The rat poly Pyrimidine Tract binding protein (PTB) interacts with a single-stranded DNA motif in a liver-specific enhancer

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ABSTRACT

We characterized and purified a protein from rat liver which specifically binds to a DNA motif present in a liver-specific enhancer of the rat tyrosine aminotransferase (TAT) gene, when offered as single-stranded DNA. Binding is highly sequence-specific and coincides with a region known to be essential for function of the enhancer. Microsequencing revealed that this protein is the rat homologue of the mouse and human poly Pyrimidine Tract binding protein (PTB), which has been shown to bind to premRNA and may participate in RNA splicing. This finding was corroborated by subsequent Western blot experiments using a PTB-specific antibody. These findings indicate a possible dual role for this protein in RNA processing and transcription.

INTRODUCTION

Gene expression in eukaryotes is regulated by the interplay between specific cis-acting elements constituting enhancers or promoters and the cognate transcription factors whose expression or activity might be cell type-specific or affected by various signal transduction pathways (reviewed in 1–3). In the case of the rat tyrosine aminotransferase gene it has been shown that liver-specific and hormone-inducible transcription depends on the activity of three different enhancer elements located at −2.5 kb, −3.6 kb and −11 kb, with respect to the transcriptional start site (reviewed in 4, and 5). The enhancer located at −3.6 kb is strictly liver cell-specific and regulated by the cAMP signalling pathway. Two distinct elements are absolutely required for activity of this enhancer (6). Whereas one of the elements has been identified as a cyclic AMP-responsive element (CRE) which binds a known transcription factor, CREB (Nichols et al., in preparation), the nature of the protein binding to the second element (called BIII) remains unclear. Multiple copies of the BIII-element are sufficient to confer strong liver cell-specific transcriptional activation onto a heterologous promoter. Mutational analysis has defined a short stretch of 10 nucleotides

essential for the activity of this element (6). An element with highly homologous sequence has been identified in the promoter of the rat transferrin gene where it contributes to the liver-specific expression of the gene (7).

We identified a protein which specifically binds to the T-rich strand of the DNA probe. We attempted to purify this binding activity. To this end we developed a novel affinity chromatography method which is based on single-stranded DNA immobilized on Sepharose. Microsequencing of 3 polypeptides purified revealed that they are related and correspond to the rat homolog of the human poly-Pyrimidine Tract Binding protein (PTB) which is known for its ability to bind RNA with a certain sequence preference (8). The potential role for this protein in the process of enhancer activation is discussed.

MATERIALS AND METHODS

Preparation of nuclear extracts

Whole cell extracts of cultured cells were prepared according to (9) with the modifications introduced in (10). Extracts from rat livers were prepared by a modified protocol according to Dignam et al. (9). Briefly, livers of male adult rats were washed in PBS, cut with scissors and homogenized in 1 volume of buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) in a motor driven Potter S homogenizer by 3 strokes with a loose pestle. Subsequently, nuclei were collected by a 20 min spin in a SS34 rotor (19000 rpm, 4°C) and resuspended in an equal volume of buffer C (20 mM Hepes pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT). 1/20 volume of a saturated NH_4SO_4 solution was added and the mixture stirred for 1 hr at 4°C. After 1 h of centrifugation at 35 000 rpm in a Beckman Ti45 Rotor the clear supernatant was precipitated by addition of 0.33 g/ml of solid ammoniumsulfate. After stirring for 1 h at 4°C the precipitate was collected by centrifugation (30′, 35 000 rpm, Ti45) and redissolved in TGK_{30} (20 mM Tris pH 7.9, 30 mM KCl, 10% glycerol, 1 mM DTT) followed by dialysis against TGK_{30} overnight with one change.

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Bandshift experiments

Single stranded DNA (200 ng) corresponding to the A-rich or T-rich strand of the BIII motif (6) which had been chemically synthesized, was 5' end-labelled with 40 µCi of ^{32}P-γATP using T4 Polynucleotide kinase. The labelled fragment was gel-purified and diluted to 10 000 Cerenkov counts/µl (0.1 ng/µl). For experiments with ds DNA either the A-rich or T-rich single strand was selectively labeled as described; after heat inactivation of the kinase an equimolar amount of the complementary strand was added and allowed to anneal. Double-stranded DNA was gel purified and diluted to 10000 cpm/µl. 1 µl of the probe was used in a 10 µl bandshift reaction containing 2 mM $MgCl_2$, 10% glycerol, 500 ng of poly dAdT and 1 to 3 µl of protein fraction. In experiments with affinity-purified protein the non-specific competitor DNA was omitted. After 10 min at room temperature the reaction was loaded on a native 6% polyacrylamide gel containing 0.3 × TBE as running buffer. Electrophoresis was for 2 hrs at 200 V/40 mA. The gels were dried and exposed to Fuji X-ray film.

