Association of the rat heterogeneous nuclear RNA-ribonucleoprotein F with TATA-binding protein[1]

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Abstract Heterogeneous nuclear ribonucleoprotein F (hnRNP-F) has been shown to be a pre-mRNA splicing factor. Recent studies have uncovered the coordination of synthesis of pre-mRNA and its processing, including post-transcriptional modification and splicing. Here, we present evidence for an association between a splicing factor, hnRNP-F, and TATA-binding protein (TBP), which is an essential factor needed for transcription initiation. An affinity detection experiment revealed hnRNP-F in the preparation of TBP-interacting proteins. HnRNP-F was associated with TBP in nuclear extracts and was capable of direct binding to TBP in vitro. These results suggest that hnRNP-F is associated with TBP in the cell. HnRNP-F was observed in abundance in the thymus, spleen and testis, and its distribution pattern was similar to that of TBP, implying a functional coordination of transcription and splicing. We assume that the splicing machinery is associated with the transcription apparatus as a prerequisite prior to transcriptional elongation.

Key words: Heterogeneous nuclear ribonucleoprotein F; Splicing factor; TATA-binding protein; Transcription factor

1. Introduction

Initiation of transcription by RNA polymerase II (RNAP II) requires functional assembly of the pre-initiation complex (PIC) at a promoter [1,2]. The PIC contains multiple general transcription factors (GTFs) and some transcriptional regulators and mediators. When nucleotide substrates are added to the PIC, RNAP II enters the elongation mode [3]. The elongating RNAP II does not contain GTFs other than TFIIF [2,3]. Nascent pre-mRNAs transform to mature mRNAs through various modification processes, including splicing, polyadenylation, and capping [4–6]. The results of several studies suggest that transcription and pre-mRNA processing do not occur independently in vivo [4–6]. It has been demonstrated that pre-mRNA processing factors physiologically interact with the transcription machinery [4,5]. The antibody against RNAP II precipitated SR proteins [7–11] and spliceosomal snRNPs from mammalian cell extracts [1,12]. McCracken et al. [13] reported that an mRNA processing factory exists on the C-terminal domain (CTD) of the largest subunit of RNAP II. These observations mean that transcription elongation and post-transcription processes are coupled.

Dantonel et al. [14] reported that the cleavage-polyadenylation specificity factor (CPSF) was associated with TFIID, which is one of the GTFs for transcription initiation composed of essential TBP (TATA-binding protein) and its associated factors, and they proposed that the pre-mRNA modification factors can be included in the PIC. It is possible that pre-mRNA processing factors may already be associated with transcription initiation factors prior to pre-mRNA synthesis. Consequently, a splicing factor may also be associated with the PIC or may bind to some GTF, though no clear evidence for this has been provided so far. Here, we present evidence of the association of a splicing factor, hnRNP-F (heterogeneous nuclear ribonucleoprotein F) with TBP.

2. Materials and methods

2.1. Histidine-tagged TBP-mediated affinity detection for TBP-interacting proteins

Bacterially expressed histidine-tagged mouse TBP (HXmTBP) was purified to near homogeneity as previously described [15]. Rat liver nuclear extract (8 mg) prepared as previously described [16] was incubated with HXmTBP (0.7 mg) at 4°C for 12 h. Ni-agarose beads (80 μl) (Qiagen) were added to the mixture and then incubated at 4°C for 1 h. The beads were washed with 1 ml of NP buffer (25 mM HEPES-KOH [pH 7.9], 0.1% NP-40, 0.1 M KCl, 0.5 M NaCl, 10% glycerol, 20 mM imidazole-HCl [pH 7.9]) [17], and bound proteins were eluted with 8 M urea and resolved by two-dimensional (2-D) electrophoresis (pH 3–9 for the first run and 10% SDS-PAGE for the second run). For protein micro-sequencing, proteins were transferred to a PVDF membrane (Millipore) and digested with trypsin, and the resultant peptides were subjected to a protein sequencer (Perkin Elmer) as described previously [15].

2.2. Immunological procedures

Anti-rat hnRNP-F was raised in rabbits by injection of the purified recombinant hnRNP-F. The procedure for preparation of the anti-TBP antibody has been described previously [15]. Preparation of whole cell extracts from rat tissues has also been described previously [18]. For immunoprecipitation, anti-TBP or anti-hnRNP-F antibodies (200 μl each) were immobilized on protein A-Sepharose (Pharmacia). Rat liver nuclear extract was dialyzed against IP buffer (25 mM HEPES-KOH [pH 7.9], 0.1 M KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40 and 10% glycerol) and incubated with antibody-coupled protein A-Sepharose at 4°C for 1 h. Subsequently, the beads were washed five times with IP buffer, and proteins were eluted with 8 M urea. The eluted proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was processed for Western blotting according to the procedure recommended by the manufacturer.

