP). A second region of the rat 100 kDa protein, containing alternating basic, mostly arginine, and acidic amino-acid residues, is structurally related to sequence motifs found in the 70K protein of the U1 small nuclear ribonucleoprotein particle (snRNP), which is involved in RNA splicing. Northern blot analysis shows that a corresponding 9.5 kb transcript is highly expressed in rat testis; lower mRNA levels are found in other tissues such as liver, kidney, lung and brain. Ontogenic studies reveal that the expression of the 100 kDa protein-encoding gene and sexual maturation are correlated, being barely detectable during early post-natal life but reaching maximal levels around the first month after birth.

INTRODUCTION

In eukaryotes, most primary gene transcripts undergo diverse modifications. In the case of mRNA, these processes include polyadenylation and splicing, both of which take place in the nucleus. Many proteins are thought to be involved in these reactions and an accumulating number have been identified in the last few years. In addition to nuclear polypeptides, cytoplasmic proteins are thought to interact with mRNAs thereby affecting their translational efficiency. One protein that is known to be involved in mRNA metabolism is the poly(A) binding protein (PABP) which binds to the poly(A) tails of RNA (1). It has been found both in the nucleus and in the cytoplasm (2), and seems to be widely distributed throughout eukaryotes (reviewed in 3).

The poly(A) binding proteins have been suggested to be involved in the transport of poly(A)⁺ RNA into the cytoplasm, the degree of mRNA polyadenylation (1,4), and hence on the mRNA half-life (1,5), and the modulation of translation initiation and/or reinitiation events by interacting with the 60S ribosomal subunit (3). Additional protein factors, acting in concert with PABP, may be required to mediate the specificity of these reactions.

The structural properties of PABP were elucidated by the cloning and sequencing of the corresponding cDNAs from different eukaryotic organisms (2,6–9). The protein, which is about 70 kDa in size, contains four functionally-independent repeats of an RNA recognition motif (RRM), each of which is 80–90 amino-acid residues in length (9). While the N-terminal two thirds of PABP containing the four RRM sequences seem to be involved in RNA binding, the C-terminal sequence may form contact sites for transient RNA-protein and/or protein-protein interactions (7). A particular motif present in the C-terminal sequence of all known PABPs have not been reported yet.

Here we report on a novel 100 kDa protein from rat that contains sequence motifs structurally related to those found in eukaryotic PABPs and in the 70K protein of the U1 snRNP, known to be involved in RNA splicing. This structural relationship suggest that the 100 kDa protein might itself have a function in maturation and/or post-transcriptional regulation of mRNA.

MATERIALS AND METHODS

Cloning and DNA Sequencing Procedures

When screening a rat olfactory bulb cDNA library with the oligonucleotide probe 5' TTCATCATCCAG(T/A)(C/G)CGAG-AAGCCCCCCCCACTACCTGGA 3' used to identify rat insulin degrading enzyme (IDE) clones (manuscript in preparation), several cDNAs of various length were obtained that encoded a protein sequence unrelated to IDE. A cDNA fragment unrelated

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to the IDE clone was used to screen a phage lambda gt11 rat testis cDNA library (Stratagene, Heidelberg, FRG). Three independent clones were further investigated. The inserts of the longest cDNA clones were isolated and the two longest cDNA inserts (3.2 and 3.1 kb) were excised with EcoR I and subcloned in both orientations into M13 mp18 vectors. DNase I deletions were prepared using HindIII as the first and SmaI as the second restriction enzyme (10). Sequencing of the subclones was performed with dye primers using an Applied Biosystems DNA sequencing machine (model 373A).

In Vitro Transcription and Translation

The 3.2 and 3.1 kb cDNA inserts were subcloned into pBluescript plasmid (Stratagene, Heidelberg, FRG) and the constructs subsequently linearized with appropriate restriction enzymes. T7 or T3 RNA polymerase (BRL, Eggenstein, FRG) was used for *in vitro* transcription according to the protocol #2 of Promega. RNA products were checked in agarose gels. *In vitro* translation of sense RNA was performed in the presence of ³⁵S-methionine (spec. activity. 1000Ci/mmol; Amersham, Braunschweig, FRG) as recommended by the manufacturer using a nuclease-treated rabbit reticulocyte lysate (New England Nuclear, Bad Homburg, FRG). Labelled products were analyzed in a 6% SDS-polyacrylamide gel (11), followed by autoradiography.

