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Molecular Cloning of a Rat 49-kDa TBP-Interacting Protein (TIP49) That Is Highly Homologous to the Bacterial RuvB

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TBP as a central component in transcriptional regulation can form complexes with various regulatory factors. Using histidine-tagged TBP for affinity-purification of TBP-bound proteins, we isolated a 49-kD protein termed TBP-interacting protein 49 (TIP49) from rat liver nuclear extracts. We cloned the entire cDNA of TIP49 encoding a novel polypeptide of 456 amino acids, and thereafter established an FM3A cell line that constitutively expressed an epitope-tagged TBP. Immunoprecipitation analysis of the cell extracts indicated that TIP49 and TBP were present in an identical complex. Interestingly, the amino acid sequence of TIP49 exhibited high similarity to those sequences of the RuvB bacterial recombination factors which direct branch migration of the Holliday junction and contain the Walker A and B motifs responsible for ATP binding and ATP hydrolysis. These findings suggest that TIP49 is a putative ATP-dependent DNA helicase. © 1997 Academic Press

Initiation of transcription by RNA polymerase II (pol II) requires the coordinate action of multiple general transcription factors (GTFs) to form the preinitiation complex (PIC) (1, 2). The PIC assembly is initiated by TATA-binding protein (TBP) (3). In addition to such a TBP-triggered sequential assembly model for PIC formation, an alternative model involving holo-pol II (holoenzyme of RNA polymerase II) has been suggested (4). The holo-pol II is a huge protein complex consisting of pol II, various GTFs including TBP, and transcriptional mediators. Pre-constructed PIC is supposed to have a merit in providing a quick response for the initiation of transcription.

TBP is not present alone in the nucleus. In addition to being complexed with holo-pol II, TBP is found as a component of GTFs such as various types of TFIIDs for pol II, SL1 for pol I, and TFIIIB and SNAPc for pol III systems (5-7). A component of TFIID has been demonstrated to be a cell cycle-associated factor (8). Marldonada *et al.* have found their holo-pol II preparation to contain various DNA repair proteins as well as transcription-related factors (9). Moreover, holo-pol II reportedly contains chromatin-remodeling factors, cell cycle-related factors, and splicing factors (10-12). Accumulating lines of evidence that TBP interacts with various regulatory factors directly or indirectly linked to pol II have led to the idea that transcription and other nuclear events are coordinately regulated and that factors involved in such processes can physically interact via TBP and/or pol II. This assumption has prompted us to study interactions between general transcription apparatuses and other nuclear factors to look for possible novel cross-talk between nuclear events.

According to the above view, we investigated physical interactions between TBP and other nuclear factors. Using an *in vitro* binding assay, we found a novel TBP-interacting protein with a molecular mass of 49-kD (TIP49), and isolated its cDNA. This protein formed a complex with TBP and had a significant homology

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with the RuvB bacterial recombination factor. The importance of this discovery of TIP49 is discussed.

MATERIALS AND METHODS

Purification and amino acid sequence determination of TIP49. TBP-interacting proteins (TIPs) were affinity-purified from rat liver nuclear extracts by use of histidine-tagged TBP, and protein sequence determination was performed as previously described (13).

cDNA cloning. On the basis of the determined peptide sequences, degenerate oligonucleotides were synthesized. To isolate cDNA clones for TIP49, we screened a rat liver cDNA library in λgt11 with the ^{32}P-labeled oligonucleotides.

Production of the recombinant TIP49 and its antibody. The recombinant TIP49 carrying an N-terminal histidine-tag was expressed in *E. coli* by use of the pET vector system (14). The recombinant TIP49 was purified with Ni-agarose and Mono Q columns, and the protein in the SDS-PAGE was recovered from the gel to generate polyclonal antibody in rabbits.

Immunoprecipitation analysis. Nuclear extracts were prepared from FM3A cells expressing a hemagglutinim (HA) epitope-tagged TBP and dialyzed against IP buffer (25 mM HEPES/KOH [pH7.9], 0.1 M KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40, 10% glycerol). Anti-HA monoclonal antibody (12CA5, BAbCo) or mouse IgG was bound to protein A-Sepharose beads. Beads were incubated with the extracts at 4 °C for 12 hr and extensively washed with IP buffer. For elution of the proteins bound to the beads, 80 μl of IP buffer containing 1 mg/ml HA peptide (MGYPYDVPDYAS) was added to the beads, and incubation was carried out for 30 min at room temperature (15). The resulting liberated proteins were analyzed by Western blotting.