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[1] The nucleotide and amino acid sequences of rat hnRNP-F appear in the DDBJ, EMBL, and GenBank databases with the following accession number: AB022209.

Abbreviations: hnRNP-F, heterogeneous nuclear ribonucleoprotein F; TBP, TATA-binding protein; RNAP II, RNA polymerase II; CTD, carboxy-terminal domain; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PIC, pre-initiation complex; GTF, general transcription factor; HXmTBP, histidine-tagged mouse TBP; CPSF, cleavage-polyadenylation specificity factor; TIP, TBP-interacting protein; GST, glutathione *S*-transferase; PMSF, phenylmethylsulfonyl fluoride

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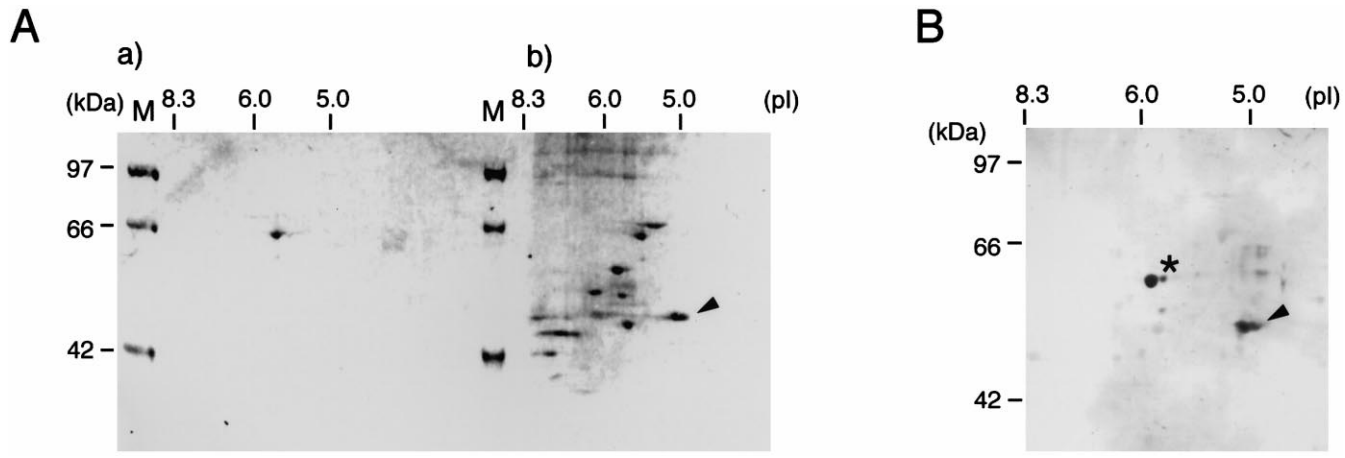

Fig. 1. Rat hnRNP-F is detected in the proteins isolated as TIPs. A: 2-D electrophoresis of TIPs. The TIPs in rat liver nuclear extracts were prepared and detected as described in Section 2. M: molecular weight marker. a: Control experiment performed without HXmTBP. b: Experiment using HXmTBP. The arrowhead indicates the 45-kDa protein with pI = 5.0 that was determined to contain rat hnRNP-F by later examinations. B: Western blot analysis of the 2-D electrophoresis membrane that is equivalent to panel A-b using anti-hnRNP-F antibody. Arrowhead: major signal. Asterisk: minor signal.

2.3. Cloning of a cDNA of rat hnRNP-F

On the basis of human hnRNP-F cDNA sequences [17], two oligonucleotides were synthesized as probes, corresponding to nucleotide positions 370–393 and 838–861 (5′-AAGGAAGAAATTGTTCAGTTCTTC and 5′-GAGTTCACAGTGCAGAGCACCACA, respectively). Using these probes, we screened a rat liver cDNA library and obtained a rat hnRNP-F cDNA (Yoshida et al., unpublished results).

2.4. Binding assay for hnRNP-F and TBP

Using a PCR technique and a GST fusion system (Pharmacia), the rat hnRNP-F coding sequence was subcloned into the pGEX-2 vector, and the fusion protein was overexpressed in *Escherichia coli*. HXmTBP (0.9 μg) was incubated with GST-hnRNP-F (0.4 μg) or GST protein (0.4 μg) together with 20 μl of glutathione Sepharose beads (Promega) in 200 μl of IP buffer at 4°C for 2 h. The beads were washed five times with 1 ml of IP buffer. Bead-immobilized proteins were eluted with 20 μl of SDS-PAGE sample buffer, resolved on 12% SDS-PAGE, and stained with silver.

