IDENTIFICATION AND CHARACTERIZATION OF NOVEL PROTEIN-PROTEIN INTERACTIONS WITH THE BASAL TRANSCRIPTION FACTOR, TATA-BINDING PROTEIN

by

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ABSTRACT

Recruitment of the TATA-Binding Protein, TBP, to the promoter is a critical, rate-limiting step that drives the initial phase of nearly all gene transcription events. Because of this, many transcriptional regulators target TBP, either to localize TBP at the promoter, or to relay signals between other promoter-bound protein complexes and the basal transcription machinery. Studies described herein were designed to identify novel protein-protein interactions with TBP. To do this, we screened mid-gestational pregnancy-associated cDNA prey libraries using two different yeast two-hybrid systems. Screens in both systems revealed both known and novel TBP interactors. B'-Related Factor 1 and Transcription Factor II A were identified in screens that used full-length TBP as bait. These proteins are known to interact with TBP and thus validated our system. In addition to known interactors, novel interactions with both the N-terminal (TBP-N) and C-terminal (TBP-C) domains of TBP were identified. Coactivator-Associated Arginine Methyltransferase 1 (CARM1), Pax Transactivation Domain-Interacting Protein (PTIP), and Lipopolysaccharide-Induced Tumor Necrosis Factor Alpha Factor (LITAF) all interacted with TBP-N. Proteins that interacted with TBP-C were Huntingtin-Associated Protein 1 (HAP1), four members of the Protein Inhibitor of Activated STAT (PIAS) family of transcriptional regulatory proteins, and Zinc Finger Protein 523 (ZFP523). The TBP interaction domains on PIAS1 and HAP1 were mapped to further define each interaction. Mapping studies revealed that TBP interacts with a single region on PIAS1, and with two separate regions on HAP1. We also show that TBP co-precipitates with PIAS1, PIAS3, HAP1, and PTIP. In conclusion, our studies, in agreement with previous published data suggest that TBP interacts with many classes of regulatory proteins, including transcriptional activators, repressors, and individual components of the transcriptional co-regulatory complexes. They also support the hypothesis that the TBP N-terminus is a protein interaction module and may provide clues to the function of the vertebrate-specific N terminus of TBP.

THE TATA-BINDING PROTEIN AND TRANSCRIPTION

TATA-Binding Protein and Transcription Initiation

Introduction to Transcription

Transcription in eukaryotes is an ordered set of processes orchestrated mainly by large, multimeric protein complexes. The purpose of transcription is to copy the genetic information from the chromosomal DNA into a messenger (m) RNA. mRNA transcripts are templates used by the cellular translation machinery to make proteins. Throughout transcription initiation, elongation, and termination, the assembly and disassembly of subsets of polypeptides regulate a progression of events that occur only as the previous step is completed. Transcription relies on the combinatorial usage of both DNA (cis-acting) and protein (trans-acting) factors to assure proper temporal and spatial expression of the genome. Since the focus of my studies is mammalian TATA-binding protein (TBP), a central player in transcription initiation, this review will revolve around the metazoan-specific transcription factors that are intimately involved in the processes underlying transcription initiation.

TBP Domains

TBP is one of the most highly characterized proteins. It can be divided roughly into two halves, a 180 amino acid C-terminal core domain that is conserved among both archaea and eukaryotes (66), and a 135 amino acid N-terminal portion that is conserved among vertebrates (10). Despite the vast knowledge concerning TBP functionality and the numerous protein complexes that assemble on its C terminus (66, 70, 103, 171), little

is known about the structure or function of the mammalian TBP N terminus (237). Work completed in our lab has shown that mice lacking 111 (amino acids 25-135) of the 135 amino acid residue N terminus of TBP ($TBP^{\Delta N/\Delta N}$) die in mid-gestation due to a placental defect which can be rescued by supplying the mutant embryo with a wild-type placenta (69). This suggests that the TBP N terminus plays an integral role in fetal survival. By removing the N terminus of TBP, we may have removed a protein interaction domain that, when bound by stage- or "situation-specific" transcription factors, functions to regulate a subset of genes that are essential for survival of the fetus.

A limited number of studies have focused on the role of the vertebrate-specific TBP N terminus. Mittal and Hernandez (141) provide evidence that the TBP N terminus is required for assembly of the Small Nuclear RNA (snRNA) Activating Protein complex (SNAPc) in humans. This complex is required for transcription of the gene U6, a snRNA involved in gene splicing processes (141). Das and colleagues (37) showed that a high mobility group protein, HMG-1, can bind the polyglutamine tract within the N terminus of human TBP. Their data indicate that the TBP/HMG-1 interaction increases the affinity of TBP for the TATA element ~20-fold, resulting in an increased rate of binding of TBP to the TATA box (37). Finally, heterozygous disruption of TBP in chicken DT40 cells implicates the TBP N terminus in controlling cellular growth rates (219). In contrast to these reports, work completed in our lab shows that removal of the TBP N terminus is inconsequential for basal cellular functions in mouse embryonic fibroblast cells (190).

TBP Function and TBP Containing Complexes

For proper initiation of gene transcription, many individual proteins must assemble in an ordered fashion on the promoter of a target gene (156). The multi-protein complex that forms is termed the pre-initiation complex (PIC). The PIC has a mass of almost two megadaltons and consists of more than 30 individual proteins (43). At the core of the PIC is TBP. TBP is a saddle-shaped molecule that binds DNA in the concave groove of the protein (87). The DNA sequence most commonly bound by TBP is the "TATA box" (consensus TATAa/tAa/t) (178). Once bound, TBP unwinds and "bends" the DNA into a conformation that allows for proper assembly of the PIC (87, 152). Second, TBP binds and recruits additional proteins that are required for transcription initiation (12, 97, 237).

TBP does not act alone in the cell (59, 150, 171). TBP is found in a number of multimeric protein complexes, the most common being Transcription Factor IID (TFIID)(59). In addition to TBP, TFIID contains 10-12 "TBP-associated factors" (TAFs) that have numerous functions. Some of the TAFs bind and recruit additional transcription factors to the promoter, while some serve as coactivators of transcription, and others bind distinct (non-TATA box) promoter DNA elements (59). Promoter elements bound by the TAFs include the pyrimidine-rich initiator (INR), centered over the transcription start site (consensus YYANt/aYY) (18) and the downstream promoter element (DPE) (15) (Fig. 1.1).

The TFIID complex is specific for transcription of messenger (m) RNA by RNA Polymerase II (RNAPII). Additionally, TBP is found in the promoter selectivity factor (SL1) and Transcription Factor IIIB (TFIIIB) complexes, which orchestrate

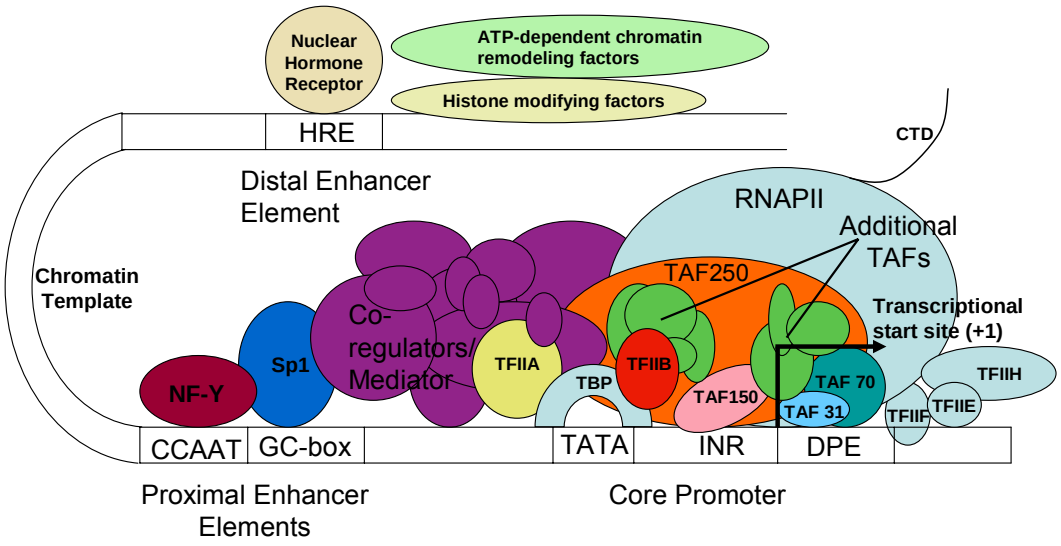


Figure 1.1: Assembly of the transcriptional initiation machinery at the promoter. Selected cis- and trans-regulatory factors are shown. Details and mechanisms are outlined in the text. Adapted from (102, 179).

transcription by RNA polymerase I (RNAPI) and RNA polymerase III (RNAPIII), respectively (66, 70). Generally, RNAPI transcribes ribosomal (r) RNA, RNAPII transcribes messenger (m) RNA, and RNAPIII transcribes transfer (t) RNA and other small, non-coding RNAs such as the snRNAs (see section on snRNA transcription). Finally, TBP is an essential component of the yeast Spt-Ada-Gcn5-Acetyltransferase (SAGA) and human $SNAP_c$ complexes. SAGA is mostly required for transcription of yeast stress response genes (75), while $SNAP_c$ directs transcription of the U6 snRNA (66).

Studies show that TBP is required for most, if not all transcription initiation events. Mice lacking both copies of the TBP gene do not develop past embryonic day 3.5 (E3.5). Survival up to E3.5 is dependent on maternal TBP stores from the egg (130). Once the maternal TBP stores are diminished, the majority of transcription stops. To be

more specific, all transcriptional events by RNAPI and RNAPIII absolutely require TBP (130), whereas, surprisingly, RNAPII-based transcription appears to require TBP for the primary transcription event only. Transcription re-initiation occurring after the primary transcriptional event by RNAPII continues even after TBP is depleted in TBP homozygous null cells (130). This phenomenon may be explained by the finding that at least one cellular protein complex has been identified that is able to support transcription, but does not contain TBP. This complex, called the TBP-free, TAF-containing complex, was shown to substitute, *in vitro,* for TFIID (229).

TBP Family

Within the last 12 years, a family of TBP-related proteins has been identified in vertebrates (70). Yeast contain only TBP and no other family members (153, 192), suggesting that the additional TBP family members found in vertebrates are involved in vertebrate-specific processes. The TBP family includes TBP-Related Factor 1 (TRF1), TRF2, and TRF3. All are homologous to the C-terminal core of mouse (m) TBP, whereas homology to the vertebrate-specific TBP N terminus is generally not seen (4). TRF1 was the first to be identified, and, to date, is only known to exist in *Drosophila* (4, 33). Like TBP, TRF1 is able to bind the TATA box, but, distinct from TBP, can also bind a T/C-rich promoter element. TRF1 preferentially directs transcription from RNAPIII-based promoters, but also plays a role in transcription of the *Drosophila* Tudor gene by RNAPII (74).

TRF2, also known as TLF/TRP/TLP, is found throughout the metazoan species. Human (h) TRF2 is 39% identical to hTBP in its C-terminal core (153). Despite this, it

does not bind the TATA box, but it does bind some of the same proteins that interact directly with TBP (172). Genetic studies show that mice lacking both copies of TRF2 are defective in spermiogenesis, resulting in male sterility (128, 129).

TRF3 is the most recent addition to the TBP family (162). It is found in most vertebrate species, including fish, frogs, and mammals (162, 235). The C terminus of human TRF3 is 93% identical to the conserved hTBP C terminus. Despite the high degree of similarity, TRF3 purifies in native complexes that are smaller than those purifying with TBP (162). This suggests TRF3 may have functions distinct from TFIID. Consistent with this, TBP is rapidly imported into the nucleus after mitosis is complete, whereas the import of TRF3 is greatly delayed (162). The delayed entry of TRF3 coincides with transcription re-initiation in the post-mitotic nucleus, and thus may control transcriptional re-activation of genes silenced during mitosis (162).