Missing contact analysis

The T-rich strand of the BIII oligonucleotide (6) was 5'-end labelled, redissolved in 50 µl TE and treated with hydrazine (30 µl) for 30 min at RT (11). 1 ml of 1-butanol was added; the solution was mixed by vortexing and the DNA recovered by centrifugation (5 min RT) in an Eppendorf centrifuge. After two reprecipitations by ethanol the DNA was dried and diluted to 30 000 cpm/µl in TE. Preparative bandshifts were performed with the modified probe; the complexes and free DNA samples were excised, electroeluted and incubated with piperidine (1 M) for 30 min at 90°C. The DNA was purified and loaded on a 20% polyacrylamide sequencing gel. The gel was dried and exposed for autoradiography.

Southwestern blotting

Protein from the nuclear extract or a derived fraction was separated on a 10% polyacrylamide/SDS gel and electroblotted onto a nylon membrane (Nytran, Schleicher & Schüll), since initial experiments with nitrocellulose and PVDF membranes gave very poor signals. The membrane was blocked by overnight incubation in binding buffer (10 mM Hepes pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol) containing 5% nonfat dry milk. The filters were washed three times in the same buffer containing 0.25% dry milk and incubated in 15 ml binding buffer containing 0.25% dry milk, 10 µg poly dAdT and 10×10^6 cpm of the labelled single-stranded oligonucleotide. After 18 hr incubation at 4°C the filters were washed in binding buffer, airdried and exposed for autoradiography.

Purification of PTB

Nuclear extract from rat liver (100 ml) was passed over a Blue Sepharose column. The column was eluted with a gradient of 900 ml ranging from 30 to 1000 mM KCl. The specific singlestrand binding protein eluted at 800 mM KCl, as monitored by the bandshift assay. The active fractions were pooled (60 ml), dialyzed and applied to a DEAE Sepharose column. From this column the protein eluted at 160 mM KCl. Active fractions were pooled (30 ml) and applied to a specific, single-stranded DNA column which had been prepared by coupling a 53 nt oligonucleotide, corresponding to two binding sites for PTB in the T-rich strand, to CNBr-activated Sepharose, following the

protocol described for ds DNA (12). By this method we were able to immobilize 60 µg of DNA per ml of resin. The protein bound very avidly to the column, it was eluted at 0.8 M KCl, thereby allowing the separation of PBT from contaminating proteins which bound to single-stranded DNA without any sequence preference (data not shown). The protein in the affinity purified fractions was stabilized by the addition of 500 µg insulin (Sigma) per ml.

Peptide sequencing

Affinity-purified protein from rat liver was concentrated to a final volume of 50 µl using the Centricon system (Amicon Inc.).

Figure 1. Identification of a sequence-specific DNA binding protein which interacts with single-stranded DNA. (A) DNA fragments used in this study. The nucleotide sequence of the BIII motif in the rat TAT gene is shown between positions −3610 and −3578, with respect to the transcriptional start site. The sequences of the single-stranded oligonucleotides used in this study are also indicated; annealing of ssA$_{WT}$ and ssT$_{WT}$ yielded the double-stranded DNA used in some experiments. (B) Partially purified protein from FTO-2B cells, eluted from Blue Sepharose at 300 mM KCl, was incubated with 5'-end labelled single-stranded DNA corresponding to the A-rich (lanes 1−3) or T-rich (lanes 8−12) strand of the BIII motif. The nucleoprotein complexes were separated on a native polyacrylamide gel. The same protein fraction was incubated with double-stranded DNA, labeled at either the A-rich (dSAWT, lanes 4−7) or the T-rich (dSTWT, lanes 13−16) strand, which was subjected to prior heat treatment as indicated. Specificity of the complexes was assayed by incubation with an 100-fold excess of unlabeled single-stranded DNA of either the wild type or mutant (M12) sequence (T-rich strand). Competition with the A-rich strand of the wild type sequence was performed at 100-fold (lane 10) and 400-fold (lane 11) molar excess. Complexes B and D represent the sequence-specific single-strand binding protein. Whereas in a denaturing polyacrylamide gel the A-rich and T-rich single-strand comigrate, the aberrant migration of the T-rich strand in the native gel (best seen by comparing the free probe in lanes 1 and 11) most likely is a consequence of a particular secondary structure formed by this DNA single strand. In fact, the T-rich single strand comigrates with the double-stranded probe DNA (compare e.g. lanes 11 and 13).

