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IDENTIFICATION OF NOVEL RNA-BINDING PROTEINS THAT INTERACT IN THE CODING REGION OF PROTEIN D SENSE RNA IN VITRO

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SUMMARY: Ultraviolet (UV)-cross-linking and sodium dodecyl sulfate(SDS) polyacrylamide gel electrophoretic (PAGE) analysis were used to identify proteins of nuclear and cytosolic (S100) origin that specifically bind to an *in vitro* transcribed mRNA sequence for protein D. The coding region of the protein D cDNA was subcloned, *in vitro* transcribed to [^{32}P]RNA, and incubated with nuclear and cytosolic extracts of enzymatically dispersed epididymal cells. As revealed by UV-cross-linking and SDS-PAGE analysis, two proteins exhibiting a molecular weight mass of ~2.5 and ~35 Kd that specifically recognize and bind to the *in vitro* transcribed mRNA sequence for protein D. Our findings suggest that the regulation of protein D gene expression in the rat epididymis may involve novel RNA-binding proteins.

Epididymal fluid is thought to play a physiological role in the maturation of spermatozoa as they pass through the epididymal duct (1). Some of these maturational changes are mediated by androgen-dependent proteins and glycoproteins that are synthesized by epithelial cells, secreted into the epididymal fluid, and subsequently bind to spermatozoa (2,3). Among the proteins found in epididymal fluid, protein D has been shown to play a functional role in the development of motility and fertility by epididymal spermatozoa (4). Protein D has been purified and characterized under a variety of names, including protein D/E (5), 32 Kd protein (6), sialoprotein [SP] (7), and acidic epididymal glycoprotein [AEG] (8). Although no enzymatic activity has been shown to be associated with protein D, the primary amino acid sequence of protein D demonstrates homology with that of yeast carboxypeptidase (9,10). This observation suggests that protein D is involved in the post-translational modification of proteins secreted into the epididymal lumen. In a previous study (11), we demonstrated regional differences in the expression of protein D at the level

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of protein and mRNA. One conclusion drawn from this study was that the expression of protein D in the corpus epididymidis may be regulated at the level of mRNA. To further explore this possibility, the techniques of *in vitro* transcription, UV-cross-linking, and electrophoretic analysis were used to identify and characterize proteins of nuclear and cytosolic origin that specifically bind to the sense RNA sequence which corresponds to the coding region of the protein D mRNA.

MATERIALS AND METHODS

Materials: The sources of the major experimental materials used in this study were as follows: A Gene Amp DNA Amplification Reagent Kit (Perkin-Elmer/Cetus), Random primers and RNasin, T_3 and T_7-RNA polymerase (Promega), Bluescript SK(+) vector (Stratagene), restriction, kinase, T_4DNA ligase, phosphatase, proteolytic enzymes, and transfer RNA (tRNA) (Boehringer Mannheim), RNAase T1 (Calbiochem), [^{32}P]CTP (>800 Ci/mmol, Amersham), [^{35}S]-dATP (>1000 Ci/mmol, New England Nuclear/-Dupont), Sequenase version 2.0 DNA Sequencing Kit (United States Biochemical Co.), glass microfiber filters (Whatman Biochem. Co.), and Geneclean Kit (Bio 101 Inc.). All other reagents used in this study were of molecular biology grade purchased from Sigma Chemical Co.

Animals: Sexually mature (>60 days) male Harlan Sprague-Dawley rats, weighing 300-400 g were used for all experiments. Four-six rats were used for each experiment which was repeated four times (N = 4 experimental replicates). Each epididymis was carefully sectioned into three anatomical segments: caput, corpus and cauda regions as described previously (11).

Plasmid Construction: Total RNA purification and polymerase chain reaction (PCR) amplification of a 670-base pair (bp) fragment corresponding to the coding region of protein D cDNA was performed as described previously (11). The 670-bp fragment was separated by electrophoresis on a 1.5% agarose gel. After electrophoresis, the 670-bp fragment was isolated from gel slices by using a Geneclean Kit. The blunt ends of the fragment were filled in and phosphorylated by using Klenow enzyme and polynucleotide kinase. The Bluescript SK(+) cloning vector was cleaved with *Hinc II* and the ends were dephosphorylated by using calf intestinal phosphatase. The cDNA fragment was inserted into the phagemids via blunt end ligation using T_4 DNA ligase and transformations performed by using the bacterial host, JM101. After identifying the positive clones, the plasmid DNA was isolated by using the alkaline lysis method (12).