Recruitment of TBP to the Promoter

As mentioned, TBP binds the TATA box and is required for most transcription. But, many promoters do not contain a TATA box (201). In this case, TBP still localizes at the promoter, albeit by slightly different mechanisms. TBP can be re-directed to non-TATA elements, depending on which proteins it is bound to. For example, at promoters lacking a TATA box, TBP still localizes at the promoter, around the -30 region where the TATA box is normally found (59). The key to targeting TBP to some TATA-less promoters appears to lie within the TAFs (1, 133). Certain TAFs, including drosophila (d) $TAF_{II}250$, $dTAF_{II}150$, and $dTAF_{II}60$ bind, or at least are in close proximity to core promoter elements, including the Initiator element (Inr) and Downstream Promoter

Element (DPE) (15, 59, 221). Moreover, these interactions appear to be critical for transcriptional efficiency at promoters lacking a TATA box (14). Even in some TATA-containing promoters, TBP only weakly binds the TATA box in the absence of the TAFs (31). Therefore, it seems that by binding core promoter elements, TAFs function to properly position and stabilize TBP at the promoter. As a result, promoter strength increases and transcriptional efficiency is greatly enhanced (1).

Another example of TBP recruitment to the promoter is observed in snRNA transcription. snRNAs are transcribed by RNAPII and RNAPIII, depending on which trans-acting (proteins) and cis-acting (DNA) components are present at the promoter (67). As detailed in two reviews by N. Hernandez (66, 67) and in additional reviews (121, 193) RNAPII transcribes U2 snRNA, whereas the U6 snRNA is transcribed by RNAPIII. In the case of U6 transcription, the promoter contains two cis-acting factors that are upstream of the transcription start site: (1) a proximal sequence element (PSE) bound by $SNAP_c$ and (2) a TATA box, bound by TBP in the TFIIIB complex. In contrast, the U2 promoter contains only the PSE. The difference in polymerase specificity is driven by the presence (or absence) of a TATA box, and whether TBP is bound to one of two homologous proteins, Brf1 or Brf2. The details of each system are as follows. For transcription of U6, the presence of a TATA box converts the promoter to one used by RNAPIII. TBP, bound to Brf2 in the TFIIIB complex binds the TATA box and orchestrates RNAPIII transcription. For RNAPII-based U2 transcription, there is no TATA box, but TBP still has to be at the promoter to initiate transcription. In this case, TBP is bound to Brf1 and assembles into the $SNAP_c$ complex, rather than TFIIIB. $SNAP_c$ localizes at the PSE within the core promoter region, resulting in proper TBP

placement at the promoter. Once bound, RNAPII is recruited to the promoter and transcription of U2 can begin (66, 121, 193). The example of snRNA transcription illustrates two important paradigms of gene expression. First, the substitution of one protein for another, in this case Brf1 for Brf2, is a common example of how the eukaryotic cell is able to achieve different transcriptional events using a minimal number of changes. Second, by using different combinations of cis-regulatory elements and the proteins that bind them, much greater control and expanded diversity of gene expression is accomplished.

General Transcription Factors

Prior to RNAPII-based mRNA transcription, a number of trans-acting factors are recruited to the promoter. The first step in this process is the binding of TFIID to the TATA box. The TFIID complex covers nearly 80 bases of DNA around the start site of transcription. Although TFIID is able, by itself, to bind the TATA box and support activated transcription (1), additional general transcription factors (GTFs) are required for transcriptional efficiency *in vivo*. Examples include TFIIA and TFIIB. TFIIA, in association with TAF40 (a component of TFIID), binds TBP and stabilizes the interaction of TBP with the DNA (97). TFIIA is also responsible for liberating TBP molecules that are bound to transcriptional repressor proteins, such as negative cofactor 2 (NC2), ultimately favoring PIC assembly (38). TFIIB functions in a similar manner. It contacts both TBP and the promoter DNA to promote transcription. In addition, the co-crystal structure of TBP, TFIIB, and DNA, along with mutation analyses *in vivo,* provides

evidence that the TBP/TFIIB interaction is necessary for transcriptional proficiency and cell viability (12).

Once the TFIID/TFIIA/TFIIB complex forms at the promoter, the remaining GTFs are recruited. These include transcription factors TFIIE, TFIIF, and TFIIH (59, 102). First, TFIIF, bound to hypophosphorylated RNAPII, integrates into the PIC (178). The main role of TFIIF is to target RNAPII, a twelve subunit enzyme complex, to the basal promoter. The addition of TFIIF and RNAPII also provides increased stability to the PIC. RNAPII can be isolated in complexes with proteins other than TFIIF, such as the mediator complex, but the TFIIF/RNAPII interaction is generally the strongest and most commonly seen (175). After RNAPII recruitment, incorporation of the last two GTFs, TFIIE and TFIIH, completes the PIC assembly. Both proteins make multiple contacts with RNAPII and the other GTFs that have already assembled at the promoter (178).

TFIIE is required for regulation of the three major enzymatic activities of TFIIH. Specifically, TFIIE mediates the kinase activity of TFIIH, the result of which is phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNAPII (134, 194, 227). Upon entry into the PIC, the RNAPII CTD, containing 52 YSPTSPS heptapeptide repeats, must become (hyper) phosphorylated for RNAPII to transcribe mRNA beyond the promoter region. Within the CTD repeat region, Ser 2,5,7 and Tyr1 serve as substrates for phosphorylation, each of which is targeted by different kinase proteins, including TFIIH (discussed in more detail later in this review) (35). TFIIH also contains subunits that have both DNA-dependent ATPase and helicase activities. The

role of these subunits is to unwind the promoter DNA, termed "promoter melting," around the start site of transcription (-9 to +2) (207).

Cofactors of Transcription

Promoter melting initiates formation of an open complex over the start site of transcription, and transcription initiation can ensue. However, before further discussion of transcription initiation and elongation can begin, a number of additional protein complexes that are pivotal in the processes leading up to formation of the open complex should be introduced. A number of reviews describe the roles of the cofactors of transcription (102, 123, 132, 179). These proteins include the sequence-specific, DNA binding activator and repressor proteins of transcription, as well as a host of supporting proteins, collectively known as the "cofactors" or "coactivators" of RNAPII transcription (70, 82, 170). The sequence-specific transcription factors bind the DNA at numerous sites and relay the information encoded in that DNA to the transcription machinery at the core promoter. To do this, the sequence-specific factors do not always directly interact with the members of the PIC. Instead, they often relay information via the cofactors of transcription, which function to bridge the gap between the DNA bound activators and repressors and the general transcription machinery (i.e. TBP and RNAPII) (80).

Transcriptional activators and repressors bind the DNA in regions upstream or downstream of the start site of transcription. The cis-regulatory sequences bound by these proteins are called enhancers. Typically, the enhancer element is responsible for controlling gene expression for a tissue- or cell-type-specific subset of genes, rather than just a single gene. In addition, synergism between enhancers and the transcription factors

that bind them is common (103). Commonly, synergism is achieved by clustering together multiple cis-regulatory regions. These clusters can be both proximal and distal to the core promoter, the overall effect being an increase in the specificity of gene regulation (80).

A well-characterized example of an activator is Specificity protein 1 (Sp1). Sp1 binds the GC-box (GGGGCGGGG) (Fig. 1.1), an enhancer element involved in regulation of myeloid lineage genes, as well as many other widely expressed genes (177). Sp1 is also reported to bind numerous proteins, including TBP (44) and some of the human and *Drosophila* $TAF_{II}s$ (23, 71, 209). The Sp1/TBP interaction, like certain TAFs, directs TBP to core promoters of some genes lacking a TATA box (77, 83). For Sp1 to activate transcription, the transcriptional cofactor, CRSP, is also required. CRSP is a multi-protein complex containing protein subunits homologous to the yeast mediator proteins (see below) (181, 182). Therefore, CRSP is required to relay information between Sp1 and the GTFs, perhaps by providing the correct structural context to the assemblage of transcription factors at the promoter. Recently, the RE-1 silencing transcription factor (REST), a transcriptional repressor that binds the cis-repressor element, RE-1, was shown to bind Sp1 (164). The Sp1/REST interaction results in inhibition of Sp1 activation of transcription (164). REST was also found to bind TBP (146). Considering this, REST interactions with Sp1 and TBP may disrupt signaling between the two proteins, resulting in improper PIC assembly and a block in gene transcription.

In addition to the Sp1/GC-box interaction, numerous other cis- and trans-regulatory elements are important for gene expression. Some of the best-characterized

are the TATA box, CCAAT box, GC box, heat shock element, and the glucocorticoid (hormone) response element. These DNA elements are bound by the trans-acting factors TBP, C/EBP, Sp1, heat shock transcription factor or HSF, and the glucocorticoid receptor (GR), respectively (reviewed by J. T. Kadonaga) (80). It is now known that many of the sequence-specific binding factors are modular. They typically contain a DNA binding domain linked to an activation domain (80). Commonly, DNA binding domains contain one or more α-helical regions that contact the DNA. These include the leucine zipper, basic helix-loop-helix, zinc and RING finger, and helix-turn-helix motifs (94). The transcriptional activation domains are more diverse. Some are α-helical and contain a negatively charged activation region. Others contain glutamine-rich regions, such as TBP and Sp1, while still others contain a hydrophobic β-sheet domain or a proline-rich region. The sequence-specific factors can also contain regulatory modules capable of binding additional polypeptide subunits that control the activity of the activator or repressor proteins. Finally, the sequence-specific factors recruit coactivators and corepressors, a necessary event for optimal control of transcription (80).

Coactivator proteins generally function to alter the chromatin structure in such a way that transcription of a gene is favored, whereas corepressors promote a more closed chromatin structure that is less permissive to transcription (202). The role of a "classical" coactivator protein is to promote association of TBP with the promoter, as was seen with the Sp1/CRSP/TBP interaction. In addition, some coactivators can modify the histone proteins that wrap the DNA (179, 203). Histones can be modified in many ways, including acetylation, phosphorylation, methylation, and ubiquitination (220). Depending on the modification state, transcriptional processes are controlled. Finally,

some cofactors can move, remove, or add nucleosomes (complexes of eight histone proteins that wrap ~200 nucleotides of DNA), helping to either block or activate gene transcription (6).

The cofactors of transcription can be roughly divided into five categories, based on their functions (102). The first group is directly associated with the core transcriptional machinery. Examples include the TAFs, TFIIA, and TFIIB. TAFs found within the TFIID complex can be gene- or cell-type-specific, or play a more general (or "basal") role in transcription. For example, $TAF_{II}105$ is expressed in a tissue-specific manner in the granulosa cells of the ovarian follicle, where it was shown to be required for proper oocyte development (51). At the opposite end of the spectrum, $TAF_{II}250$ is a general activator of transcription with several activities, including the ability to acetylate histone tails, bind the core promoter Inr element, as well as post-translationally modify other general transcription factors (226).

The second group of cofactors is commonly associated with activator or repressor proteins and does not appear to possess chromatin modifying activities. One example is the the B cell-specific transcriptional coactivator, OCA-B (102). OCA-B is recruited to the promoter by octamer binding protein 1 (Oct1), a transcriptional activator. The resulting OCA-B/Oct1 heterodimer provides additional surfaces for protein-protein interactions that result in immunoglobin gene expression in B cells (120, 202).

Third, a class of cofactors exists that is found to be free of both transcriptional activator/repressor proteins and the core transcriptional intiation machinery. Termed mediators, they provide a scaffold for additional protein-protein interactions, such as "moderating" the action of linking a DNA-bound activator to the general transcription

machinery (i.e. RNAPII) with the ultimate goal of correct assembly of the PIC (104). The Mediator (Med) complex was originally identified and purified in yeast (90). More recently, evidence of a ubiquitously expressed mammalian Med complex arose (11). Quickly thereafter, a number of "mediator complexes" were identified, including the thyroid hormone receptor-associated proteins (TRAP) (47, 48), and the cofactor required for Sp1 activation (CRSP) (181, 182) ((also known as "positive cofactor 2 (PC2)), the vitamin D receptor interacting proteins (DRIP) (173, 174). Further investigation ultimately led to isolation of a highly purified, consensus mammalian Med complex, which was found to be composed of greater than 20 protein subunits, and contained most of the subunits of previously identified "mediator" activity complexes, such as TRAP, CRSP, and DRIP (26, 184). Mammalian Med, as mentioned, is an adaptor complex, which relays both positive and negative signals between the DNA-bound trans-regulatory molecules and the general transcription initiation machinery and RNAPII. The Med complex shows significant conservation from yeast to mammals (26). Med plays many roles, including recruitment and stabilization of TFIID and RNAPII at the promoter (79, 233), perhaps to provide "a stable platform for reiterative assembly of the RNAPII preinitiation complex" (28). Many of the individual Med subunits provide unique interaction surfaces for specific proteins and transcriptional activation domains (26, 28). Essentially, Med is now thought to be a requirement for activated transcription in mammals, although many questions still remain unanswered concerning its exact mechanisms of action both as a whole and as individual subunits.