Proteins were separated on a 10% SDS-PAGE as shown in fig. 3 C. The gel was stained with Coomassie brillant Blue; the three bands, corresponding to 5 μg each, were precisely excised from the gel. The gel slices were cut in pieces (about 1 × 1 mm) and incubated in water with frequent changes for 16 hr. The washed gel pieces were then immersed in 200 μl of 100 mM ammonium hydrogen carbonate (pH 8.5) containing trypsin at an enzyme/protein mass ratio of approximately 1:5. Following incubation at 37°C for 8 h, the protein fragments produced were eluted from the gel by shaking the pieces twice for three hours with an equal volume of 0.1% trifluoroacetic acid in water. Residual water was extracted from the gel matrix by treatment with acetonitrile. The concentrated eluates were extracted twice with isoamyl alcohol/heptane (1:4) to remove traces of SDS (13). Separation of the tryptic peptides was performed by reversed-phase high-performance liquid chromatography on a Vydac 218TP5 column (1.6 × 250 mm). Peaks were selected from the elution diagram and the material was subjected to gas-phase sequence analysis according to Gausepohl et al. (14).
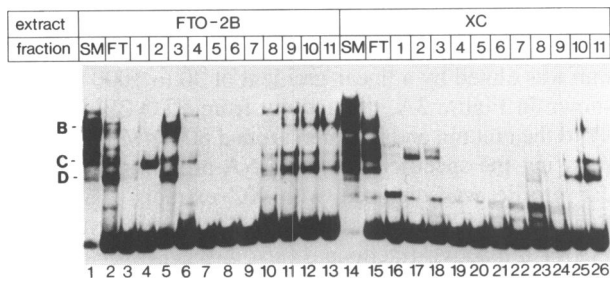
Figure 2. Purification of the single-stranded DNA binding protein from FTO-2B and XC cells on Blue Sepharose. Crude extracts from FTO-2B cells and XC cells were passed over Blue Sepharose and eluted with a linear gradient of 11 fractions containing 30 to 1000 mM KCl. 2 μl of extract (starting material, SM), the column flowthrough (FT) and each fraction were tested in the bandshift assay as detailed in Material and Methods, using the 5′-end labelled T-rich strand of the BIII motif. Designation of nucleoprotein complexes as in Figure 1 B.

Western blot experiments

Proteins were separated on a 10% SDS-polyacrylamide gel and blotted to nitrocellulose. The filters were blocked and treated with the antipeptide serum and preimmune serum, respectively. Antibody recognition was monitored by a secondary biotinylated antibody and streptavidin-coupled phosphatase from the Amersham kit, following the suppliers instruction. For approximate size estimation, we used biotinylated MW markers (Bio-Rad) which are costained by the detection procedure.

Denaturation/Renaturation experiments

Proteins were separated on a 10% SDS polyacrylamide gel. MW ranges were inferred from the positions of the bands of a prestained MW marker (Bio-Rad) run in parallel on the same gel. Gel slices were denatured and renatured following the protocol of Hager and Burgess (15).