RESULTS AND DISCUSSION

Cloning and Expression of the Rat 49-kD TBP-Interacting Protein

We previously reported the identification of many TBP-binding proteins from rat liver nuclear extracts by use of histidine-tagged TBP (HXmTBP) as a ligand and referred to these as TBP-interacting proteins (TIPs) (13). To further isolate these proteins, we performed two-dimensional electrophoresis and found at least 10 major discrete spots ranging from 40 ~ 90-kD (data not shown). The 49-kD protein was directly excised from the gel and subjected to microsequencing. The amino acid (a.a.) sequences of three tryptic peptides and the N-terminal region of the protein were determined (underlined in Fig. 1). On the basis of those sequences, we synthesized oligonucleotide probes and used them to screen a rat liver cDNA library. Finally, a 1.6-kb cDNA containing a 3′ poly (A) tail was cloned. Northern blot analysis of this clone detected 1.8-kb mRNA species in rat various tissues (data not shown). The DNA sequence revealed an open reading frame with 456 a.a., and all determined a.a. sequences were found within the identical open reading frame (Fig. 1). The calculated molecular mass of the protein was 50 kD.

To confirm the identity of the cDNA-encoded product,

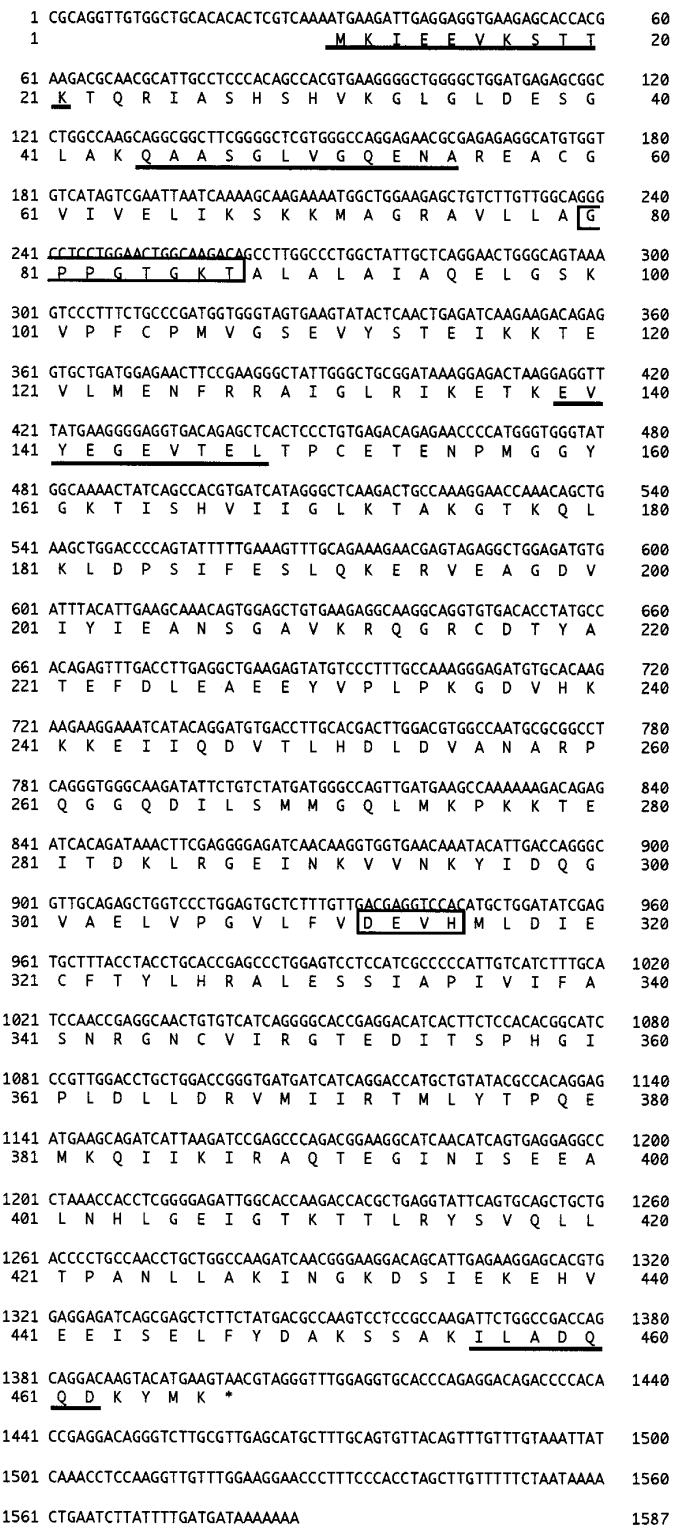

FIG. 1. Nucleotide and predicted amino acid sequences of the rat TIP49. Underlined amino acids represent peptide sequences derived from microsequencing of the purified TIP49. Boxes indicate Walker A and B motifs. An asterisk indicates an in-frame stop codon.

we appended a histidine tag at the N-terminus of TIP49, overexpressed the protein in *E. coli* via the pET vector system (Fig. 2A, lane 2) and purified to near homogeneity (lanes 3 and 4). Western blot analysis revealed that anti-TIP49 antibody reacted with the affinity-purified protein and with the endogenous protein in the nuclear extract as well as with the recombinant protein (Fig. 2B, lanes 1-5). The recombinant TIP49 was indistinguishable from that of the endogenous TIP49 protein in the SDS-PAGE. From these results, we concluded that the cloned 1.6-kb cDNA encoded the full-length TIP49.