Northern Blot Analysis

Total RNA from Wistar rats was extracted from pulverized frozen tissue (brain, testis) with guanidinium isothiocyanate and purified by CsCl-centrifugation (12). Glyoxylated total RNA (20 µg) was separated in 1% (w/v) agarose gels (13), and transferred to GeneScreen Plus membranes (Du Pont, Bad Homburg, FRG) according to the instructions of the supplier. Hybridization and washing conditions were carried out as described (14) with ³²P-labelled fragments (see Result section) of the 3.2 kb cDNA. For controls, blots were stripped and successively hybridized (14) with cDNAs encoding immunoglobulin heavy chain binding

protein (a gift from Dr. I. Haas, Cologne, FRG) and chicken beta-actin (a gift from Dr. H. Arnold, Hamburg, FRG).

Mapping of mRNA 3' and 5' ends with RNase H

Two oligonucleotides, complementary to nucleotides 412–432 (OL5') and 2851–2873 (OL3') of the 3.2 kb cDNA (sense strand), were used. 100 pmol of each oligonucleotide was incubated with 25 µg rat testis total RNA in 20 µl H₂O for 2 min at 50°C. After addition of 5 µl 0.25 M KCl, hybridization was performed for 10 min at 21°C. RNA/DNA hybrids were digested with 2.5 U RNase H (Gibco-BRL) in 50 µl 20 mM Tris-HCl buffer (pH 7.5, containing 10 mM MgCl₂, 125 mM KCl, 0.1 mM DTT and 50 g/l sucrose) for 1 h at 37°C. Reactions were stopped by the addition of 5 µl 250 mM EDTA.

The RNA was extracted with phenol, and precipitated with ethanol prior to glyoxylation and Northern blot. To investigate the poly(A) tail length of the mRNA, RNase H digestions were also performed in the presence of oligo dT (100 pmol, 12–18 mer).

RESULTS AND DISCUSSION

DNA Sequence and *In Vitro* Translation of the 3.2 kb cDNA

From rat brain and testis cDNA libraries the former initially screened for clones encoding a cytosolic protease (insulin degrading enzyme, IDE; 15), a cDNA of 3180 bp, termed t32, was isolated coding for a novel protein totally unrelated to IDE. The cDNA contains an open reading frame coding for 889 amino-acid (aa) residues with a calculated molecular mass of 100 kDa (Fig. 1). Its 3' end is characterized by a stretch of adenosine residues that are preceded by a canonical polyadenylation signal (not shown). The first in-frame translation initiation codon starts at nucleotide position 94 and fulfils the general rule that a purine should be present at position –3 (16).

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MMSARGDFLNIALSLMRSHNDEHSDVLPVLDVCSLKHVAYVFQALYIWK 50
AMNQQTTLDTPLERKRTRELLGIDNEDSEHENDDDTSQSATLNDKDD 100
ESLPAETGQNHFFRRSDSMTFLGCIPNPFEVPLAEAIPLADQPHLLQF 150
NARKEDLFGRPSQGLYSSAGSGKCLVEVTMDRNCLEVLPTKMSYAANLK 200
NVMNQNRQKKAGEDQSMLEAEADSSKPGPSAHDVAAQLKSSLLAEIGLT 250
ESEGPPLTSFRPQCSFMGMVISHDMLGRWRLSLELFGRVFMEDVGAEPG 300
SILTELGGFEVKESKFRREMEKLRNQSRDLSLEVDRDRDLIQQTMRL 350
NNHFGRRCATTPMAVHRVKVTFKDEPGEGSGVARSFYTAIAQAFLSNEKL 400
PNLDCIQNANKGTHSLMQRLNRNCRDREREREREMRRSSGLRAGSR 450
RDRDFRRQLSIDTRFPRASEGNPSDDPDPLPAHAAASERIVIVVAY 500
APAFITRERERESPAQLLLLLASEDSLRAVEEAMELIVAHGRENGAD 550
SILDGLLLDSSEKVENRKRHGSSRSVVDMDLDDTDDGDDNAPLFYQPGK 600
RGFYTPRPGKNTARLNCFRNIGRILGLCLLQNELCPITLNRHVIVKVLG 650
RKVNWHDFAFDPVMYESLRQLILASQSSDADAVFSAMDALFAVDLCKEE 700
GGGQVELIPNGVNIPVTPQNVYEVYRKYAEHRMLVVAEQPLHAMRKGLLD 750
VLPKNSLEDLTAEDFRLLVNGCGEVNVQMLISFTSFNDESGENAEKLLQF 800
KRWFWSIVERMSMTERQDLVYFWTSSPSLPASEEGFQPMPSITIRPPDDQ 850
HLPTANTCISRLVPLVYSSKQILKQKLLLAIKTKNFGFV 889

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Figure 1: Deduced protein sequence of the 3.2 kb cDNA. The PABP-like domain is shown in black, and the arginine-rich mixed-charge regions are boxed. Proline-rich segments and two highly-acidic regions are underlined. A putative protein kinase A phosphorylation site is indicated by an asterisk.