RESULTS

In an attempt to characterize proteins binding to the BIII element of the enhancer located at −3.6 kb of the TAT gene, we performed bandshift experiments with a double-stranded DNA fragment of 32 bp centered around the essential 10 bp-motif defined by mutational analysis of the enhancer (Figure 1 A; reference 6). The DNA fragment was 5′-end labelled and incubated with nuclear extracts derived from either rat liver, cultured hepatoma cells (FTO-2B) or fibrosarcoma cells (XC). In further experiments fractionated extracts were tested by the same technique. We were able to identify a specific nucleoprotein complex which was competed by the wild type DNA much better than by the M12 mutant. While this complex was observed initially in experiments designed to identify binding to a double-stranded DNA fragment, we realized that it actually represents binding to single-stranded DNA which arose accidentally by melting of the double-stranded probe. This observation was confirmed by a control experiment, where the bandshift experiment was performed using either strand as a labelled probe; alternatively, the double-stranded DNA, labelled at either the A-
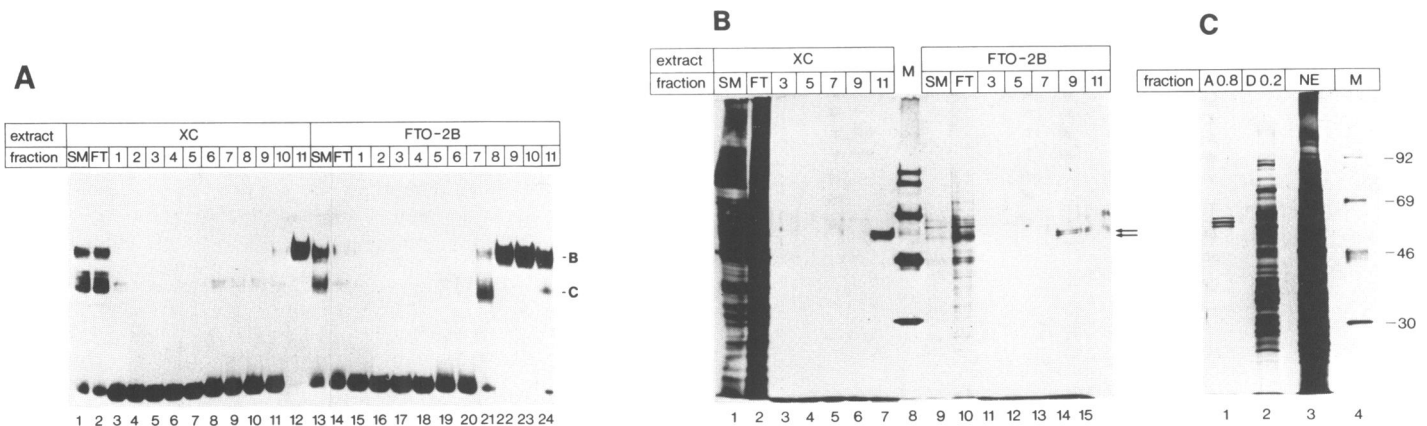
Figure 3. Affinity chromatography of the single-stranded DNA binding protein. (A) The active fractions derived by chromatography on Blue Sepharose from FTO-2B cells and XC cells were passed over the column containing the T-rich strand of BIII attached to Sepharose. The column was eluted by a gradient from 30 to 1000 mM KCl, in 11 fractions each. 2 μl of each fraction were tested in the bandshift assay. Designation of complexes as in Figure 1 B. (B) Aliquots (50 μl) of the fractions shown in panel A were separated by SDS/PAGE on a 10% gel, which was subsequently stained by silver nitrate. M: MW marker, as in panel C. (C) Single-stranded DNA binding protein from rat liver was purified on Blue Sepharose, where it eluted at 0.8 M KCl, on DEAE Sepharose where it eluted at 0.2 M KCl (D 0.2), and finally, on the the specific single-strand DNA column where it eluted at 0.8 M KCl (A 0.8). Aliquots of the nuclear extract and the active fractions of each chromatography were separated by 10% SDS/PAGE followed by silver staining. Sizes of the molecular weight marker proteins are given in kD.

rich or T-rich strand, was used with or without a prior heat treatment, which denatured the probe. As shown in Figure 1 B, several nucleoprotein complexes (designated A, B, C, and D) with partially purified protein from FTO-2B cells are readily formed on the T-rich single strand (ssT*$_{WT}$, lane 8), while only very little complex formation occurs on the complementary, A-rich strand (ssA*$_{WT}$, lane 1). The double-stranded DNA fragment labelled at the T-rich strand does not bind any protein in this assay (lane 13), whereas a strong bandshift is observed when the probe is heat-denatured prior to the assay (lane 14). The double-stranded DNA probe labelled at the A-rich strand does not bind any protein even after heat denaturation (lane 5). This observation confirms that binding of the proteins characterized in this study is highly selective for the T-rich single strand. Specificity of two distinct nucleoprotein complexes (Bands B, D) is demonstrated by their titration with a 100-fold molar excess of the wt sequence but not the M12 mutant when added as single-stranded DNA (lanes 9,12 and lanes 15,16). Addition of the A-rich single strand as unlabeled competitor in high excess interferes with the formation of all complexes (lanes 10, 11). Since the A-rich strand did not display binding (lanes 1−3), we assume that under these conditions the T-rich single strand anneals with the complementary strand; the resulting double-stranded DNA is not capable of binding the protein (cf lanes 4, 13). Taken together, these data suggest that there is a protein(s) present in FTO-2B cells which recognizes the T-rich strand of the BIII element of the TAT enhancer and that binding is abolished by the M12 mutation.

Extracts were prepared from FTO-2B and XC cells, passed over a Blue Sepharose column and eluted by a linear gradient from 30 to 1000 mM KCl. DNA binding activity in each fraction was monitored by the bandshift assay, using the 5'-end labelled

T-rich strand of the BIII oligonucleotide as a probe. It appears that several distinct DNA binding proteins can be separated by this technique. A major DNA binding protein, characterized by complexes B and D, elutes from the Blue Sepharose column at 300 mM KCl (fraction 3) in the case of the FTO-2B extract, whereas similar complexes are formed with the column flow-through in the case of the XC cells (Figure 2). The specificity of complexes B and D from FTO-2B cells has been shown in Figure 1 B. Identical results were obtained, when complexes B and D from XC cells were analyzed by a similar competition experiment (data not shown, see below). While some additional minor shifts do not vary between the cell lines, the different elution profile for the major DNA binding protein has been observed repeatedly and may thus represent a cell-specific modification of this particular protein.