The fourth group contains chromatin remodeling activities, which can be either activating or repressive to transcription. Included are those proteins that contain histone

acetyltransferase activity, such as p300/CBP, a global transcriptional coactivator (204), $TAF_{II}250$, a component of the PIC (142), and histone deacetylases HDAC-1 and HDAC-2 (204). These cofactors are required for controlling the state of the chromatin by covalently modifying histone side chains, as well as other transcription factors. Acetylation of histone tails reduces their positive charge which is presumed to reduce their affinity for negatively charged DNA or other chromatin proteins. Therefore, the nucleosome structure is destabilized, leading to conformational changes that favor a more "open" chromatin state. The open complex facilitates transcription by allowing the transcription machinery to access the promoter regions in DNA (65, 204). In contrast, histone deacetylation favors a more tightly packed chromatin structure that is generally non-permissive to transcription (60).

The final group of cofactors includes ATP hydrolyzing proteins such as the SWI/SNF complex. These proteins are recruited to the promoter by transcriptional activators, and "remodel" the chromatin in an energy-requiring reaction (102, 163). The SWI/SNF complex is able to bind both DNA and nucleosomes, and its remodeling activities induce conformational changes of both DNA and the histones. The result is an altered chromatin state with disrupted histone-DNA interactions. In a less condensed state, the DNA is much more accessible to the transcription machinery.

Transcription Initiation

Once the "open complex" forms at the promoter, transcription can progress. The first step in this process is the synthesis of the first phosphodiester bond by RNAPII. The polymerase forms a clamp over the DNA, unwinds the DNA duplex in its active site, and

catalyzes mRNA polymerization. Nucleotides (nt) funnel into RNAPII where they are used to synthesize mRNA that is complementary to the DNA template (32, 56). In its earliest phases, transcription is "abortive." With each nucleotide addition to the nascent transcript, there is a tendency for RNAPII to dissociate from the DNA and cease transcription. Because of this, RNAPII synthesizes numerous short transcripts of only a few nucleotides in length at the start of transcription (42, 84). It is becoming clear that upon formation of a 4-nt nascent RNA, RNAPII becomes committed to promoter escape. At this point, RNAPII undergoes a conformational change and is able to continue synthesis of transcripts up to 10-14-nt in length, but the transcription complex is still relatively unstable until mRNAs reaching ~15-nt are made. Once the RNA reaches this critical length, protein-RNA interactions fully stabilize the polymerase (42). As long as the necessary co-factors of transcription are present, the polymerase is set to transition from the initiation to elongation stage of transcription.

Transcription Elongation

As the polymerase moves away from the promoter and begins elongating transcripts beyond the 15-nt length, a number of important events must take place. First, the phosphorylation state of the RNAPII CTD is greatly increased(122). Two proteins that play a major role in this event are the TFIIH-associated Cdk7/cyclin H kinase subunit and pTEFb kinase (127), preferentially phosphorylating Ser-5 and Ser-2 of the CTD repeat region, respectively (36). Hyperphosphorylation of the CTD is the defining step for the beginning of elongation (36) and it sets off a number of events that are critical for further elongation of the transcript.

Perhaps the most important event in the transition between intiation and elongation is the exchange of transcription initiation factors for those required by the elongation competent polymerase. Notably, the transition from transcription initiation to early elongation is highly dependent on the TFIIH complex. In addition to phosphorylation the CTD, TFIIH subunits containing helicase activity unwind the DNA in front of the polymerase (27, 42). The melting of the DNA template by TFIIH also helps to dissociate TFIIF from the promoter DNA (27). This is an important step for promoter clearance since the TFIIF interaction with the DNA in front of the polymerase may impede promoter escape (27) .

In recent years, *in vivo* experiments have redefined the components of the elongating polymerase. Early, *in vitro* experiments proposed roles for some of the transcription initiation factors in the elongation process, but this no longer seems to be the case (165). In fact, one of the most recent studies of promoter occupancy and the elongating yeast RNAPII complex shows that the mediator complex, as well as most, if not all GTFs associate only with RNAPII at the promoter and are not found in the elongating RNAPII complex (165, 198). Loss of the initiation factor complexes clears the way for the elongation-specific proteins to load onto the polymerase. These include the Positive Transcription Elongation Factor b (P-TEFb)/Negative Elongation Factor (NELF)/DRB Sensitivity-Induced Factor (DSIF) system (223) and the Elongator and FAcilitates Chromatin Transcription (FACT) complexes (222).

To reiterate, arrest of transcription is common. Many things can cause this, including inadequate phosphorylation the CTD (197). There is also a delay in early elongation to allow ample time for recruitment of the capping enzymes (see below for

description of the capping process). DSIF and NELF are key molecules in the capping process, and their association with RNAPII at the promoter is inhibitory to elongation (124). Once capping is complete, P-TEFb, in a kinase-dependent manner, disrupts DSIF and NELF interactions with the polymerase and productive elongation can proceed (166, 197).

As was the case with transcription initiation, the elongating polymerase must contend with the chromatin structure. Two complexes that help to clear a path for the elongating polymerase are Elongator and FACT (198). The role of these complexes is to modify the state of the nucleosomes to facilitate passage of the polymerase through the chromatin. Elongator contains Histone Acetyltransferase (HAT) activity to acetylate histones H3 and H4 (230) and was recently shown to be associated, *in vivo*, with both the polymerase (86) and the nascent RNA (55). The FACT complex, in addition to playing a role in relief of DSIF and NELF inhibition of elongation (222), is able to interact with and remove histone H2A and H2B proteins from the DNA (157, 198).

As the nascent transcript protrudes out from the polymerase, a number of RNA maturation events occur co-transcriptionally. These include capping, splicing, and polyadenylation of the mRNA, all of which are interconnected and augment one another (169). Capping occurs early in transcription, when the polymerase is still in a partially phosphorylated state and the nascent transcript is ~20-30-nts long (195). The cap is a N7-methylated guanosine residue that is covalently attached to the first nucleotide of the mRNA (169). Once capped, the mRNA is bound by the Cap-Binding Complex (CBC). The CBC stabilizes the mRNA, protects it from degradation, facilitates mRNA export out

of the nucleus, and recruits the translation initiation factor, eIF-4E, initiating the translation process (169, 195).

The second mRNA maturation event is splicing. Genes serving as templates for mRNA transcription contain both coding (exonic) and non-coding (intronic) sequences. Production of a mature transcript requires the removal of the introns from the transcribed pre-mRNA (64). The removal procedure is termed "splicing" and involves numerous splice factor proteins, such as the SR (Serine-Rich) proteins (58) and snRNAs (64). As a whole, the splicing factors assemble into a multimeric RNA processing machine known as the spliceosome. The spliceosome recognizes sequences in the pre-mRNA that flank both the 5' and 3' ends of the introns. It is at these splice sites that introns are cut out of the pre-mRNA by the spliceosome. Once excised, the the spliceosome ligates the 5' end of the upstream exon to 3' end of the downstream exon (25). Since most genes are composed of numerous exons and introns, this process occurs progressively down the pre-mRNA until all introns are gone, leaving only the re-connected exons that compose the mature mRNA.

Transcription Termination

Once the elongating RNAPII nears the end of the gene, transcriptional termination ensues. The termination process involves addition of a poly(A) tail to the message, release of the transcript from the polymerase, and release of the polymerase from the DNA (13). Termination of transcription begins with polymerase recognition of unique DNA sequences near the end of the gene. The major termination-specific sequences recognized by the polymerase include the poly(A) signal (AATAAA) and the

downstream G/T rich sequence and termination tract sequences (24, 168, 169, 214). The polymerase, with help from additional termination factors such as cleavage stimulation factor, cleavage/polyadenylation specificity factor, and cleavage factors 1 and 2, recognizes and transcribes through the poly(A) signal. Ultimately, the mRNA will be cleaved 20-30-nt downstream of the poly(A) signal, and upstream of the G/U rich motif (24). But, the transcript doesn't end directly after the poly(A) signal. Rather, it extends hundreds of bases further downstream until the polymerase reaches the termination tract sequences. As the polymerase reaches the termination tract region, cleavage of the mRNA occurs at numerous sites within the termination tract, as well as at the poly(A) site (169). Recent studies have shown that depletion of exonuclease proteins Rat1p in yeast or Xrn2 in human cells leads to a defect in termination. These 5' to 3' exonucleases, which attach to the 5' end of the cleaved nascent mRNA (at either the poly(A) signal or to co-transcriptional cleavage sites) and cleaves the RNA, eventually catching the elongating polymerase. The result is destabilization of the polymerase and eventual termination of transcription (89, 228). After the cleavage events, the mRNA is polyadenylated by poly(A) polymerase which adds approximately 200 adenosine residues to the 3' end of the message. The poly(A) tail stabilizes the message, increases translational efficiency, and aids in export of the mature message into the cytoplasm, where it is translated into protein (24).

In conclusion, transcription is a highly complex process. The last 20 years have vastly increased our understanding of how transcription occurs. However, when considering the great number of proteins and complexes that are intimately involved in transcription, it becomes evident that we have yet to identify all the pieces of the puzzle.

Elucidation of new interactions is occurring on a regular basis. To this end, my research seeks to further our understanding of transcription by discovering a few more of these novel protein interactions with one of the most central players in transcription.

INTERACTION OF PROTEIN INHIBITOR OF ACTIVATED STAT (PIAS) PROTEINS WITH THE TATA-BINDING PROTEIN

Introduction

TBP functions in transcription initiation by all three nuclear eukaryotic RNA polymerases (29, 66). TBP containing complexes include SL1, TFIID, and TFIIIB, which function with RNA polymerase I (RNAPI), RNAPII, and RNAPIII, respectively. RNAPII requires TFIID for promoter-targeted assembly of the PIC (171). TBP is also an essential component of the human SNAPc complex, which functions in transcription initiation at small nuclear RNA genes by both RNAPII and RNAPIII (140), and the yeast SAGA complex (216).

Recruitment of TBP to the core promoter is regulated by both positive and negative factors (171). Some activators of transcription bind TBP or the TBP-associated factor (TAF) components of the TFIID complex and direct TFIID to the promoter (1, 150). TBP function can also be up- or down-regulated through interactions with BTAF1/MOT1 and the NC2α/β subunits of the NC2 complex (92, 160). Recently, ZNF76, the human ortholog of mouse zinc finger protein 523 (ZFP523) and of frog Staf (149), was shown to function via direct interaction with TBP (238). The interaction of ZNF76 with TBP is blocked by PIAS1-dependent sumoylation of ZNF76 (238).

PIAS proteins are found in all eukaryotes. The human and mouse family of PIAS proteins consists of PIAS1, PIAS3, PIASx, and PIASy proteins (189). The *piasx* gene encodes two splice-variants, PIASxα/ARIP3 (androgen receptor-interacting protein-3) (143, 205) and PIASxβ/Miz1 (Msx-interacting zinc finger-1) (232), the difference being

in their C termini. *pias3* and *piasy* also each encode two isoforms, PIAS3/PIAS3β and PIASy/PIASyE6^{-}, as a result of alternative splicing. The PIAS3β isoform contains an insertion of 39 amino acids in its amino-terminal region and PIASyE6^{-} lacks exon 6 (200). In total, seven different PIAS proteins are expressed in mammals, each of which likely differs in which cell types and conditions favor its expression.