Association of TIP49 with TBP in Nuclear Extracts

We performed immunoprecipitation analysis to investigate whether TIP49 is associated with TBP under physiological conditions. Unfortunately, anti-TIP49 antibody were unable to immunoprecipitate the recombinant TIP49 protein (data not shown). When immunoprecipitation was carried out by anti-TBP antibody, high background proteins including IgG prevented the detection of TIP49 (e.g., IgG heavy chain and TIP49 have almost the same size). To overcome these difficulties, we established a mouse FM3A-derived cell line constitutively expressing a hemagglutinin (HA) epitope-tagged TBP (HA-TBP) (15) by using retroviral vectors (K. Yamamoto, manuscript in preparation), since a HA epitope-tagged protein can be specifically precipitated by commercially available monoclonal antibody and the resulting protein can be specifically eluted by the synthetic peptide (HA peptide, see MATERIALS AND METHODS). We found that a low level of HA-TBP was expressed compared with the level of wild-type TBP (Fig. 3A, lane 1) and that total amounts of TBP in the transformed cells were comparable with those in the original FM3A cells (data not shown). These results suggest that the HA-TBP was present in the cells at nearly a physiological concentration (15).

Nuclear extracts were prepared from those FM3A cells and incubated with protein A-Sepharose-linked anti-HA monoclonal antibody. After extensively washing under stringent conditions, the bound proteins that eluted by HA peptides were detected by Western blot analysis. Figures 3A and 3B show that HA-TBP was effectively depleted from the nuclear extracts (lane 5) and was recovered in the eluate (lane 6). When beads were linked to mouse IgG as a control, HA-TBP was neither depleted nor eluted (Figs. 3A and 3B, lanes 2 and 3). In lane 6 of Fig. 3A, we found endogenous TBP together with HA-TBP. This is probably due to self-association between TBP-containing complexes, because TBP and TFIID are known to exist as a dimer (16, 17). As shown in Fig. 3C, TIP49 was detected in the eluate (lane 6), but not in a parallel experiment using control mouse IgG (lane 3). These observations