Figure 2: *In vitro* translation of sense RNA derived from the t32 cDNA. *In vitro* translation was performed using nuclease-treated reticulocyte lysate in the presence of ³⁵S-methionine. Lanes 1 and 2: control reactions without added RNA (1) or with yeast RNA (2). Lane 3: translation of sense RNA corresponding to the 3.2 kb cDNA. Lane 4: translation of sense RNA corresponding to the 3.1 kb cDNA devoid of the predicted translation initiation codon. Products were analyzed by SDS-PAGE and autoradiography. The apparent molecular masses of reference proteins are indicated.

To confirm the predicted size of the encoded protein, *in vitro* transcribed sense RNAs were translated in the reticulocyte lysate system. Translation of RNA derived from the 3.2 kb cDNA yields a product with an apparent molecular mass of 108 kDa (Fig. 2, lane 3). The difference between the predicted (100 kDa) and apparent (108 kDa) molecular weights may be explained by the presence of regions that have a high density of charged amino acids in the protein (see below). Highly charged domains have been shown to be responsible for the retarded mobility of various RNA binding proteins in SDS-PAGE (17).

The 108 kDa product did not appear when a shorter RNA from the 3.1 kb cDNA was used in the lysate system. This cDNA lacks 120 bp of the 5'-end of the 3.2 kb cDNA and, therefore, is devoid of the putative translation start codon supporting the notion that the first AUG in the open reading frame is indeed used as the translation start point.

Predicted Structural Features of the Protein

The predicted protein contains relatively high amounts of acidic (14%) and strongly basic (R and K, single letter amino-acid code, 12%) residues and has a pI of 5.7. Hydropathy plots revealed that the protein is generally hydrophilic; it does not display extended hydrophobic regions such as membrane-spanning domains or an N-terminal leader peptide (data not shown). This suggests that the protein is neither inserted into membranes nor secreted. It also lacks putative N-glycosylation sites.

Searches of protein databases (NBRF, release 30 and Swissprot, release 18) did not reveal overall homology to any known protein. However, two regions were identified that exhibit a high degree of similarity to two different RNA binding proteins. Most striking is the similarity (residues 485–514, Fig. 1) to a 30 amino-acid region of the poly(A) binding protein (PABP). Using computer alignments of only the C-terminal parts of eukaryotic PABPs, we found this hitherto undetected motif to be conserved between yeast and higher organisms (Fig. 3A). This motif is absent, however, in the sequence of *Drosophila* PABP.

Figure 3: Domains of the rat 100 kDa protein are similar to regions in PABPs and in U1 70K protein. **A:** Amino acids 485–514 of the rat protein are aligned with parts of the human (aa 556–585)(7), *Xenopus* (aa 558–587)(8), and yeast (aa 506–537)(2) PABPs. Two additional residues present in the yeast sequence are indicated. Vertical lines between the human and rat sequences denote identical residues, and colons indicate conservative amino-acid substitutions. A consensus, derived from the four sequences, is shown below the alignment. X marks non-conserved residues. **B:** Similar regions between the 100 kDa protein and PABP or U1 70K protein are indicated by boxes. The precise positions within the 100 kDa protein are aa 485–514 for the PABP-like domain and aa 426–454 for the U1 70K-like domain.

A homology search for this 30 amino-acid stretch in the protein databases failed to detect other proteins. Comparison of the 100 kDa protein region with that from human PABP revealed 67% sequence identity, and predominantly conservative amino-acid substitutions (8 out of 10) at non-identical positions. Additional alignments with the sequences of *Xenopus* and yeast PABPs show that two subregions (R/K Q A/M L G E and K I T G M L/I L E/D L/I) are particularly conserved (Fig. 3A). Although the functional significance of this sequence motif in PABPs remains to be unravelled, its presence in the rat 100 kDa protein is remarkable and may suggest a functional role in protein-RNA interaction. It should be noted that the 30 amino-acid region does not represent the RNP consensus sequence found in various RNA binding proteins (17, 22).