3. Results

To address the question how a transcription initiation factor is physically associated with splicing factors, we focused on TBP. TBP binds to a TATA box to initiate PIC formation and remains at a promoter after RNAP II leaves [1,2]. First, we attempted to find a TBP-associating splicing factor by the TBP-interacting protein (TIP) detection system that we have developed [15]. By applying this procedure followed by 2-D electrophoresis and CBB staining, we detected multiple TIPs in the rat liver nuclear extract (Fig. 1A-b). By comparison with the control experiment (Fig. 1A-a), we were able to identify at least 10 specific spots of TIPs. These TIPs were recovered dependent on the C-terminal conserved region of TBP (data not shown).

We determined the partial amino acid sequences of almost

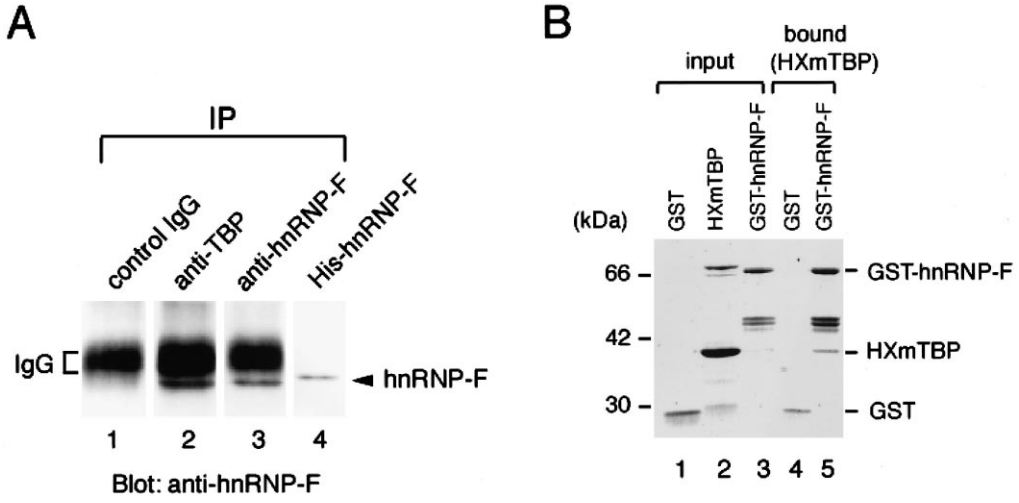

Fig. 2. Association of rat hnRNP-F and TBP. A: Association of hnRNP-F and TBP in rat liver nuclear extracts. The extract was immunoprecipitated with control IgG (lane 1), anti-TBP antibody (lane 2) or anti-hnRNP-F antibody (lane 3). The precipitated proteins and recombinant histidine-tagged hnRNP-F (lane 4) were analyzed by Western blotting with anti-hnRNP-F antibody. Positions of hnRNP-F and IgG heavy chain are indicated. B: Direct interaction of hnRNP-F with TBP. Bacterially expressed GST tag alone (lane 1), HXmTBP (lane 2), and GST-hnRNP-F (lane 3) were partially purified and used in the experiment. GST (lane 4) and GST-hnRNP-F (lane 5) were assayed for their ability to bind to TBP. Positions of each protein are indicated.

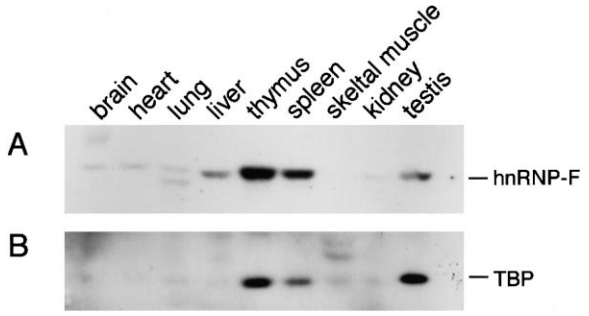

Fig. 3. Tissue distribution of rat hnRNP-F. Proteins (20 μg) extracted from each rat tissue were analyzed by Western blotting for (A) hnRNP-F and (B) TBP. Proteins in the extracts were determined carefully by protein assay (Bio-Rad). Protein extraction efficiencies were estimated to be equivalent according to Western blotting using control antibodies (our unpublished data).