PIAS proteins regulate the activities of transcription factors including the signal transducer of activated transcription (STAT) family of proteins (95, 116, 117, 189, 199). PIAS proteins have SUMO E3-ligase activity and interaction of PIAS proteins with transcription factors often results in sumoylation of that protein. Ligation of SUMO-1 to most transcription factors represses activity, although the mechanisms that underlie regulation differ (78, 139). In addition to sumoylation, PIAS proteins can regulate gene expression by blocking the interaction of a transcription factor with its target DNA, by recruiting co-repressors and co-activators of transcription, and by targeting proteins to nuclear bodies (118).

The conserved N-terminal region of PIAS proteins contains several well characterized domains (200). The SAF-A/B, Acinus, PIAS (SAP) domain binds A/T-rich DNA and may be involved in targeting PIAS proteins to the nuclear scaffold (155). The SAP domain encompasses an LXXLL motif that is required for transcriptional repression (116). The RING-finger-like zinc-binding domain (RLD) mediates the SUMO-E3-ligase activity of PIAS proteins (95) and binds directly to Ubc9, the SUMO E2 enzyme (81). Most PIAS proteins also contain a PINIT motif, which plays a role in nuclear retention (41).

The C termini of PIAS proteins are more diverse; however all contain an acidic domain preceded by several serines (Ser/Ac). Within the acidic domain, a SUMO-1 Interaction Motif (SIM) exists, although deletion of SIM does not abolish PIAS-mediated sumoylation of interacting proteins (95, 183). Also, a serine- and threonine-rich region (S/T) is present in the C termini of all PIAS proteins except PIASγ. The function of this region is unknown (200).

Here, we show that mouse TBP interacts with ZFP523, the mouse ortholog of human ZNF76. In addition, we report the novel interaction of TBP with PIAS1, PIAS3, PIASx, and PIASy proteins. The TBP/PIAS interaction is shown to occur between *in vitro*-translated proteins, suggesting the interaction is direct, and it is detected between endogenous proteins in nuclear extracts, suggesting it occurs *in vivo*. Our results suggest that PIAS proteins might modulate transcriptional signaling at the TBP interface.

Materials and Methods

TBP Bait Constructs and cDNA Prey Libraries

Two bait vectors were used for two-hybrid screens and confirmatory interactions: pGBKT7 (BD Bioscience, Palo Alto, CA) and MP34 (R. Brazas, Mirus Bio). pGBKT7 places the Gal4 DNA binding domain (DBD) upstream of the bait in the fusion protein, while MP34 places the DBD downstream of the bait (Fig. 2.1A). In both vectors, expression is from the ADH1 promoter, replication uses a 2μ origin, and the TRP1 marker is used for selection in yeast. The vectors also encode ampicillin resistance for selection in bacteria. cDNA fragments encoding mouse TBP full length (TBP-FL) and TBP N terminus (TBP-N, amino acids 1-136), were generated by PCR from a plasmid

containing the predominant mouse somatic TBP cDNA (154, 206) using the following primer sets (Table 1): TBP-FL, TBP-N-start primer and TBP-C-end primer; TBP-N, TBP-N-start primer and TBP-N-end primer. PCR-amplified TBP cDNA fragments were cut with *Sal* I/*Not* I and were ligated into *Sal* I/*Not* I-cut MP34. The TBP C terminus (TBP-C) was amplified using TBP-C-start primer and TBP-C-end primer, digested with *Sal* I and *Not* I and inserted into a pGBKT7 vector modified by digesting with *Bam* HI, filling with Klenow, and ligating to itself to shift the reading frame one base in the +1 direction, allowing for in-frame insertion of TBP-C into the modified pGBKT7^{+1} vector. All bait clones and vector modifications were verified by sequencing.

The pGADT7 prey plasmid (BD Bioscience) was altered to allow efficient directional cloning of oligo(dT)-primed cDNA libraries with *Sal* I and *Not* I 5' and 3' linkers, respectively, as follows. The *Not* I site was cut, filled with Klenow, and re-ligated to kill the site, generating pGADT7$^{\Delta Not\ I}$. A linker containing internal *Sal* I and *Not* I sites and 5' *Bam* HI and 3' *Xho* I overhangs was ligated into *Bam* HI/*Xho* I-cut pGADT7$^{\Delta Not\ I}$. The modified prey plasmid was named pGADT7^{SN}.

Oligo(dT)-primed cDNA prey libraries were constructed and inserted into the pGADT7^{SN} vector as follows. Total RNA was extracted and CsCl-purified (191) from embryonic day 10.5 (E10.5) wild-type C57Bl/6J whole pregnant uteri or placentas[2]. In each case, either whole pregnant uteri or placentas were obtained from four pregnant dams to generate a pool of RNA. Poly(A+) mRNA from these samples was purified using Oligo(dT)$_{25}$ Dynabeads (Dynal Biotech ASA, Oslo, Norway) following the

Table 2.1. Oligonucleotide primer sequences for construction of bait and prey cDNAs.

name	sequence[1]
TBP-N-start	5'-atc*gtcgact***atggaccagaacaacagccttcca**-3'
TBP-N-end	5'-tat*gcggccgccc***agagctctcagaagctggtgt**-3'
TBP-C-start	5'-tat*gtcgac*caccatgg**cgagctctggaattgtaccgcag**-3'
TBP-C-end	5'-tat*gcggccgc***gtggtcttcctgaatccctttaa**-3'
TBP-C160-forward primer	5'-at*ctcgag***cattgcacttcgtgcaagaaatgctg**-3'
TBP-C201-forward primer	5'-tat*gtcgac*caccatg**gccaagagtgaagaacaatcc**-3'
TBP-C210-reverse primer	5'-tat*gcggccgc***gctagtctggattgttcttcactc**-3'
TBP-C251-forward primer	5'-tat*gtcgac*caccatg**gtgctgacccaccagcagttc**-3'
TBP-C263-reverse primer	5'-tat*gcggccgc***tctggctcatagctactgaact**-3'
PIASxβ-forward primer	5'-tat*gtcgac*catgaat**atggtttctagttttagggtttc**-3'
PIASxβ-reverse primer	5'-tat*gcggccgc*ctttagtccaaagagatgatgtc-3'
PIASy-forward primer	5'-tat*gtcgac*gctagtggccaag**atggc**-3'
PIASy-reverse primer	5'-tat*gcggccgc***tcagcacgcgggcaccaggcct**-3'
PIAS1-C452-reverse primer	5'-tat*gcggccgcctcgag***gtatttgaggactggttgtggg**-3'
PIAS1-C491-reverse primer	5'-tat*gcggccgc***acttagtggtgacgtaggagacag**-3'
PIAS1-C562-reverse primer	5'-tat*gcggcc**gc***tagcagggaggtgttgtaatg**-3'
PIAS1-C605-reverse primer	5'-tat*gcggccgc***gttggtggctggcagagatgtgct**-3'
PIAS1-N1-forward primer	5'-tat*gtcgacc***atggcggacagtgcggaactaaagcaaatggttatgagc**-3'
PIAS1-N484-forward primer	5'-tat*gtcgac***cctgtctcctacgtcaccactaagt**-3'
PIAS1-N555-forward primer	5'-tat*gtcgac***gcattacaacacctccctgctag**-3'
PIAS1-N598-forward primer	5'-tat*gtcgac***gagcacatctctgccagccaccaa**-3'
pGADT7-reverse primer	5'-gaaagaaattgagatggtgcac-3'

[1]Endogenous protein-coding sequences are in bold and engineered restriction sites are in italics.

manufacturer's protocols. Each cDNA library was constructed using 2.5 μg of poly(A+) mRNA and the Superscript plasmid system for cDNA synthesis and cloning (Invitrogen, Carlsbad, CA), which yields cDNAs containing 5' *Sal* I and 3' *Not* I overhangs. cDNAs were ligated into *Sal* I/*Not* I-digested pGADT7^{SN}. The whole pregnant uteri library contained ~3.0 x 10^6 independent recombinants with 82% bearing inserts; the placental library contained ~2.6 x 10^6 independent recombinants with 95% bearing inserts. The average insert size in both libraries exceeded 1 kb (Figure 1B). Both libraries and all plasmids are freely available on request.

Yeast Two-Hybrid System

All interactions were tested in *Saccharomyces cerevisiae* strain AH109 (BD Bioscience), which contains the Ade2, His3, and LacZ reporters, each under the control of a different promoter. For library transformations, a culture of AH109 containing the TBP bait construct was grown at 30ºC for 48 hours in liquid synthetic complete medium (SC) lacking tryptophan (SC-W) (Q-BIOgene, Irvine, CA). This culture was used to seed 300 ml of 2X yeast extract/peptone/adenine/dextrose (YPAD) at 5 x 10^6 yeast/ml, which was determined by counting on a hemacytometer. The culture was grown at 30ºC for ~5 hours to a density of 2 x 10^7 yeast/ml. Yeast were collected by centrifugation, washed once with water and once with 100mM LiAc and transformed with 14.4 ml 50% PEG (average m.w. 3350), 2.16 ml 1.0 M LiAc, 0.3 ml 10 mg/ml sheared denatured salmon sperm DNA, and 120 μg of the cDNA library in pGADT7^{SN}. Single- or two-component transformations of bait and/or prey plasmids into yeast used a standard PEG/LiAc protocol (54).

Two-hybrid screens were performed on SC medium lacking leucine, tryptophan, and histidine (SC-L-W-H). Both two-hybrid screens used TBP-FL as bait to screen either the whole pregnant uteri (screen 1) or placental (screen 2) cDNA prey libraries. Primary transformants were transferred onto a new SC-L-W-H plate and clones that grew well on these plates were transferred to a SC-L-W plate, grown for 48 hours at 30ºC, and replica-plated to higher stringency selection medium, such as SC-L-W-H + 2.5 mM 3-aminotriazole (3AT), a competitive inhibitor of the His3 gene product (3) or SC-L-W-H also lacking adenine (SC-L-W-H-A). Yeast were also replica-plated to SC-L-W plates containing X-α-gal (Glycosynth, Cheshire, England) to identify clones that activated the

LacZ reporter gene. Prey plasmids from those clones that grew under higher stringency selection and showed strong LacZ expression were isolated from yeast by glass bead lysis (72). Recovered plasmids were transformed into bacteria, clones were selected, and inserts were sequenced to determine the cDNA identity. Isolated prey plasmids were re-transformed into AH109 with the bait and grown on SC-L-W-H, SC-L-W-H + 2.5mM 3-AT, and SC-L-W-H-A plates to verify the interaction.

To identify the domain of TBP that interacted with prey proteins, TBP bait constructs in combination with the empty pGADT7 prey plasmid (auto-activation test) or with a prey plasmid containing novel or known TBP interactors were co-transformed into AH109 and plated onto SC-L-W. Individual colonies that grew on SC-L-W plates (containing both the bait and prey plasmid) were grown overnight in YPAD at 30ºC, pelleted, and washed three times in sterile water. Yeast pellets were resuspended in 0.5 ml water and their concentration determined by counting on a hemacytometer. Standard amounts of yeast were plated on SC-L-W-H to verify that bait plasmids were not auto-active or to test interactions of TBP sub-domain baits with each prey. Prey plasmids were also tested for auto-activation by co-transforming yeast with prey plasmids and empty bait plasmid.

Clone Isolation and Interaction Tests

PCR primers were designed to allow in-frame insertion of cDNAs into either pGBKT7^{+1} bait plasmid (for TBP deletions) or pGADT7^{SN} prey plasmid (for full-length PIASxβ, PIASy, and truncated PIAS1 mutants). RT-PCR amplification-based cloning of PIASxβ and PIASy used oligo(dT)-primed first-strand cDNA from either adult C57Bl/6J testis or E10.5 C57Bl/6J placentas, respectively, for PIASy and PIASx and the following

primer sets: PIASxβ, PIASxβ-forward primer and PIASxβ-reverse primer; PIASy, PIASy-forward primer and PIASy-reverse primer (Table 2.1).