Analysis of DNA Products: Double stranded DNA was sequenced by the dideoxynucleotide chain method (13) using a Sequenase 2.0 kit. DNA products generated by the sequencing reaction were resolved by electrophoresis on a 6% polyacrylamide/8 M urea gel in 0.089 M Tris/0.089 M boric acid/0.002 M EDTA buffer, pH 8.0. The sequence corresponding to the 670-bp fragment was verified by comparing it to the known cDNA sequence (9,10). The sequence of the 670-bp fragment amplified from caput, corpus and cauda total RNA was 99% homologous among the three epididymal regions.

***In Vitro* Transcription:** Radiolabeled and nonradiolabeled RNA transcripts were prepared from the caput, corpus and cauda protein D cDNA cloned in pBluescript plasmids using the Promega *in vitro* transcription system (Promega Technical Manual), with some modifications. These modifications included the following: (a) 2.0 µl of linearized template DNA, (b) 2.5 µl of 5'[α-^{32}P]CTP (50 mCi/ml), and (c) an incubation time of 1 hr. Incorporated radioactive CTP into protein D RNA was measured by using microfiber filters (2.4 cm x 22 cm) and scintillation counting.

Preparation of Nuclear and Cytosolic (S100) Fractions: Epithelial cells were recovered from the caput, corpus and cauda regions of the epididymis by enzymatic dispersion using the method of Skudlarek and Orgebin-Crist (14). The nuclear and cytosolic (S100) fractions were prepared from hypotonically lysed epithelial cells as outlined by Ausubel et al. (15). The purity and structural integrity of isolated nuclei was monitored by light and phase contrast microscopy. Nuclear and cytoplasmic contamination was monitored by assaying the activity of RNA polymerase, an enzyme of nuclear origin (16) and N-acetyl-β-D-hexosaminidase, an enzyme of cytoplasmic origin (17). Protein content of samples was determined by the Lowry method (18), with bovine serum albumin (BSA) as the standard.

RNA-binding Assay: Optimal conditions for the binding reaction were empirically determined and based on conditions established previously (19), with some modifications. These modifications included: (a) 100,000 cpm of [^{32}P]RNA transcript,(b) a reaction volume of 25 µl and (c) 20 µg of total protein. To assess the effect of salt concentration on the specificity of protein-RNA interaction, the binding reaction was performed in the presence of different concentrations of KCl (40 mM to 400 mM). The specificity of binding was assessed by incubating the nuclear and cytosolic fractions (20 µg of total protein) in the presence of 50, 100, and 200-fold excess unlabeled RNA followed by incubation in the presence of labeled protein D mRNA sequence. Nonspecific binding was assessed by incubating the binding reaction in the presence of different concentrations (0.1µg, 1.0 µg and 10 µg) of calf liver tRNA. To covalently cross-link proteins bound to the radiolabeled protein D mRNA sequence, the reaction mixture was exposed (30 min. at 10 cm) to a short-wave length (254 nm) of UV-light. Protein-RNA complexes were resolved by SDS-PAGE using a 15% uniform slab gel (17).

RESULTS AND DISCUSSION

Previously (11), we reported the use of PCR to demonstrate a significant reduction (>82%) in the level of expression for corpus protein D mRNA as compared to the caput and cauda regions. We suggested that the reduction in the level of expression of corpus protein D mRNA was the result of insufficient primer binding to protein D mRNAs. In the present study, we provide evidence that the reduced expression of corpus protein D mRNA may be due to a protein and/or peptide bound at or near the primer binding site. To test this possibility, total RNA was purified from the caput, corpus and cauda regions of the epididymis, subjected to proteinase K treatment and PCR amplified (Fig. 1). Proteinase K treatment of corpus total RNA increased mRNA expression to a level that was equal to that of the caput and cauda regions, indicating that a protein-like factor was associated with the corpus protein D mRNA (Fig. 1, lower panel)

To further investigate the interaction of proteins with RNA, an RNA-binding assay was used to identify proteins that bind to an *in vitro* transcribed protein D mRNA sequence. Two protein-RNA complexes (M_r = ~2.5 and ~35 Kd) were resolved electrophoretically (Fig. 2, see arrows). The banding intensity of these two complexes was greater in the nuclear fraction than in the cytosolic fraction (Fig. 2, lanes 2-4). Previous studies have shown that pre-mRNA molecules exist in the nucleus in association with specific protein complexes to form ribonucleoprotein (RNP) particles (20, 21). Thus, the possibility may