For further characterization of the protein we purified it by means of DNA affinity chromatography. Since the protein binds specifically to single-stranded DNA of the BIII sequence, we prepared a column containing a tandem copy of the T-rich strand of the BIII oligonucleotide immobilized on CNBr-activated Sepharose. The active (Blue Sepharose) fractions derived from either the FTO-2B (fraction 3) or the XC (FT) extract were dialyzed and passed over the single-stranded DNA column. The column was eluted by a linear gradient of 30 to 1000 mM KCl. As shown in Figure 3A, the activity from FTO-2B cells binds avidly to the column and is eluted around 800 mM KCl, further emphasizing the specificity of the DNA-protein interaction. A similar profile was obtained with XC extracts. The activity involved in formation of complex D was not recovered after dialysis of the active fractions.

Analysis of the column fractions on a silver-stained SDS polyacrylamide gel reveals that the DNA binding activity copurifies with several polypeptides in the range of 50−55 kD molecular weight (Figure 3 B). Purification of the protein involved in formation of complex B from rat liver was achieved by a similar protocol, where the protein was chromatographed over Blue Sepharose, DEAE sepharose and finally, the specific
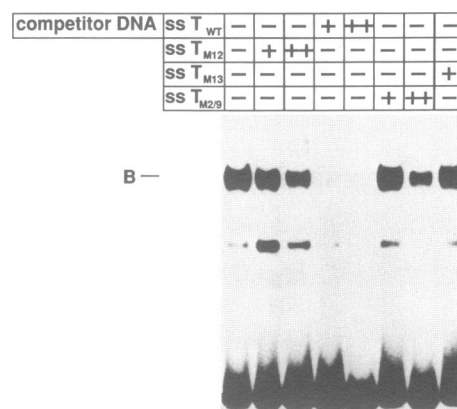

Figure 4. The single-strand DNA binding proteins from different sources interact with the same sequence motif. Bandshift experiments were performed with affinity purified protein from FTO-2B, XC and rat liver, using 5'-end labelled single-stranded DNA which had been modified by hydrazine (ref. 8, see text). Piperidine cleavage of the free (F) and bound (B) DNA was followed by denaturing gel electrophoresis. The contact points of the protein are indicated by missing bands in the bound (B) fraction (open circles).

competitor DNA	ss T$_{WT}$	−	−	−	+	+++	−	−	−
	ss T$_{M12}$	−	+	+++	−	−	−	−	−
	ss T$_{M13}$	−	−	−	−	−	−	−	+
	ss T$_{M2/9}$	−	−	−	−	−	+	+++	−

Figure 5. Binding of the protein to single-stranded DNA is sensitive to mutations M12, M13 and the M$_{2/9}$ mutant. The single-stranded DNA binding protein, purified from rat liver by ssDNA affinity chromatography, was incubated with the 5'-end labelled T-rich strand of the BIII element in the bandshift assay. To test for binding specificity, formation of the nucleoprotein complex was challenged by a 20-fold (+) and 100-fold (+ +) excess of unlabeled DNA of the wt sequence or the M12, M13 and M2/9 mutants as indicated. Designation of complexes is as in Figure 1 B.

single-stranded DNA column. In this case, the affinity-purified fraction also contains essentially three polypeptides in the range of 50 to 55 kDa (Figure 3C).

To further address the specificity of the DNA-protein interactions, we wished to establish the contact points of the protein on the single-stranded DNA probe. Experiments applying the DMS interference technique potentially detecting G and A contacts did not reveal any specific contact in the BIII sequence (data not shown). Therefore, we attempted to monitor the involvement of particular pyrimidines in formation of the specific complex (11). To this end, pyrimidines of the probe DNA were cleaved off by limited hydrazine treatment; this probe was then used in a preparative bandshift experiment. The DNA in the specific nucleoprotein complex and unbound DNA were isolated, cleaved with piperidine, and analyzed on a denaturing gel. Missing of a band in the bound fraction indicates that the protein cannot bind the probe due to a missing pyrimidine contact at that site. The contact points obtained for the protein purified from FTO-2B cells, XC cells and rat liver were virtually indistinguishable (Figure 4). Chemical modification of either T or C residues in the TCT motifs which occur twice in the binding site TCTTTGATCT interferes with complex formation. This result confirms the specificity of the DNA-protein interaction; it is worth to note that the contacts established for this particular protein map precisely within the mutation-sensitive domain of the enhancer (6).