The longest clone of PIAS1 isolated from the two-hybrid screen began at amino acid six of the open reading frame. To generate a clone encoding the full-length PIAS1 protein ($PIAS1_{1\text{-}651}$), primers PIAS1-N1-forward and pGADT7-reverse were used (Table 2.1) with the $pGADT7^{SN}$-$PIAS1_{6\text{-}651}$ plasmid clone from the library as template. Construction of truncated TBP-C and PIAS1 cDNAs used plasmid DNA containing either a TBP-FL or $pGADT7^{SN}$-$PIAS1_{6\text{-}651}$, respectively, as the template for PCR amplification. PCR primer sets for amplification of each truncated cDNA were: TBP-$C_{135\text{-}263}$, TBP-C-start primer and TBP-C263-reverse primer; TBP-$C_{135\text{-}210}$, TBP-C-start primer and TBP-C210-reverse primer; TBP-$C_{201\text{-}316}$, TBP-C201-forward primer and TBP-C-end primer; TBP-$C_{251\text{-}316}$, TBP-C251-forward primer and TBP-C-end primer; TBP-$C_{160\text{-}263}$, TBP-C160-forward primer and TBP-C263-reverse primer; $PIAS1_{6\text{-}452}$, T7 primer and PIAS1-C452 reverse primer; $PIAS1_{6\text{-}491}$, T7 primer and PIAS1-C491-reverse primer; $PIAS1_{6\text{-}562}$, T7 and PIAS1-C562-reverse primer; $PIAS1_{6\text{-}605}$, T7 and PIAS1-C605-reverse primer; $PIAS1_{598\text{-}651}$, PIAS1-N598-forward primer and pGADT7-reverse primer; $PIAS1_{555\text{-}651}$, PIAS1-N555-forward primer and pGADT7-reverse primer; $PIAS1_{484\text{-}651}$, PIAS1-N484-forward primer and pGADT7-reverse primer (Table 2.1). PCR products were digested with *Sal* I/*Not* I and were inserted into the bait or prey vectors. The $PIAS1^{1\text{-}651\Delta 453\text{-}483}$ clone was constructed by digesting the $PIAS1^{484\text{-}651}$ clone with *Sal* I/*Not* I and inserting it into *Xho* I/*Not* I-digested $pGADT7^{SN}$-$PIAS1_{1\text{-}452}$. Four clones of each truncated protein were isolated and separately co-transformed with either TBP-FL (for PIASxβ, PIASy, and PIAS1 mutants) or $PIAS1_{6\text{-}651}$ (for TBP-C mutants)

into yeast as above. Yeast were plated onto SC-L-W agar plates and grown at 30ºC. Resultant colonies were suspended in water and streaked onto SC-L-W-H and SC-L-W-H-A to test each protein interaction pair.

Antibodies, Animals, and Nuclear Extract Preparation

Mouse anti-cMyc monoclonal antibody was purchased from Amersham-Pharmacia (Piscataway, NJ); mouse anti-FLAG M2 monoclonal antibody was purchased from Sigma (St. Louis, MO); rabbit anti-TFIID (SI-1), goat anti-PIAS 1 (C-20) and goat anti-PIAS1/3 (N-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); HRP-conjugated donkey anti-rabbit antibodies were purchased from Pierce (Rockford, IL); HRP-conjugated sheep anti-mouse was purchased from Amersham-Pharmacia; HRP-conjugated rabbit anti-goat antibody was purchased from Invitrogen. Rabbit-anti-TBP N_C antiserum was raised against recombinant His_6-tagged mouse TBP amino acids 72-135. For this later antiserum, specificity was verified by western blots using recombinant TBP, wildtype mouse nuclear extracts, and nuclear extracts from mouse cells homozygous for the $tbp^{\Delta N}$ mutation (69, 190) (described below, mutation eliminates antibody-reactive domain, not shown).

For nuclear extract preparation, young adult male wildtype and $tbp^{\Delta N}$ mice were used. The $tbp^{\Delta N}$ mice are heterozygous for a targeted mutation that exchanged the endogenous *tbp* gene for a version that replaced 111 amino acids within the vertebrate-specific N terminus of TBP with two copies of the FLAG epitope tag (69, 190). The mutant protein contains the entire TBP_{CORE} region of the protein and western blots confirm that this FLAG-tagged TBP accumulates to wildtype levels in all tissues (69,

190). Heterozygous animals are healthy and fertile (69). Survival of animals homozygous for this mutation to E9.5 (100%), birth (9%), and adulthood (~1%) indicates that the FLAG-tagged protein is a functional replacement for most TBP activities (69).

Nuclear extracts were prepared similarly to procedures described previously (57, 234). Briefly, liver and spleen were harvested into ice-cold PBS, blotted, weighed, minced, and homogenized ice-cold under final conditions of 7.5% wt/vol tissue, 0.5% wt/vol non-fat dry milk, in 1.85 M sucrose, 8.4% vol/vol glycerol, 8.4 mM HEPES, pH 7.6, 12.6 mM KCl, 0.13 mM spermine, 0.42 mM spermidine, 1.7 mM EDTA, 100 μM PMSF, 1X protease inhibitors (Sigma), 5 mM DTT using a motor-driven Teflon/glass Dounce homogenizer. Homogenate was layered onto 10 ml cushions of 2.0 M sucrose, 10% glycerol, 10 mM HEPES, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 100 μM PMSF, 1X protease inhibitors, 5 mM DTT and centrifuged in an SW28 rotor at 24,000 r.p.m., 1h, 4^{o}C. Pelleted nuclei were resuspended in 10 mM HEPES, pH 7.6, 100 mM KCl, 10% glycerol, 0.1 mM EDTA, 3 mM $MgCl_2$, 10 μM PMSF, 0.1X protease inhibitors, 5 mM DTT. Nuclei were adjusted to 0.3 mg nucleic acid/ml with dialysis buffer (25 mM HEPES, pH 7.6, 10% glycerol, 40 mM KCl, 0.1 mM EDTA) containing 10 μM PMSF, 0.1X protease inhibitors, 1 mM DTT. While gently mixing, 1/10th volume of 4.0 M $(NH_4)_2SO_4$ was added and tubes were incubated on ice 1h. Chromatin was pelleted by centrifugation for 1h at 40,000 r.p.m., 4^{o}C, in a Ti50 or Ti60 rotor. Supernatent was collected and proteins were precipitated by adding 0.3 g/ml dry $(NH_4)_2SO_4$ and incubating with gentle mixing for 1h after solid dissolved. Proteins were collected by centrifugation for 1h at 40,000 r.p.m., 4^{o}C, in a Ti50 or Ti60 rotor, and were resuspended in dialysis buffer containing 10 μM PMSF, 0.1X protease

inhibitors, 1 mM DTT. Nuclear extracts were dialyzed 2 x 2h against dialysis buffer containing 0.2 mM DTT (reduced DTT to preserve antibody disulfides during immunoprecipitations), insoluble material was removed by brief centrifugation, protein concentrations were determined, and aliquots were snap-frozen in liquid nitrogen. Nuclear extracts were verified by EMSA assays for NF-Y and Oct proteins (data not shown) prior to use in immunoprecipitations.

In Vitro Translation, Transient Co-Transfection, and Co-Immunoprecipitation

In vitro transcription/translation reactions were performed using the "TNT" system (Promega, Madison, WI) following the manufacturer's protocols with the plasmid templates and conditions detailed in the figure legends. For transient transfection/immuno-co-precipitation assays, TBP-FL was inserted into pCMV-HA (BD-Bioscience), which fused the HA epitope tag to the N-terminus of TBP. $PIAS1_{1-691}$, $PIAS1_{1-452}$, $PIAS1_{6-691}$, $PIAS3_{325-628}$, $BRF1_{264-676}$, or $ZFP523_{202-568}$ were inserted into pCMV-MYC (BD-Bioscience), which fused the c-myc epitope tag to the N-terminus of each protein. Human embryonic kidney 293 cells (h293) were plated onto 60mm dishes at ~30% confluence. The next day, cells were washed once with serum-free, drug-free Dulbecco's Modification of Eagle's Medium (SFDF DMEM) (Mediatech, Herndon, VA). Transfection mixes contained 300 µl SFDF DMEM, 1 µg of each plasmid, and 10 µg of Novafector (Venn Nova, Inc., Pompano Beach, FL). Transfection mixes were added to washed cells in dishes containing 2 ml of SFDF DMEM and incubated for 5 hours at 37ºC, 7.5% CO_2. After incubation, 2 ml of DMEM containing 20% newborn calf serum (Invitrogen), 4% fetal bovine serum (HyClone, Logan, UT), and 2X antibiotic-

antimycotic solution (Mediatech) was added to each plate. Approximately 14 hours later, the medium was replaced. At 48 hours post-transfection, cells were washed 2 times in ice-cold PBS and 500 µl of lysis buffer (50mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.8% deoxycholic acid, 10% glycerol, 1 mM EDTA, 1mM PMSF, 5 µg/ml leupeptin-pepstatin-aprotinin, 0.15 mM $NaVO_3$, and 1 mM DTT) was added to each plate. Plates were rocked for 15' at 4ºC to allow cells to detach from the plate. The contents of each plate were transferred to a 1.5 ml tube and placed on ice for 30' with brief mixing at 5' intervals. Lysates were clarified at 10,000 x g for 20' at 4ºC. Lysates from each set of plates were pooled and a portion of each lysate (~50 µl) was stored at -80ºC for use as transfection controls. The remaining 950 µl of each lysate was pre-cleared with 20 µl of protein G plus/protein A agarose (Calbiochem, San Diego, CA) and 5 µl of non-specific antibody or anti-serum for two hours at 4ºC on a rotator. Samples were centrifuged to pellet the agarose and 450 µl of each supernatant was used for immunoprecipitations.

For co-immunoprecipitations, protein samples (either 450 µl pre-cleared transfected cell lysate, 100 µl of nuclear extract diluted to 1.0 ml with 1X TBS [50 mM Tris, pH. 7.5, 150 mM NaCl], or 50 µl of TNT lysates from *in vitro* co-translation reactions diluted to 500 µl with binding buffer, 20 mM Tris pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 5 µg/ml inhibitors), were transferred to tubes containing specific or non-specific monoclonal antibody (2 µg), specific or non-specific goat polyclonal antiserum (5 µl), or anti-FLAG M2 resin (40 µl, Sigma), as indicated in figure legends. Binding reactions were incubated overnight at 4ºC on a rotator and 30-40 µl of protein G plus/protein A agarose was added to each tube (except those containing

anti-FLAG m2 resin) and rotated for an additional hour at 4ºC. For transfected cell lysates, the agarose was pelleted at 750 x g for 1', washed once with ice-cold lysis buffer, four times with first wash buffer (50 mM Tris, pH 7.5, 0.3 M NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1mM PMSF, and 0.15 mM $NaVO_3$), and one time with a final wash buffer that contained the same components as the first wash buffer, except that the NaCl concentration was lowered to 0.1 M. For *in vitro* translation IPs, washes were performed with binding buffer containing 0.1% IgePAL-CA-630 detergent (Sigma) in place of Triton X-100, and for FLAG IPs, washes were performed with 1X TBS containing 0.1% Triton X-100. Pellets were resuspended in 1X loading buffer (99), boiled 5', and separated by electrophoresis through a 12% SDS-polyacrylamide gel. Proteins were transferred to supported nitrocellulose, probed with the indicated primary and secondary antibodies, and visualized using Supersignal-West chemiluminescence (Pierce) and X-ray film.