The second region (aa 420–457) of the rat 100 kDa protein that has significant sequence similarity to another RNA binding protein has been found in the 70K protein of the U1 snRNP, that is known to be involved in RNA splicing (17,19). This domain located in the 100 kDa protein some 50 amino-acid residues N-terminal of the PABP-like sequence and separated from the latter by a proline-rich segment (Fig. 1) is remarkable firstly for its abundance of arginine residues (45%), secondly for the presence of two mixed-charge sequences composed of alternating basic and acidic residues (ERDRERERERE and RDRDRD), and thirdly for the occurrence of a putative protein kinase A recognition signal (RRSS) residing between the two mixed-charge subdomains (Fig. 1). The mixed-charge region of the 70K U1 protein (Fig. 3B) is more extended (150 aa residues) and interestingly interrupted five times by putative protein kinase A phosphorylation sites. Assuming an involvement in RNA binding, phosphorylation of the serine residues within this area would be predicted to affect (by virtue of electrostatic repulsion and/or steric hindrance) RNA binding affinity, which could have a regulatory function.

Arg-Asp (RD) repeats have also been observed in a protein of currently unknown function that was initially identified as the predicted product of a highly-conserved gene within the major histocompatibility complex (20). Its mixed-charge sequence is almost exclusively composed of RD repeats ('RD protein') revealing 50% identity in a 42 amino-acid span of the 100 kDa protein. Though the function of the RD protein is unknown, an identified RNA recognition motif (21) suggests that it may *in vivo* be associated with RNA. In contrast to the RNA recognition motif common to many RNA binding and single-stranded DNA binding proteins (17,22), this RD region may allow more specific and/or transient interactions. This would be in line with a recent report demonstrating the essential role of arginine side chains in the recognition of specific RNA structures (23). A homology search for the mixed-charged sequence of the 100 kDa protein (residues 420–457) revealed only the 70K U1 snRNP and the RD proteins to have significant sequence similarity.

Northern Blot Analysis Reveals the Existence of a Single Transcript of 9.5 kb

To identify the corresponding RNA species, total RNA from rat testis and brain was analyzed by Northern blotting using the t32 cDNA as a hybridization probe. In both tissues a single band of about 9.5 kb was detected (Fig. 4). From densitometric quantification, the mRNA level appears to be six-fold higher in testis than in brain. Examination of other tissues (liver, kidney, lung, ovary) revealed small but significant amounts of the 9.5

Figure 4: Northern blot analysis of brain and testis RNA. Twenty μ g of total RNA from rat brain and testis were analyzed. The t32 cDNA, labelled with 32 P-dATP by random-primed synthesis, was used as a probe. The positions of co-electrophoresed glyoxylated RNA standards are indicated.

Figure 5: Localization of transcript sequences not present in the cDNA. Samples (25 μ g) of total RNA from rat testis were analyzed on a Northern blot after digestion with RNase H (see methods) in the presence of different oligonucleotides. OL3' and OL5' are complementary to nucleotides 412–432 and 2851–2873, respectively, of the t32 cDNA. (dT)_n is oligo (dT) 12–18mer. The same blot was successively hybridized with a 3' fragment comprising nucleotides 2874–3167 (A) and with a 5' fragment comprising nucleotides 1–404 (B) of the t32 cDNA. The lengths of the 3' and 5' ends of the mRNA, after RNase H digestion, were calculated by comparison with co-electrophoresed RNA standards (M1 is the 9.5–0.24 kb ladder and M2 is the 1.77–0.16 kb ladder, from Gibco BRL). A schematic representation showing the RNase H cleavage sites in the 9.5 kb RNA as well as the products identified by hybridization with the 3' (a) or with the 5' (b) probe, respectively, is indicated. Several minor RNA species (OL5' and OL5' + (dT)_n) might be caused by hybridization of the OL5' oligonucleotide to sequences within this long undefined mRNA region.