As the most prominent interference occured over the central C of either TCT motif, i.e. at positions 2 and 9 in the decanucleotide, we designed a specific point mutation $M_{2/9}$ which changes these two cytosines into adenosine. The binding of the affinity purified protein to the mutant sequences M12, M13 and $M_{2/9}$ (see Figure 1 A) was assayed in a competition

experiment, where binding to the wild-type oligonucleotide probe was challenged by an excess of unlabeled probe of the wild-type or mutant sequence. The experiment shows that, while addition of a 20-fold molar excess of the wild-type probe completely wipes out the specific complex, much higher concentrations of the M12, M13 or $M_{2/9}$ fragment are required to obtain a similar effect (Figure 5). These results, which are in good agreement with the contact point analysis, further emphasize the high selectivity of binding.

Since the affinity chromatography revealed very similar protein banding patterns starting from three different sources, and since purification was achieved to near homogeneity, it seems very likely that the polypeptides in the range of 50−55 kD actually represent the DNA binding activity. This was confirmed in two control experiments. First, semi-purified protein from rat liver, present in the active fraction of the DEAE sepharose column (D0.2), was separated by SDS-PAGE, transferred to a nylon membrane and probed by 5′-end labelled single-stranded DNA of either the wild type or the mutant ($M_{2/9}$) sequence ('South Western blot'). Two bands in the range of 52−55 kDa were specifically bound by the wild type but not the mutant probe. A protein of about 70 kDa bound both probes with similar affinity suggesting the presence of a non-specific single-strand binding activity. The additional bands of lower apparent MW may represent proteolytic products of the major protein (Figure 6A). In a second experiment protein of the same fraction was separated by SDS-PAGE; the gel was cut into slices corresponding to a molecular weight of >120 kD, 106−120 kD, 90−106 kD, 65−90 kD, 48−65 kD, 40−48 kD, 30−40 kD and < 30 kD. Protein was renatured from the gel slices (17) and tested for binding of the single-stranded probe. Only the gel slice corresponding to a MW of 48 to 65 kD gave rise to a specific
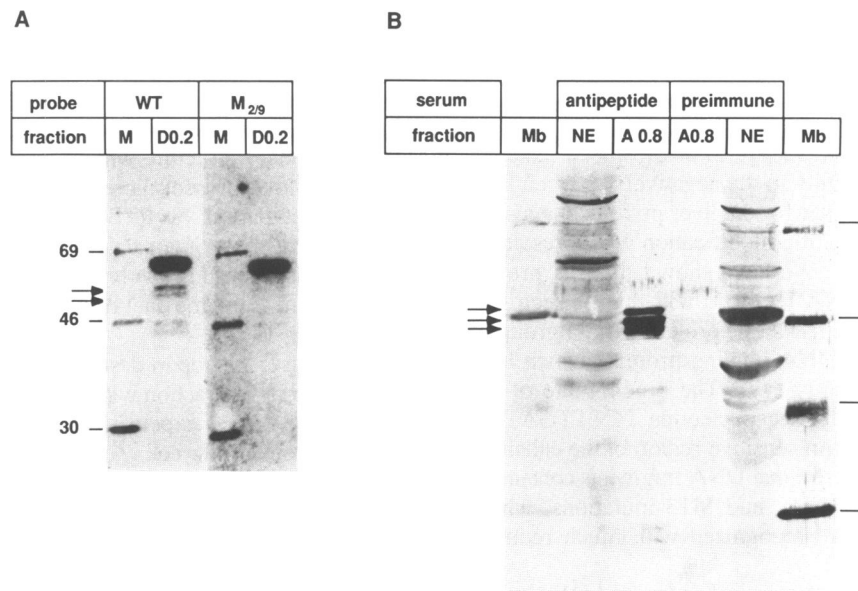

A

probe	WT		$M_{2/9}$	
fraction	M	D0.2	M	D0.2

B

serum		antipeptide		preimmune		
fraction	Mb	NE	A 0.8	A0.8	NE	Mb

69 —

46 —

30 —

Figure 6. The single-stranded DNA binding protein is the rat homologue of mouse PTB. (A) 50 μl of the active fraction of the DEAE sepharose chromatography were separated on a 10% SDS-poly acrylamide gel and transferred to a Nylon membrane. The membrane was incubated with 5′-end labelled single-stranded DNA of either the wt or the $M_{2/9}$ sequence. M: ^{14}C-labelled size markers. Arrows point to the major polypeptides recognized specifically by the wt probe. (B) Affinity purified protein from rat liver was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with the rabbit antiserum raised against a 12 aa peptide from rat PTB (16) or the corresponding preimmune serum. The blot was stained by alkaline phosphatase coupled to a secondary antibody via biotin/streptavidin. Arrows point to the three bands which are recognized by the specific antiserum. Biotinylated marker proteins (M_b, obtained from Bio-Rad) were used to control transfer of the proteins.

bandshift which was competed by an excess of unlabeled wild type but not mutated probe (data not shown).