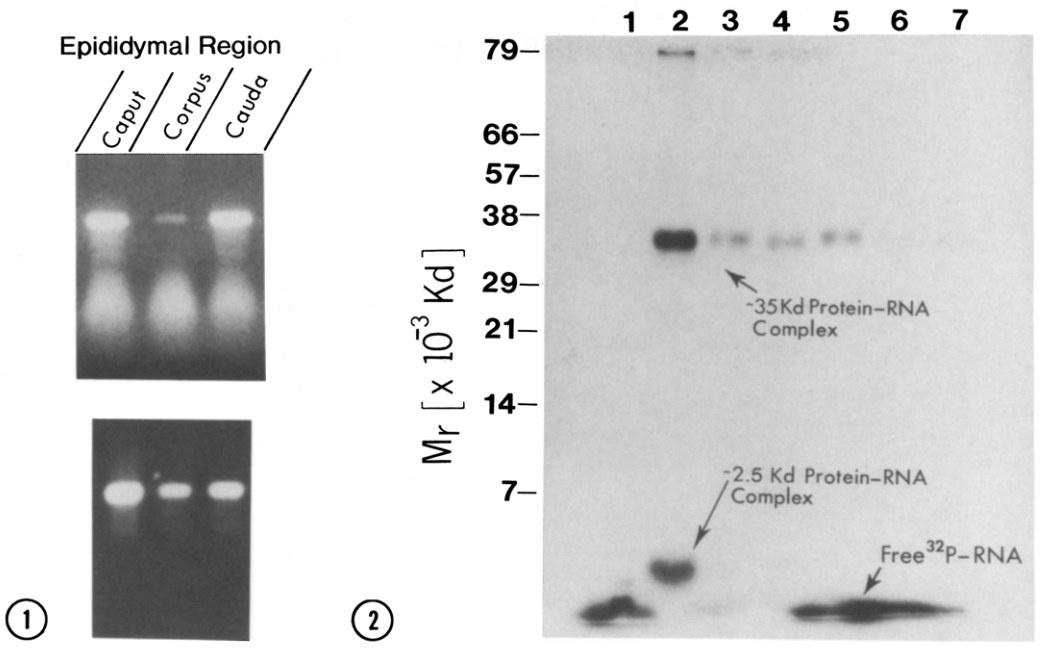

Figure 1. shows the effects of proteinase K treatment on the amplification of purified caput, corpus and cauda total RNA. Amplification before (**upper panel**) and after (**lower panel**) treatment.

Figure 2. SDS-PAGE analysis of UV-cross-linked proteins associated with the nuclear and cytosolic extract of caput, corpus and cauda epididymal cells to *in vitro* transcribed protein D mRNA sequence. Lanes 1 = control (radiolabeled protein D mRNA sequence with no extract), 2 = caput nuclear extract, 3 = corpus nuclear extract, 4 = cauda nuclear extract, 5 = caput cytosolic extract, 6 = corpus cytosolic extract, and 7 = cauda cytosolic extract.

exist that these proteins may be important for the biogenesis of mRNAs for specific proteins involved in process of sperm maturation within the epididymis.

To assess whether the protein-RNA interaction was mediated through tight or weak binding, the reactions were performed in the presence of different concentrations of KCl (Fig. 3). The ~2.5 and ~35 Kd protein-RNA complexes were stable in the presence of 0.4 M KCl (Fig 3, lanes 4,8 and 12), suggesting that the binding was extremely tight and that these proteins have a strong affinity for the protein D mRNA sequence *in vitro* .

To establish the specificity of the protein-RNA interactions, competition experiments were performed in the presence of tRNA and unlabeled protein D RNA (Fig. 4). In the presence of tRNA, the ~2.5 and ~35 Kd complexes were resolved electrophoretically (Fig. 4A, see arrows). The competition experiment using unlabeled sense and antisense RNA demonstrated a marked reduction in the banding intensity of the ~2.5 and ~35 Kd com-

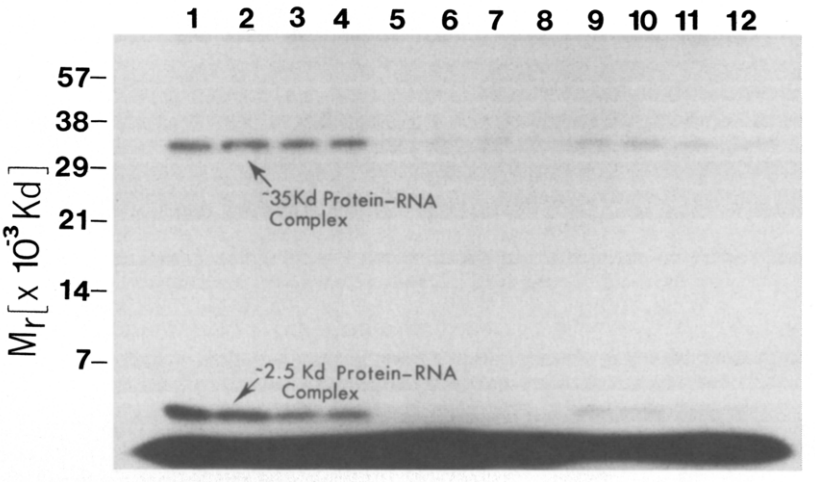

Figure 3. shows the effects of different KCl concentrations on the binding of proteins in the nuclear extract of caput, corpus and cauda epididymal cells to *in vitro* transcribed protein D mRNA sequence. Lanes 1-4 = caput nuclear extract at 40, 100, 200 and 400 mM KCl, 5-8 = corpus nuclear extract at 40, 100, 200 and 400 mM KCl, and 9-12 = cauda nuclear extract at 40, 100, 200 and 400 mM KCl.