all of those TIPs (data not shown), and found that one peptide derived from a 45-kDa protein (Fig. 1A-b) had the same internal sequence (GPYDRPGTA) as that already reported in human hnRNP-F [17]. We isolated a rat hnRNP-F cDNA and generated an anti-rat hnRNP-F polyclonal antibody (data not shown). Western blotting of the 2-D membrane revealed two significant signals (Fig. 1B). The major signal (arrowhead in Fig. 1B) coincided with the position of the 45-kDa protein. Though the structure of the minor signal was not determined, it might represent the structurally related hnRNP-H [19] because of its significant homology with hnRNP-F and its molecular mass. From these results, we suggest that a 45-kDa TIP contains rat hnRNP-F.

We investigated whether rat hnRNP-F is associated with TBP (Fig. 2A). The rat liver nuclear extract was subjected to immunoprecipitation with anti-TBP antibody and Western blotting with anti-hnRNP-F antibody. HnRNP-F was detected in the anti-TBP antibody-directed immunoprecipitates (Fig. 2A, lane 2), whereas no significant signal was observed when a control antibody (IgG) was used (Fig. 2A, lane 1). Next, we carried out a GST pull-down assay using GST-tagged hnRNP-F (GST-hnRNP-F) to examine the direct interaction of hnRNP-F and TBP. The GST tag alone did not yield any positive TBP band (Fig. 2B, lane 4). However, GST-hnRNP-F and TBP were found to be co-precipitated together with glutathione beads (Fig. 2B, lane 5). These results suggest that hnRNP-F interacts directly with TBP.

We investigated the tissue distribution pattern of hnRNP-F protein in rat tissues by Western blotting (Fig. 3). Although hnRNP-F was detected ubiquitously, it existed in abundant amounts in the thymus, spleen and in moderate amounts in the testis and liver (Fig. 3A). We also determined the TBP level in the same tissues and found that TBP was also concentrated in the thymus, spleen and testis (Fig. 3B).

4. Discussion

In this work, we searched for a splicing factor that can interact with TBP, and we eventually determined an hnRNP-F sequence in a 45-kDa protein isolated by our TIP-detection system (Fig. 1B). We believe that this protein represents rat hnRNP-F because its immunological identity (Fig. 1C) and the physical parameters (pI = 5.0 and 45 kDa) were consistent with those calculated from the sequence data of the rat hnRNP-F cDNA.

Heterogeneous nuclear ribonucleoproteins are pre-mRNA-binding proteins and they include more than 20 individual proteins [20]. Some of them are included in the spliceosome and are related to pre-mRNA splicing [21]. HnRNP-F is a poly(G)-specific RNA-binding protein [17,22] and has been found to be involved in the regulation of neuron-specific splicing of the c-*src* gene [23] in addition to more general splicing events [24]. We showed that hnRNP-F was associated with TBP in rat liver nuclear extract (Fig. 2A) and that it did bind directly to TBP (Fig. 2B). These observations suggest that hnRNP-F can associate with TBP in the cell. From the above results, it is possible to imagine that hnRNP-F is involved in transcriptional regulation. Another hnRNP protein, hnRNP-K [25], was reported to regulate transcription of the mouse c-*myc* gene through binding to the CT element [26,27]. However, hnRNP-F is not structurally related to hnRNP-K and has no typical DNA-binding motif [28,29]. Hence, it is likely that hnRNP-F has no transcription regulation function.

It is thought that transcription is coupled with pre-mRNA processing events and that transcription factors and RNAP II are associated with pre-mRNA processing factors (see above). Dantonel et al. [14] reported that CPSF was contained in proteins immunopurified with an anti-TBP antibody. They suggested that CPSF is associated with PIC via TFIID prior to transcription elongation. Interestingly, they observed that TBP weakly bound to one of the CPSF components. No evidence has so far been provided for association of a splicing factor with the PIC components. In the present study, we were able to demonstrate the association of hnRNP-F with TBP. We speculate that hnRNP-F is associated with TBP in the PIC prior to the transcription/pre-mRNA splicing-coupled reaction. The similar tissue distribution profiles for hnRNP-F and TBP (Fig. 3) support this notion. Another hnRNP, hnRNP-H, might be included in the TIP preparation (see Section 3) in addition to hnRNP-F. Association of TBP with hnRNP-F might stabilize the pre-mRNA modifying machinery at a transcription start site and stimulate the subsequent modification process. HnRNP-F may be associated with the mRNA factory described by McCracken et al. [13].

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