Taken together, these data clearly demonstrate that indeed the polypeptides of 50−55 kDa represent the DNA binding species. We purified about 15 µg of the protein from rat liver, separately excised the three bands from a Coomassie Brillant Blue-stained gel and digested the protein with trypsin (14). The resulting peptides were separated by HPLC where the patterns were almost undistinguishable for the three protein bands. The amino acid sequence was established for six different peptides: NNQFQ, VTNLLM, DYTRPD, IAIPGL, VTPQSLFI, and GQPIYIQF. Identical sequences were obtained for all three polypeptide bands. When we screened the EMBL/Swiss Prot databank with these sequences, we found a 100% match with the amino acid sequence of a mouse nuclear protein, which is known as poly-Pyrimidine Tract Binding Protein (PTB, A. Bothwell et al., manuscript submitted). The identity of our protein with PTB was further confirmed by a Western blot experiment using an antibody, a gift of Mario Zakin, raised against an additional peptide (12 aa) from the C-terminus of a protein from rat liver which has been cloned by the group of Mario Zakin and shown to be 95% homologous to murine PTB (16). As shown in Figure 6B, all three polypeptides obtained from rat liver are specifically recognized by the specific antiserum, but not the corresponding preimmune serum. These results clearly indicate that indeed the rat homolog of murine PTB recognizes the T-rich strand of the BIII motif present in the TAT enhancer at −3.6 kb.

DISCUSSION

We have purified a DNA binding activity from rat liver nuclei, which interacts with single-stranded DNA from the enhancer located at −3.6 kb of the rat tyrosine aminotransferase gene. This activity copurifies with a set of three related polypeptides in the range of 50 to 55 kDa. The protein has been found in several cell lines and tissues of different origin; however, the protein isolated from rat liver or cultured hepatoma cells displays a different behavior in the purification procedure, as compared to the protein from fibroblasts (Figure 2) or rat spleen (data not shown). Specifically, the affinity to the negatively charged Blue Sepharose matrix is much higher for the liver protein, suggesting the occurrence of a tissue-specific modification which results in a higher positive net charge. We have shown that the protein binds the single-stranded DNA template with high selectivity, a finding that clearly discriminates this protein from a group of well known single-stranded DNA binding proteins which bind to DNA without any specificity (17). The binding site of the protein has been mapped to the decanucleotide TCTTTGATCT which coincides with a mutation-sensitive region of the enhancer (6). Furthermore, we have shown that DNA fragments containing the previously characterized M12 and M13 mutations, which destroy enhancer activity, are recognized with much reduced affinity.

Recently, it has been shown that a single-stranded DNA motif in the rat transferrin promoter is recognized by pYBP, a sequence-specific single-stranded DNA binding protein isolated from rat liver (16). When we tested binding of the protein we isolated from rat liver to the PRI motif of the transferrin promoter (7), we observed that the T-rich single strand of this motif was readily bound, albeit with much reduced affinity, as compared to the oligonucleotide derived from the TAT BIII motif (data not

shown). Interestingly, 9 bases of the essential decanucleotide are conserved in the PRI sequence, as the only alteration the T in position 8 is changed into a C. This observation further confirms the high sequence selectivity of the protein. We have shown by peptide sequencing that the protein we purified from rat liver corresponds to the rat homolog of the mouse poly Pyrimidine Tract Binding protein (PTB) which has recently been cloned (Bothwell et al., submitted). The human homolog of this protein has been shown to bind to cellular RNA which is a substrate for the splicing apparatus (8, 18, 19). Therefore, one function of PTB may be to participate in mRNA maturation. It has been shown that binding of PTB to premRNA shows considerable sequence specificity; in addition, binding to RNA can be competed by single-stranded DNA oligonucleotides of identical sequence (20). In this report, we show that PTB binds to single-stranded DNA derived from an enhancer motif, a finding that potentially implicates this protein in the process of transcriptional acitvation.

Whereas the existence of bona fide transcription factors binding to single-stranded DNA has not been unambiguously established so far, proteins which bind to single-stranded DNA of particular enhancers have been described: the human estrogen receptor has been reported to bind the 'coding' strand of the estrogen responsive element of the vitellogenin gene with much higher affinity than the corresponding double-stranded DNA (21); more recently, it has been reported that the purified receptor does not bind to the ERE with high affinity, but that binding of the receptor is greatly facilitated by a single strand DNA binding protein which displays considerable sequence specificity (22). Motifs that are recognized by single strand DNA binding proteins have been identified in the enhancers of some muscle-specific genes (23) as well as some adipocyte-specific genes (24). In the case of the SV40 early promoter, a protein has been identified which binds to single strand DNA of the promoter and greatly stimulates transcription in a cell-free system (25). From these reports, it seems likely that transcriptional activation in eukaryotes is controlled not only by the more canonical transcription factors which bind double-stranded DNA motifs; instead there may exist another level of control which involves regulatory features like DNA secondary structure which imply temporal melting of the DNA. While at present there is no conclusive evidence for an active participation of PTB in the process of either RNA processing or transcriptional activation, it cannot be excluded that a single gene product is involved in different regulatory pathways, the precedent being the dual role of NF-I/CTF in replication and transcription (26).