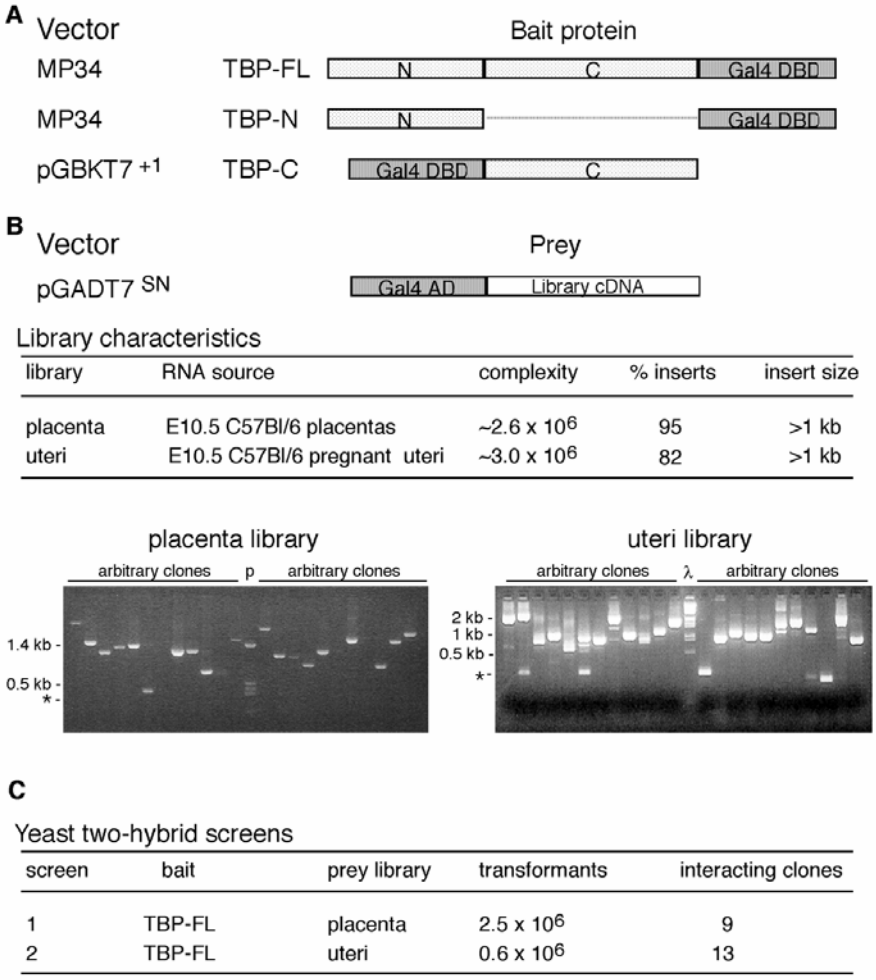
Results

Identification of TBP Interacting Proteins by Yeast Two-Hybrid

To identify mouse proteins that interacted with TBP, we screened both a placental cDNA library (screen #1) and a whole pregnant uteri cDNA library (screen #2) with mTBP-FL in the MP34 bait plasmid (Fig. 2.1). MP34-mTBP-FL did not grow on SC-L-W-H medium in the presence of an empty prey plasmid (Fig. 2.2, sector 1), confirming that the bait was not auto-active.

In screen #1 (placenta library), ~2.5 x 10^6 primary transformants (yeast containing both bait and prey plasmids) (Fig. 2.1C) were plated onto SC-L-W-H medium. Fifty-eight colonies grew and all were transferred to a SC-L-W-H plate containing X-α-gal. Thirty-seven clones grew well and expressed Lac Z. These were transferred to a SC-L-W plate, grown for 48 hours, and replica plated to SC-L-W-H-A medium to test for combined expression of both the HIS3 and ADE2 reporter genes. In this round of selection, nine clones grew well. After isolating the prey plasmid from each, we re-tested the interactions by co-transforming new yeast with the cDNA-containing prey plasmid and TBP-FL bait plasmid. All nine clones re-grew on SC-L-W-H + X-α-gal medium and all expressed Lac Z (not shown).

In screen #2 (whole pregnant uteri library; Fig. 1C), of ~0.6 x 10^6 primary transformants, ~100 colonies grew on SC-L-W-H plates, of which 41 were selected for further analysis. These were transferred to SC-L-W-H-A medium containing X-α-gal. Thirteen clones grew well and expressed Lac Z following retransformation.



Library characteristics

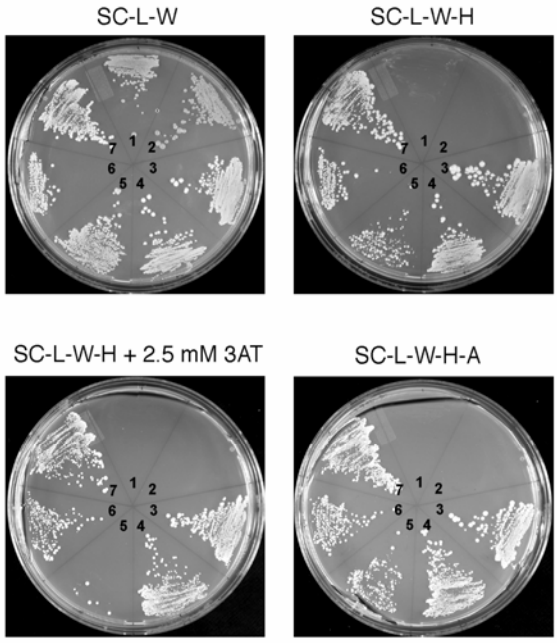
library	RNA source	complexity	% inserts	insert size
placenta	E10.5 C57Bl/6 placentas	~2.6 x 10^6	95	>1 kb
uteri	E10.5 C57Bl/6 pregnant uteri	~3.0 x 10^6	82	>1 kb

C

Yeast two-hybrid screens

screen	bait	prey library	transformants	interacting clones
1	TBP-FL	placenta	2.5 x 10^6	9
2	TBP-FL	uteri	0.6 x 10^6	13

Figure 2.1: Yeast two-hybrid bait constructs, prey libraries, and screens. **(A)** Bait constructs. TBP-FL and TBP-N were expressed from the MP34 plasmid, which fused the Gal4 DBD downstream of TBP. TBP-C was expressed from pGBKT7^{+1}, which fused the Gal4 DBD upstream of TBP. N and C designate the vertebrate-specific N terminus and the pan-eukaryotic C terminus of TBP (TBP$_{CORE}$), respectively. **(B)** Two prey libraries were constructed and inserted into pGADT7^{SN}, which fused prey cDNAs downstream of the Gal4 AD. Characteristics of each library are indicated. Below is shown PCR analysis of arbitrary clones from each library using a primer pair that spans the multiple cloning site of the vector. Lane "p" contained *Hinf* I-cut PBS$^+$ plasmid markers; lane "λ" contained *Hind* III/*Eco* RI-cut λ-phage DNA markers. Landmark band sizes are indicated at left of gels; asterisks denote the size of the PCR product arising from empty prey vector. **(C)** The results of the two yeast two-hybrid screens performed are shown. In both screens, TBP-FL was used as bait to screen either the placental or pregnant uteri library for interacting proteins. TBP-interacting prey library clones were subsequently identified by sequencing.



sector	bait	prey
1	TBP-FL	empty vector
2	pLAM C	SV40 T-Ag
3	p53	SV40 T-Ag
4	TBP-FL	TFIIAα
5	TBP-FL	ZFP523
6	TBP-FL	$PIAS1_{6-651}$
7	TBP-FL	$PIAS3_{325-628}$

Figure 2.2: Yeast two-hybrid screens for proteins that interact with TBP. Bait and prey plasmid combinations that were contained within each yeast clone are indicated below. Sector designations correspond to those on plates. Growth of yeast on SC-L-W indicated that all clones contained both the bait and prey plasmid. Sector 1 is a negative "auto-activation" control showing that yeast bearing only the TBP bait vector and an empty prey plasmid (no insert) did not grow on SC-L-W-H, SC-L-W-H +2.5 mM 3AT, or SC-L-W-H-A. Sector 2 was a negative interaction control that was supplied with the Matchmaker III system encoding non-interacting bait and prey proteins. Sector 3 was a positive interaction control supplied with the Matchmaker III system, and accordingly, yeast in this sector grew well under all conditions tested. In sector 4, the interaction of TFIIA and TBP is shown. Growth of this clone under all conditions tested indicated a strong interaction between TFIIA and TBP, and served as a positive control, since TFIIA is known to interact with TBP (53, 210). Sector 5 shows the interaction of ZFP523 with TBP. This interaction may be weaker than the TFIIA:TBP interaction since growth of yeast on the SC-L-W-H + 2.5mM 3-AT plate was reduced. However, in comparison to negative controls in sectors 1 and 2, the TBP:ZFP523 interaction was scored as strongly positive. Sectors 6 and 7 show the interaction of PIAS1 and PIAS3, respectively, with TBP. Both of the PIAS clones interacted strongly with TBP as compared to control interactions in sectors 3 and 4.

Sequence analysis of the prey cDNAs from screen #1 revealed that seven of the nine clones encoded PIAS1, one clone encoded PIAS3, and one encoded the a-subunit of transcription factor IIA (TFIIA), a known TBP interacting protein (39, 53, 210). Analysis of TBP interacting prey cDNAs from screen #2 identified three more PIAS1 clones. We also obtained three clones encoding B'-related factor 1 (BRF1), another known TBP interactor (39, 53, 210, 225). Additionally, we identified ZFP523, the mouse homologue of a recently identified novel TBP interacting protein, hZNF76 (238). The remaining six clones were all unique, although no obvious physiological connection to TBP was evident. The interactions of TBP with TFIIA, ZFP523, PIAS1, and PIAS3 in the yeast two-hybrid system are shown in Figure 2.2.

For verification, the interactions between TBP and proteins identified by two-hybrid screens were tested by co-immunoprecipitation in cell culture. Both PIAS1 and PIAS3 co-precipitated with TBP when co-expressed in h293 cells (Fig. 2.3A). Additionally, ZFP523 and BRF1, both known TBP interactors, co-precipitated with TBP (Fig. 2.3B).

In Vitro Co-Translated PIAS1 and TBP Co-Immunoprecipitate

The PIAS/TBP interaction was identified using yeast, which contain homologues of most mammalian transcription machinery components. Verification used transfected human cells, which contain the mammalian machinery. It was possible that, in both systems, PIAS and TBP did not interact directly, but rather, PIAS proteins assembled into a stable TBP containing complex via interactions with other endogenous TBP interacting proteins. To distinguish these possibilities, we co-translated the two proteins *in vitro*