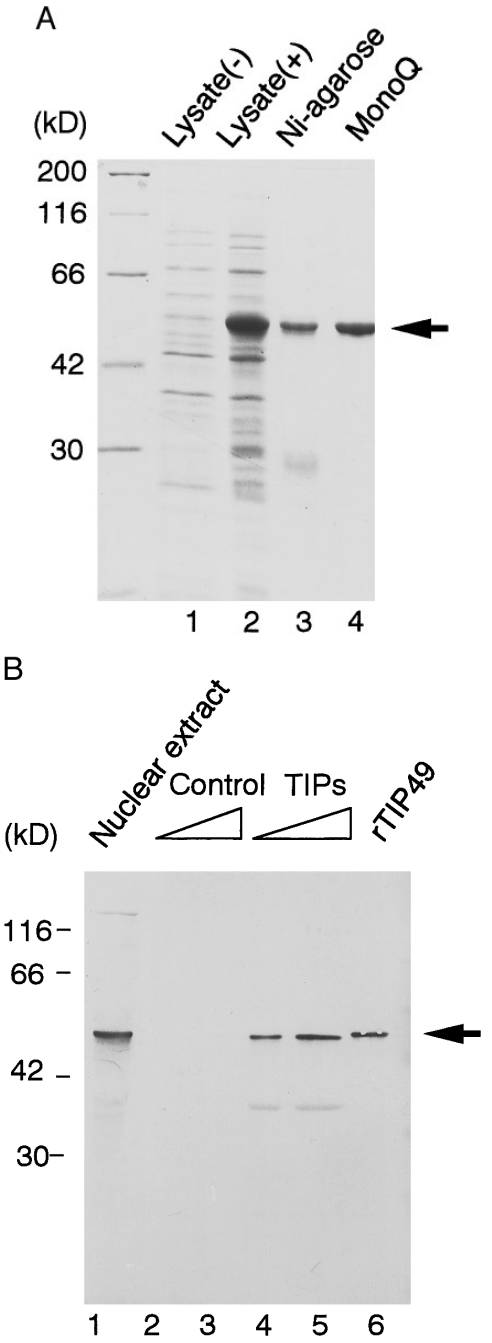

FIG. 2. Identification of the cloned TIP49. (A) Expression and purification of the recombinant TIP49. Histidine-tagged TIP49 was overproduced in *E. coli* and purified as described in Materials and Methods. Crude lysates of *E. coli* (lane 1), crude lysates of IPTG-treated *E. coli* (lane 2), eluate of the Ni-agarose column (lane 3), and eluate of the Mono Q column (lane 4) were analyzed by SDS-PAGE and CBB staining. The arrow indicates the position of the recombinant TIP49 (B) Western blot analysis of the recombinant and endogenous TIP49. Nuclear extracts from rat liver (lane 1; 10 μg), affinity-purified TIP49 by using histidine-tagged TBP (lanes 4 and 5; 10 ng and 20 ng, respectively), and the recombinant TIP49 (lane 6; 10 ng) were analyzed by Western blotting using anti-TIP49 antibody. As a control, proteins bound to Ni-agarose beads in the absence of histidine-tagged TBP were used (lanes 2 and 3). The arrow indicates the position of TIP49.

suggest that TIP49 is complexed with TBP in nuclear extracts. However, upon incubation of the recombinant TIP49 with TBP, no direct interaction between TIP49 and TBP was detected (data not shown), suggesting that the association between TIP49 and TBP is indirect and may involve additional cellular factors. A question arises as to how TIP49 was affinity-purified by use of histidine-tagged TBP. It is possible that HXmTBP binds to the TBP moiety in the native TBP/TIP49-containing complex, since TBP can self-associate as described above.

Structure of TIP49

It is interesting that a database search with the BLAST program demonstrated high homologies between TIP49 and several bacterial RuvB proteins. RuvB is a DNA damage-inducible protein involved in DNA repair and recombination (18, 19). It promotes the branch migration of the DNA crossover known as the Holliday junction. RuvB works as a DNA helicase together with RuvA that provides DNA-binding specificity by targeting RuvB to the Holliday junction. The a.a. sequence of TIP49 was aligned with those sequences of bacterial RuvB proteins with the BLAST + BEAUTY program (20) (Fig. 4). There were multiple

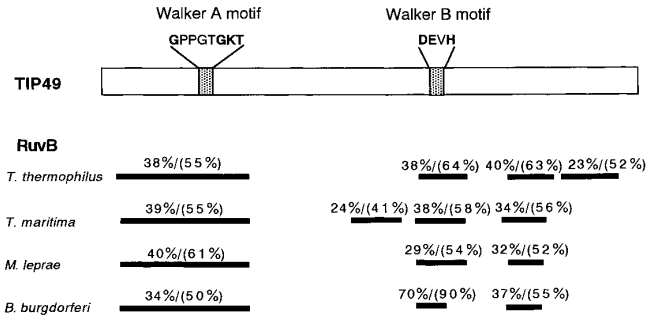

FIG. 4. Comparison of the amino acid sequence among rat TIP49 and bacterial RuvBs. The amino acid sequence of TIP49 was aligned with those sequences of *Thermus aquaticus thermophilus* (25), *Thermotoga maritima* (25), *Mycobacterium leprae* (GenBank accession no. U00011), and *Borrelia burgdorferi* (GenBank accession no. Y08885) RuvBs. Bars show conserved regions between TIP49 and RuvBs. Percentage of the identity (similarity) for each homologous region against TIP49 is indicated. Walker A and B motifs in TIP49 are indicated, and conserved amino acids are shown with bold characters.