Figure 6: Transcript levels in rat testis during development. Twenty μ g of total RNA from different postnatal stages (1 day to 6 months) were hybridized to the 32 P-labelled t32 cDNA (A). For control, the same blot was stripped and hybridized to a cDNA encoding the immunoglobulin heavy chain binding protein (BiP) (26) (B) or to a chicken beta actin cDNA probe (C). The observed increase in the amount of BiP mRNA between d14 and d28 probably reflects the enlargement of the endoplasmic reticulum during differentiation (26). The slight decrease of the main actin signal during development rules out the possibility that RNA from the early stages simply had not been transferred to the blot. An additional actin signal appearing from day 28 onwards presumably represents the rat analogue of a mouse testis-specific actin mRNA that is expressed only in postmeiotic cells (25).

kb RNA (data not shown). The discrepancy in length between the cDNA (3.2 kb) and the hybridizing RNA (9.5 kb) suggested 5'- and/or 3'-terminal extensions of the transcript. To determine the length and the position of additional mRNA sequences, the testis RNA was digested with RNase H in the presence of specific oligonucleotides complementary to either nucleotides 412–432 (OL5') or nucleotides 2851–2873 (OL3') of the t32 cDNA (sense strand). The resulting RNA fragments were analyzed on Northern blots by hybridization to a 5' (nucleotides 1–404) or 3' (nucleotides 2874–3167) t32 cDNA fragment (Fig. 5). After hybridization with the 3' cDNA probe (Fig. 5A), RNA species of 0.4 kb (OL3') and 2.8 kb (OL5') were detected both of which are roughly 60 nucleotides longer than the corresponding 3' terminus of the t32 cDNA. Removal of the mRNA poly(A) tract (OL5' + (dT)_n) leads to the detection of a slightly shorter RNA of about 2.7 kb, indicating that the 3' ends of the transcript and the cDNA are identical except for additional terminal adenylate residues present in the mRNA.

After hybridization with the 5' cDNA probe, RNA species of 9.1 (OL3') and 6.7 kb (OL5') were detected (Fig. 5B), both of which exceed the length of the corresponding cDNA by about 6.3 kb. This difference fully accounts for the length discrepancy between the 9.5 kb transcripts and the t32 cDNA, indicating that the mRNA has either an unusually long 5'-untranslated sequence or represents a partially spliced version of the 100 kDa protein-encoding gene. The unusual long 5'-untranslated region of the 100 kDa-encoding mRNA could imply that initiation of translation occurs at an internal site rather than at the capped 5' end via the so-called ribosomal scanning model (24). Recently, the leader region of the mammalian immunoglobulin heavy chain binding protein (BiP)-encoding mRNA has been shown to confer an internal ribosomal binding site to a dicistronic messenger construct, thereby permitting translation of the second cistron in HeLa cells (25). The extended leader region of the 9.5 kb mRNA, therefore, does not exclude *in vivo* translation of the 100 KDa protein.

Developmental Upregulation of the 9.5 kb RNA in Testis

The high levels of the 9.5 kb transcript in testis prompted us to examine whether this is related to the functional maturation of the tissue. Total testicular RNA from rats of 1, 7, 14, 28 days (d) and 3 and 6 months (m) old was, therefore, analyzed on a Northern blot (Fig. 6A). At all developmental stages, a single RNA of 9.5 kb hybridized with the cDNA probe. However, the levels of the transcripts clearly differed at early (1–14 d) and later stages (28 d–6 m). While only a very faint signal was detected at the early stage of development, the 9.5 kb RNA level increased about thirteen-fold between day 14 and day 28. This is the time at which sperm cell differentiation commences and this is accompanied by testis-specific gene activation (26). Attempts to identify the testicular cell type expressing the 100 kDa protein-encoding mRNA by *in situ* hybridization failed which was probably due to relatively low levels of the respective mRNA.

The enhanced expression of the 100 kDa protein-encoding transcript during testicular maturation might indicate an involvement in RNA processing and/or translational control. Because transcription ceases long before the differentiation of sperm cells is completed, an array of mRNAs is stored for protein synthesis which occurs at a later stage of spermiogenesis (26). The change in translational activity is combined with the shortening of mRNA poly(A) tails (26). Currently, the causal link between changes in poly(A) tail length and translational efficiency is not understood. There is no question, however, that regulatory proteins, which are capable of interacting with RNA or RNA binding proteins have to be involved in this phenomenon.

Taken together the rat 100 kDa protein shares a sequence motif of 30 amino-acid residues present in eukaryotic poly(A) binding proteins. In close proximity to the PABP-like domain of the 100 kDa protein a second structural motif of mixed-charge sequences is found displaying similarity to another RNA binding protein, the 70K U1 snRNP polypeptide and to the so called RD protein family. Based on the presence of these sequence motifs the 100 kDa protein appears to belong to the class of RNA binding proteins that may be involved in the production of mature mRNA or in regulating mRNA translation rates.

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