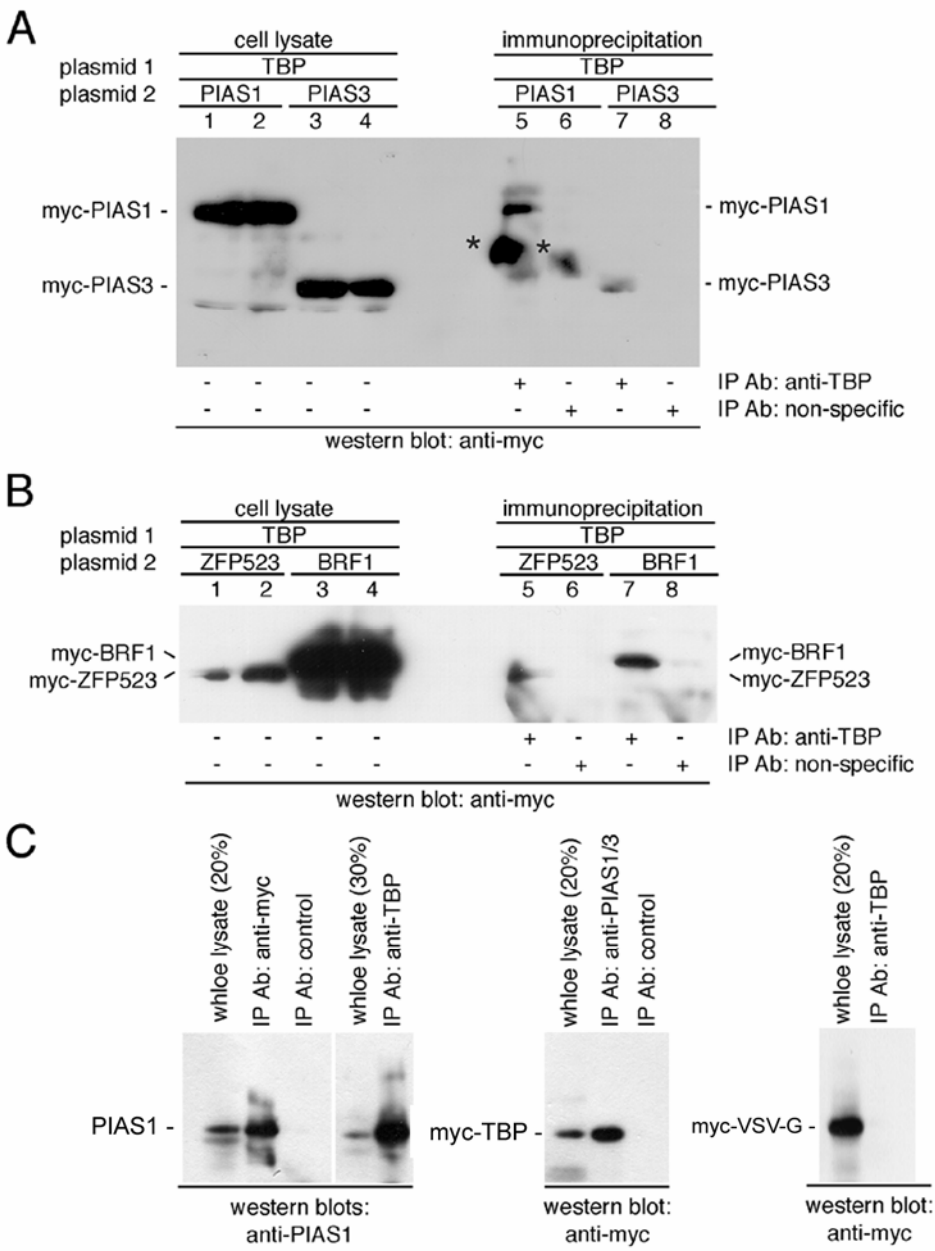


Figure 2.3: Co-immunoprecipitation assays. **(A)** PIAS/TBP interactions in co-transfected cells. h293 cells were co-transfected with pCMV-HA-TBP-FL and pCMV-MYC-PIAS1 or pCMV-HA-TBP-FL and pCMV-MYC-PIAS3, as indicated. Whole cell lysates (lanes 1-2, PIAS1; lanes 3-4, PIAS3) or immunoprecipitated samples (lanes 5-6, PIAS1; lanes 7-8, PIAS3) were assayed using western blots and anti-myc antibody. PIAS1 and PIAS3 co-precipitated with TBP in the presence of the anti-TBP antibody (lanes 5 and 7, respectively), but not with the non-specific antibody (lanes 6 and 8). Asterisks designate non-specific cross-reactive materials. **(B)** TBP-ZFP523 and TBP-BRF1 interactions in co-transfected cells. ZFP523 co-precipitatated with TBP using the

anti-TBP antibody (lane 5), but not with the non-specific antibody (lane 6). As a positive control, a BRF1 clone isolated from our yeast two-hybrid screens using TBP-FL as bait was included, since BRF1 is known to directly interact with TBP (225). BRF1 co-precipitated with TBP in the presence of the anti-TBP antibody (lane 7), but not with the non-specific antibody (lane 8). **(C)** *In vitro*-translated TBP and PIAS1 interact in reticulocyte lysates. Transcription/translation reactions were performed in standard 50µl TNT reactions charged with either 1.0 µg each of *Not* I-linearized pGBKT7^{+1}-TBP-FL (encodes myc-tagged full length mouse TBP protein) and *Not* I-linearized pGADT7^{SN}-PIAS1$_{6-651}$ for PIAS1:TBP reactions, or 1.0 µg each of *Not* I-linearized pGADT7^{SN}-TBP-FL and pCDNA3.1-VSV-G (a gift from M. Hardy) (encodes a myc/HIS-tagged full-length VSV-G protein) for TBP:VSV-G reactions. Immunoprecipitations were performed with anti-myc, anti-TBP-N$_C$ (designated anti-TBP, left blots), anti-PIAS1/3 (N18), anti-TFIID (designated anti-TBP, right blot), or a matched negative control antibody, as indicated. Western blots were loaded with immunoprecipitate or whole reticulocyte lysate corresponding to the indicated percent of the amount of extract used in the immunoprecipitation and were probed with the indicated antibody. PIAS1 co-precipitated with TBP using either the anti-myc or anti-TBP-N$_C$ antibody (left blots); TBP co-precipitated with PIAS1 using the anti-PIAS1/3 antibody (middle blot); TBP did not co-precipitate with VSV-G (negative interaction control) using the anti-TFIID antibody (right blot).

using a reticulocyte-based system and tested whether PIAS1 and TBP co-immunopreciptated. Immunoprecipitation of either protein pulled down the other; however non-specific antibody did not pull down either (Fig. 2.3C). Also, immunoprecipitation of TBP did not pull-down myc-VSV-G, a negative interaction control protein (Fig. 2.3C). Thus, the PIAS/TBP interaction does not require other nuclear proteins, but rather, is very likely the result of direct PIAS/TBP contacts.

Identification of the TBP Interacting Domain on PIAS1

Sequence analysis of the ten TBP interacting PIAS1 clones isolated in the two-hybrid screens revealed that six were of differing lengths. The longest clone encoded amino acids 6 to 651 (PIAS1$_{6-651}$); the shortest encoded amino acids 453 to 651 (PIAS1$_{453-651}$) (Figs 2, 4). Other clones encoded amino acids 135-651, 359-651, 430-651, and 439-651 of PIAS1 (Fig. 2.4B). The single PIAS3 clone encoded PIAS3 amino acids

325-628. Because libraries were oligo(dT)-primed, all clones used their natural stop codon.

The shortest PIAS1 clone isolated from the two-hybrid screens ($PIAS1_{453-651}$) contained the last 199 amino acids of PIAS1. To more precisely identify the domain that interacted with TBP, we constructed more 5'-truncated versions, which encoded amino acids 484-651, 555-651, and 598-651, and 3'-truncated versions of PIAS1, which encoded amino acids 6-452, 6-491, 6-562, 6-605 (Fig. 2.4B). Each PIAS1 mutant was tested for interaction with TBP-FL in the two-hybrid system (Fig. 2.4A).

Only clones containing the region from 453 to 491 interacted with TBP, as indicated by growth on SC-L-W-H selective medium (Fig. 2.4A). Yeast that contained the $PIAS1_{6-491}$ prey grew less well (Fig. 2.4A, bottom panel, sector 1), suggesting that this truncation might have weakened but not ablated the TBP interaction domain. Sectors 7-10 are TBP interaction and auto-activation controls for $PIAS1_{6-651}$ and $PIAS1_{453-651}$, respectively. A clone that lacked amino acids 453-483 of PIAS1 ($PIAS1^{1-651\Delta453-483}$) was also constructed to test whether loss of most of the TBP interaction domain on PIAS1 would disrupt the TBP-PIAS1 interaction. Indeed, PIAS1 clones that lacked amino acids 453-483 did not interact with TBP-FL by yeast two-hybrid (not shown). Our data suggest that the TBP interaction domain of PIAS1 requires the 39 amino acids from positions 453 to 491, which includes the Ser/Ac domain (Fig. 2.4B).

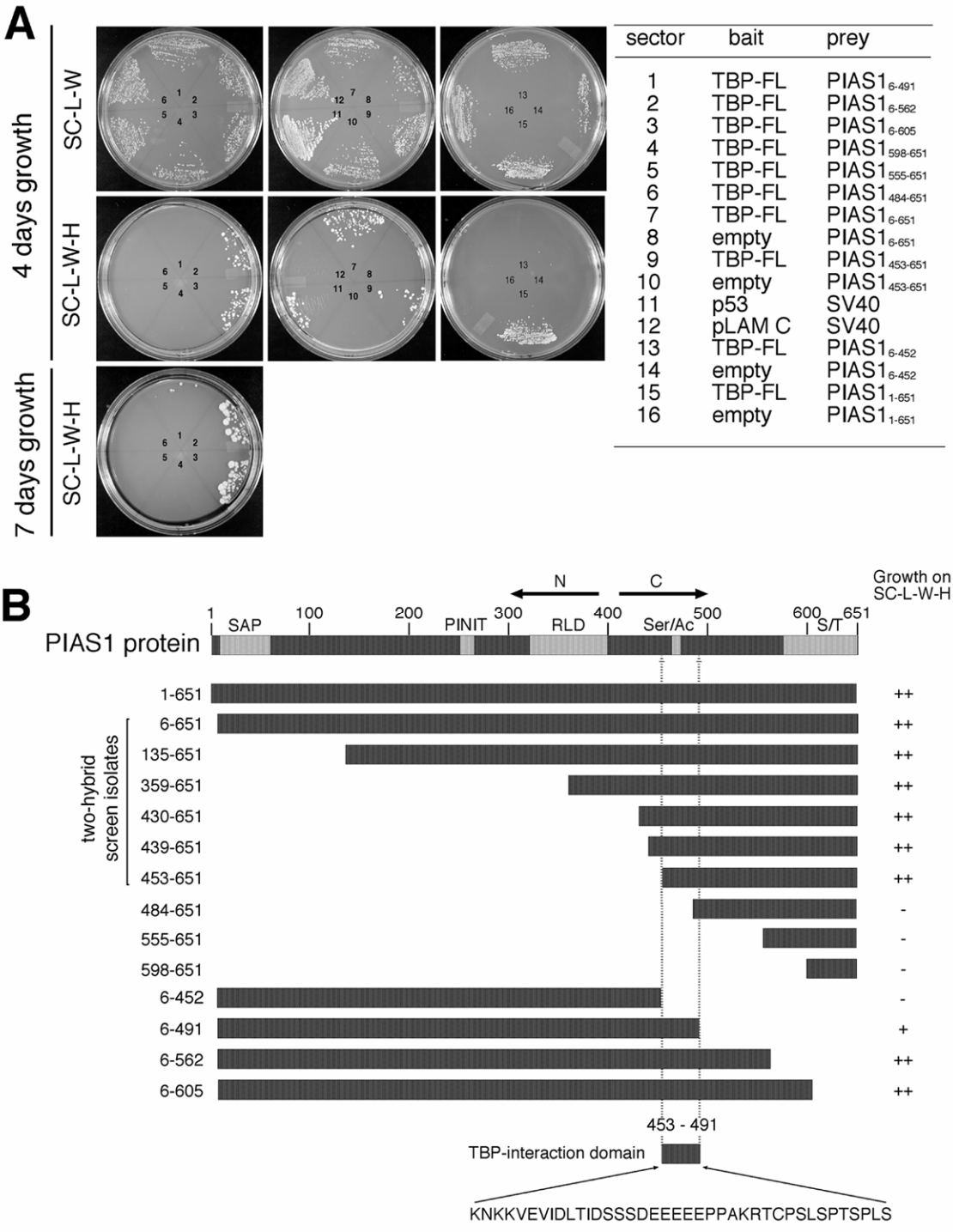


Figure 2.4: Identification of the PIAS1 domain required for interaction with TBP. **(A)** Interaction of truncated PIAS1 clones with TBP. Yeast containing the indicated bait and prey plasmids (listed at right) were grown on SC-L-W (top panel), which selects for the presence of the bait and prey plasmids, and SC-L-W-H (middle and bottom panels), which selects for the interaction between bait and prey. In sectors 1-10 and 13-16, all

baits were expressed from the MP34 plasmid and all truncated PIAS1 preys expressing the indicated amino acids were expressed from the pGADT7^{SN} plasmid. Clones in sectors 11 and 12 were positive and negative controls, respectively, supplied with the Matchmaker III system. All 4 isolates of PIAS1$_{6-491}$ (clone 1) grew at a slower rate and lower density compared to the more robustly growing clones in sectors two and three. Therefore, the bottom panel shows the same plate photographed above which had been allowed to grow for an additional three days to reveal growth of the more slowly growing clone in sector 1. Sectors 7 and 9 were the longest and shortest PIAS1 clones, respectively, that were isolated directly from yeast two-hybrid screens using TBP-FL as bait. Sectors 8 and 10 were controls confirming that PIAS1$_{453-651}$ and PIAS1$_{6-651}$, respectively, were not auto-active. The absence of growth of clones 4-6 and 13 on SC-L-W-H suggested that these clones lacked the TBP-interaction domain. Four independent clones of all PIAS1 truncations were tested and each gave the same result; one representative set is shown. None of the truncated PIAS1 fragments tested were able to grow when supplied with the empty bait plasmid (not shown), confirming that the prey plasmids were not auto-active. **(B)** Deletion analysis of the TBP-interaction domain of PIAS1. The full-length PIAS1 protein is represented at top, including the location of known domains. PIAS1 amino acids expressed by each cDNA clone in pGADT7^{SN} are shown at left. The six different PIAS1 clones isolated from two-hybrid screens as TBP-interacting proteins are indicated. Approximate relative two-hybrid growth rates with the TBP-FL bait vector are represented by ++, +, and -, at right. Results suggested that the TBP-interacting region of PIAS1 was between amino acids 452 and 492, which included the Ser/Ac domain.