In summary, this report describes the purification of PTB from rat liver and its interaction with the TAT enhancer, and provides the basis for further experiments to definitely establish the role of this protein in the cell.

REFERENCES

1. Dynan, W.S. (1989) *Cell* **58**: 1–4.
2. Johnson, P.F. and McKnight, S.L. (1989) *Ann. Rev. Biochem.* **58**: 795–839.
3. Mitchell, P.J. and Tjian, R. (1989) *Science* **245**: 371–378.
4. Nitsch, D., Stewart, A.F., Boshart, M., Mestril, R., Weih, F. and Schütz, G. (1990) *Mol. Cell. Biol.* **10**:3334–3342.
5. Nitsch, D., Ruppert, S., Kelsey, G., Schedl, A., Weih, F., Stewart, A.F., Strähle, U., Schmid, W., DeVack, C., Reik, A., Boshart, M. and Schütz, G. (1991) In Cohen, P. and Foulkes, J.G. (eds.), The hormonal control regulation of gene transcription. Elsevier Science Publishers B.V., pp. 223–234.
6. Boshart, M., Weih, F., Schmidt, A., Fournier, R.E.K. and Schütz G. (1990) *Cell* **61**:905–916.
7. Brunel, F., Ochoa, A., Schaeffer, W., Boissier, F., Guillou, Y., Cereghini, S., Cohen, G.N. and Zakin, M.M. (1988) *J. Biol. Chem.* **263**: 10180–10185.
8. García-Blanco, M.A., Jamison, S.F. and Sharp, P.A. (1989) *Genes & Dev.* **3**:1874–1886.
9. Dignam, J. D., Lebovitz, R.M. and Roeder, R.G. (1983) *Nucleic Acids Res.* **11**: 1475–1489.
10. Boeuf, H., Reimund, B., Jansen-Dürr, P., and Kédinger, C. (1990) *Proc. Natl. Acad. Sci. USA* **87**: 1782–1786.
11. Brunelle, A., and Schleif, R.F. (1987) *Proc. Natl. Acad. Sci. USA* **84**: 6673–6676.
12. Baeuerle, P.A., and Baltimore, D. (1989) *Genes & Dev.* **3**: 1689–1698.
13. Bosserhoff, A., Wallach, J. and Frank, R.W. (1989) *J. Chromatograph.* **473**: 71–77.
14. Gausepohl, H., Trosin, M. and Frank, R.W. (1986) In Wittmann-Liebold, B., Salnikow, J. and Erdmann, V.A. (eds.), Advanced methods in protein microsequence analysis. Springer Verlag, Berlin, pp. 149–160.
15. Hager, D.A. and Burgess, R.R. (1980) *Anal. Biochem.* **109**: 76–93.
16. Brunel, F., Alzari, P.M., Ferrara, P. and Zakin, M.M. (1991) *Nucleic Acids Res.* **19**: 5237–5245.
17. Cox, M.M. (1987) *Ann. Rev. Biochem.* **56**: 229–62.
18. Gil, A., Sharp, P.A., Jamison, S.F. and Garcia-Blanco, M.A. (1991) *Genes & Dev.* **5**: 1224–1236.
19. Patton, J.G., Mayer, S.A., Tempst, P. and Nadal-Ginard, B. (1991) *Genes & Dev.* **5**: 1237–1251.
20. Wang, J. and Pederson, T. (1990) *Nucleic Acids Res.* **18**: 5995–6001.
21. Lannigan, D.A. and Notides, A.C. (1989) *Proc. Natl. Acad. Sci. USA* **86**:863–867.
22. Mukherjee, R. and Chambon, P. (1990) *Nucleic Acids. Res.* **18**: 5713–5716.
23. Santoro, I.M., Yi, T.-M. and Walsh, K. (1991) *Mol. Cell. Biol.* **11**: 1944–1953.
24. Wilkison; W.O., Min, H.Y., Claffey, K.P., Satterberg, B.L. and Spiegelman, B.M. (1990) Control of the adipsin gene in adipocyte differentiation. *J. Biol. Chem.* **265**:477–482.
25. Gaillard, C. and Strauss, F. (1990) *J. Mol. Biol.* **215**:245–255.
26. Santoro, C., Mermod, N., Andrews, P.C. and Tjian, R. (1988) *Nature* **334**: 218–223.