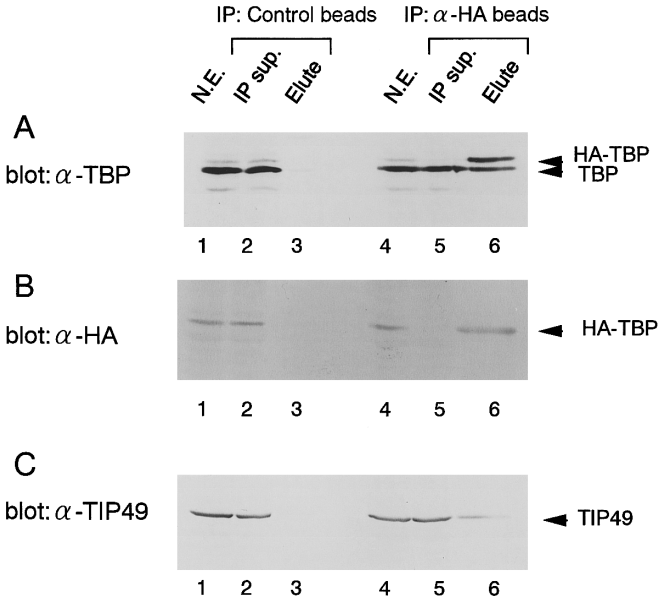

FIG. 3. Association of TIP49 with TBP in nuclear extracts. In each panel, proteins from nuclear extracts of FM3A cells expressing a hemagglutinin epitope-tagged TBP (HA-TBP) were immunoprecipitated with control IgG (lanes 2 and 3) or anti-HA monoclonal antibody (lanes 5 and 6). Nuclear extracts (N.E.; lanes 1 and 4), supernatant of the immunoprecipitation (lanes 2 and 5), and proteins eluted from antibody-coupled beads by the HA peptide (lanes 3 and 6) were analyzed by SDS-PAGE and Western blotting with (A) anti-TBP antibody, (B) anti-HA antibody, and (C) anti-TIP49 antibody.

conserved regions between TIP49 and RuvBs. In the case of *T. thermophilus* RuvB, homologous regions were 23∼40% identical; and when conservative amino acid substitutions were taken into account, the sequence similarity rose to 52∼64% (Fig. 4).

Most of the DNA/RNA helicases generally contain several highly conserved motifs. Walker A (Gx4GKT) and B motifs are involved in ATP binding and ATP hydrolysis, respectively (21, 22). In regard to the Walker B motif, two families of DNA/RNA helicases, i.e., the DEAD box family and DEAH/DExH box family, have been reported (22). TIP49 also contained both the Walker A and B motifs in the RuvB-homologous regions, *i.e.*, 70-GPPGTGKT-77 and 302-DEVH-305 (Figs. 1 and 4), thus suggesting that it belongs to the DExH box family. These sequence similarities and the presence of conserved motifs lead us to an assumption that TIP49 could play a role in biological processes such as recombination and repair. A eukaryotic counterpart of the bacterial *ruv*B has not been reported so far. It is generally surprising that a eukaryotic (rat) protein would exhibit extensive homology to a bacterial one. Thus, it is of particular interest to examine whether TIP49 has a DNA unwinding activity and promotes the branch migration of Holliday junction as does RuvB.

Unwinding of nucleic acid duplexes is considered to be essential to the process of replication, transcription, repair, and recombination (23). If TIP49 actually has a helicase activity, what is the biological role of the complex that includes TIP49 and TBP? Several studies have demonstrated that TBP and GTFs are associated with RNA polymerase II and other transcription factors to form holo-pol II *in vivo* (10, 11). Most recently, human holo-pol II has been shown to contain DNA-

repair proteins and splicing factors (9-12). These findings suggest that transcription can be coupled with other biological processes. This idea is also supported by the fact that TFIIH or some components of TFIIH are involved in basal transcription as well as in nucleotide-excision repair (24). Further study of the association between TBP and TIP49 could provide new clues for understanding of the cross-talk between transcription and recombination/repair.

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