plexes (Fig. 4B, lanes b-g). These data suggest that the proteins complexed to the *in vitro* transcribed RNA were specific for a unique sequence in the coding region of the mRNA. Although the proteins complexed to the mRNA sequence were found in the cytosolic fraction (Figs. 2-4), it is unlikely that the presence of these proteins in the cytosolic fraction was due to contamination. No evidence of RNA polymerase activity (I, II or III) was detected in the cytosolic fraction, suggesting that these RNA-binding proteins are located in the nucleus and cytosol of the cell. Since previous studies (20, 21) have demonstrated that proteins of nuclear and cytosolic origin exhibit RNA-binding specificity (20, 21), it is possible that these RNA-binding proteins may have a physiological role in regulating the expression of protein D genes at the level of transcription and/or translation in cells of the rat epididymis.

The concept of proteins as regulators of mRNA transcription and translation is further supported by studies in other eukaryotic cell systems (22-25), which demonstrated that proteins of nuclear and cytosolic origin can recognize and bind to specific RNA sequences of hnRNA, pre-mRNA and mRNA. These studies suggest that nuclear and cytosolic RNA-binding proteins may regulate gene expression through alternative splicing and/or control of mRNA translation. While the control of gene expression at the level of transcription has been well documented in eukaryotic systems (26), there are not many examples of translational control of gene expression in eukaryotes. The most well-studied form of translational

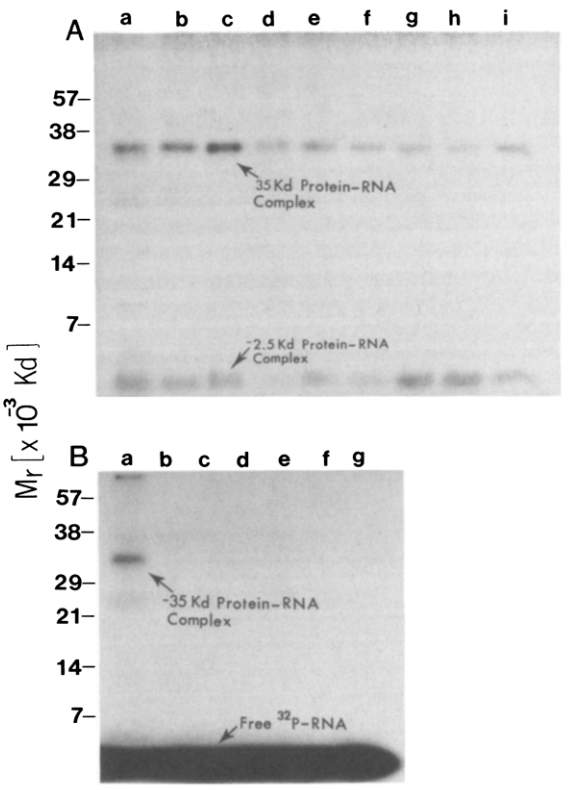

Figure 4. SDS-PAGE analysis of competition experiments. (**A**)Competition with 0.1 μg, 1.0 μg and 10 μg of tRNA; lanes a-c = caput nuclear extract, d-f = corpus nuclear extract, g-i = cauda nuclear extract. (**B**) Competition with 50, 100 and 200-fold excess of unlabeled sense and antisense *in vitro* transcribed protein D mRNA sequence; lanes a = caput nuclear extract with radiolabeled RNA alone, b-d = caput nuclear extract with radiolabeled and unlabeled sense protein D mRNA sequence, and e-g = caput nuclear extract with radiolabeled and unlabeled antisense protein D mRNA sequence.

control of gene expression involving a cytosolic protein is exemplified by iron-mediated control of ferritin, an iron storage protein (25). Considering that RNA-binding proteins have been implicated in the regulation of eukaryotic gene expression at the level of transcription and translation (22-26), it is of paramount importance to determine the physiological role that these proteins play in regulating the expression of proteins involved in the maturation of sperm in the epididymis. These studies are currently under investigation.

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