PIAS1 and PIAS3 Interact with the TBP$_{CORE}$

To determine whether the PIAS clones that were isolated in two-hybrid screens interacted with the conserved C-terminal TBP$_{CORE}$ or the vertebrate-specific N terminus (10), we co-transformed yeast with the following baits and preys: TBP-N/ PIAS1$_{453-651}$; TBP-C/ PIAS1$_{453-651}$; TBP-N/PIAS1$_{6-651}$; TBP-C/PIAS1$_{6-651}$; TBP-N/PIAS3$_{325-628}$; TBP-C/PIAS3$_{325-628}$. Growth of all clones on SC-L-W verified the presence of both the bait and the prey plasmid. As controls, we tested the interactions of TBP-N or TBP-C with TFIIAα, (Fig. 2.5A) (53, 210). Neither the TBP bait clones nor the PIAS and TFIIA prey clones were auto-active (Fig. 2.5A), confirming the validity of the assay. We found that

all PIAS clones and the TFIIA clone interacted with the TBP_{CORE} (indicated by growth on SC-L-W-H in Fig. 2.5A, right panels), but not with the TBP N terminus (Fig. 2.5A).

We also constructed truncated TBP_{CORE} mutants to further define the domain of TBP that interacted with PIAS1. Only one of our mutants, TBP-$C_{135-263}$, encoding approximately the first two-thirds of the TBP_{CORE}, allowed growth with $PIAS1_{6-651}$ (Fig. 2.5B, sector 1).

Association of Endogenous PIAS1 and TBP in Mouse Nuclear Extracts

Our results indicated that PIAS1 could interact with the TBP_{CORE} both *in vivo* under conditions of overexpression (two-hybrid and transient transfection) and in reticulocyte extracts. To gain insights into whether endogenous PIAS1 and TBP proteins in normal cells might also interact, we tested whether we could detect physical association of the endogenous proteins in mouse nuclear extracts. Mice bearing a targeted mutation at the *tbp* locus that replaces most of the vertebrate-specific N-terminal domain with two copies of the FLAG tag ($tbp^{\Delta N}$ mice) provided a unique resource in which a functional TBP_{CORE} expressed at wildtype levels is epitope-tagged (69, 190).

Nuclear extracts were prepared from livers and spleens of adult wildtype and heterozygous ($tbp^{\Delta N}$) mice. Immunoprecipitation using anti-PIAS1 antibody brought down TBPDN protein from heterozygous nuclear extracts; absence of an anti-FLAG antibody-reactive band in wildtype extracts confirmed the specificity of the FLAG antibody/tag combination (Fig. 2.6A).

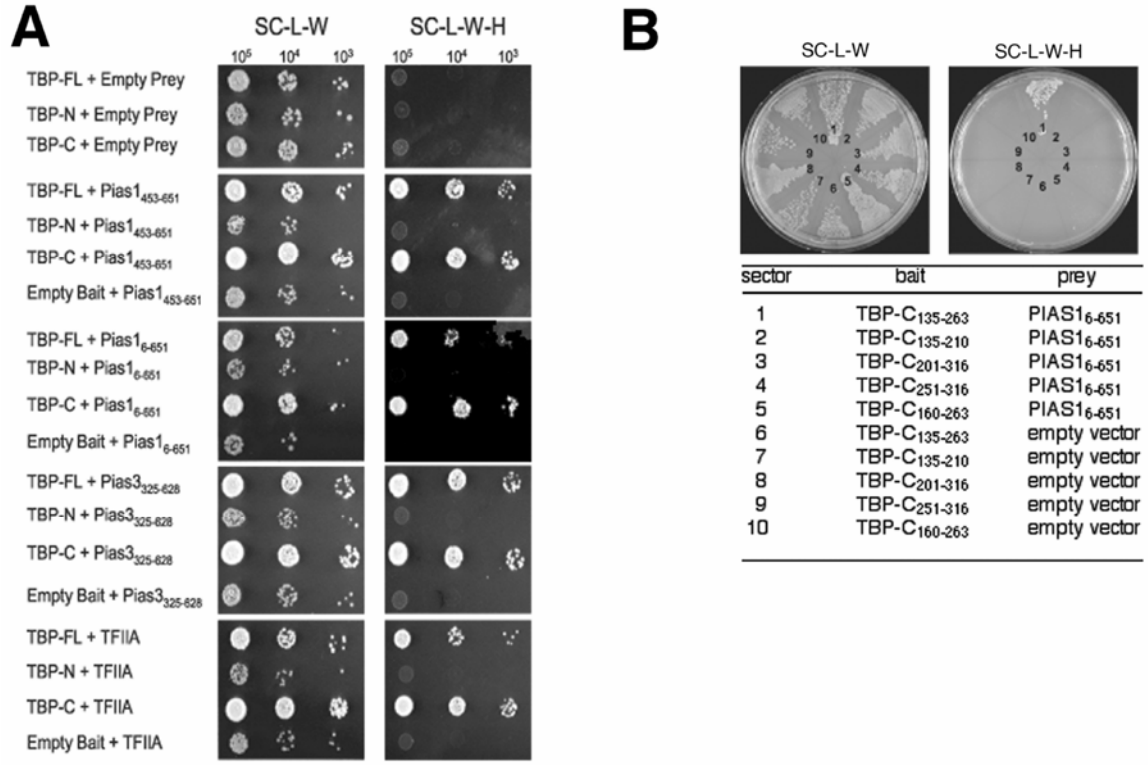


Figure 2.5: PIAS1 and PIAS3 interact with the TBP_{CORE}. **(A)** TBP-FL, -N, and -C baits were tested for auto-activation and for two-hybrid interactions with short (amino acids 453-651) and long (amino acids 6-651) clones of PIAS1 and with a clone of PIAS3 (amino acids 325-628). As controls, interactions of the TBP-FL and the N- and C-terminal TBP sub-domains with TFIIA, a known TBP_{CORE} interactor (53, 210), are shown. Serial dilutions of yeast containing the plasmid combinations indicated were spotted onto SC-L-W (left column) and SC-L-W-H (right column) plates. Growth of clones on the SC-L-W plate verified that yeast contained both the bait and prey plasmid. In the top right panel, the absence of growth indicated that none of the TBP bait clones were auto-active when supplied with the empty prey plasmid. The bottom right panel is a control showing that TFIIA interacted with TBP-FL and TBP-C, but not with TBP-N. Right panels 2, 3, and 4 show that all PIAS clones interact with both TBP-FL and TBP-C, but not with TBP-N. The bottom rows of right panels 2, 3, 4, and 5 are auto-activation controls for each prey plasmid, none of which grew on SC-L-W-H, and thus were not auto-active when supplied with the empty bait plasmid. **(B)** Truncated versions of the TBP_{CORE} were constructed to more accurately identify the sub-domain that is required for interaction with $PIAS1_{6-651}$. Only the $TBP\text{-}C_{135-263}$ protein (Sector 1), encoding approximately the first two-thirds of TBP-C, was able to maintain the interaction with PIAS1. This suggested that an extended conformation of TBP-C is required for the interaction with PIAS1.

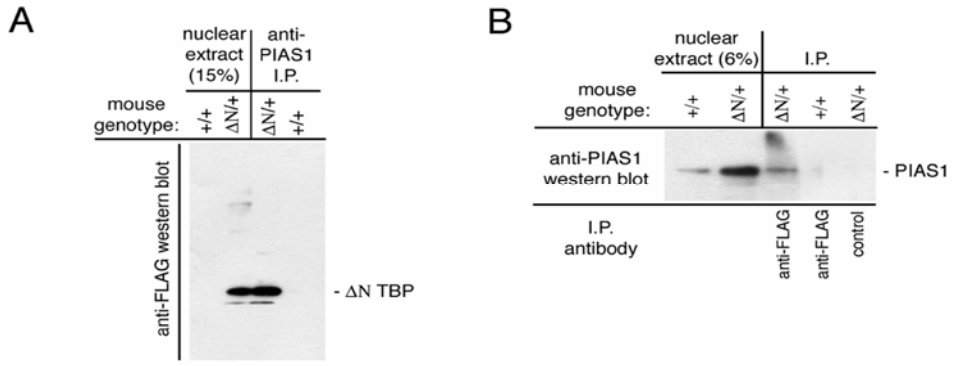


Figure 2.6: Endogenous PIAS/TBP interaction. Co-immunoprecipitation of endogenous PIAS1 and TBP from mouse nuclear extracts were performed with the indicated antibodies on nuclear extracts prepared from livers and spleens harvested from wildtype or $tbp^{\Delta N/+}$ mice. **(A)** ΔN-TBP co-immunoprecipitates with PIAS1. Nuclear extracts from wildtype (+/+) or heterozygous (ΔN/+) mice were analyzed by western blotting with anti-FLAG antibody before or after immunoprecipitation with anti-PIAS1 antibody. Results showed that the ΔN/+ nuclear extracts contained a single major anti-FLAG-reactive protein (ΔN-TBP) and that this protein co-immunoprecipitated with PIAS1. **(B)** PIAS1 co-immunoprecipitates with ΔN-TBP. Nuclear extracts were immunoprecipitated with anti-FLAG or an irrelevant negative control mouse monoclonal antibody and probed western blots with goat anti-PIAS1 (C20) antibody. Results showed that a portion of the endogenous PIAS1 co-precipitated with endogenous FLAG-tagged TBP_{CORE} from heterozygous but not wildtype nuclear extracts; the non-specific antibody did not bring down PIAS1.

Nuclear extracts were then immunoprecipitated with the anti-FLAG antibody or a negative control antibody and western blots were probed with anti-PIAS1 antibody (Fig. 2.6B). The anti-FLAG antibody, but not the control antibody, brought down PIAS1 protein from heterozygous but not wildtype extracts. These results indicated that a portion of the endogenous PIAS1 in mouse liver and spleen nuclear extracts was complexed with TBPΔN.

TBP Interacts with PIASx and PIASy

Amino acid alignment of all known isoforms of PIAS proteins from all four *pias* genes (200) revealed that all shared the TBP interaction domain (Fig. 2.7 and data not

shown) identified here (Fig. 2.4A and B). The remainder of the C-terminal region shows little identity between family members. In fact, PIASy does not contain approximately 100 amino acids that are found in the other PIAS family members. Based on the amino acid conservation in the TBP interaction domain between all PIAS proteins, we wished to determine whether proteins from the *piasx* and *piasy* genes also functionally interacted with TBP. Full-length cDNAs for PIASxβ and PIASy were isolated by RT-PCR and were inserted into the two-hybrid prey vector (pGADT7^{SN}) to test for interaction with either MP34-TBP-FL or the empty bait plasmid in two-component two-hybrid assays. Both proteins interacted strongly with TBP-FL; neither prey was auto-active when tested with empty bait (MP34) vector (Fig. 2.8).

In conclusion, our data suggest that TBP interacts with ZFP523 and with all members of the PIAS family of proteins through the conserved TBP_{CORE}. Using PIAS1 as a family representative, we show that that PIAS/TBP interaction involves a conserved 39 amino-acid region within the PIAS C-terminal region and very likely occurs through direct contacts between PIAS and TBP proteins. Moreover, using nuclear extracts from mice bearing an epitope-tagged TBP_{CORE}, we present evidence suggesting that the endogenous proteins interact as well. These results lend insights into the gene-regulatory interplay between TBP, ZNF523, PIAS proteins, and other transcriptional regulators, as discussed below.

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PIAS1.    -MADSAELKQMVMSLRVSELQVLLGYAGRNKHGRKHELLTKALHLLKAGCSPAVQMKIKE 59
PIAS3.    -MAELGELKH....F.........F.....S.......A.......SS.A.S....... 59
PIASxβ.   ----------..S.F.........F.....S....D..MR......S.......I..R. 50
PIASy.    MAAELVEAKN....F......M...FV..S.S.L....VTR..Q.VQFD...ELFK.... 60

PIAS1.    LYRRRFPQKIMTPADLSIPN----------VHSSPMPPTLSPSTIPQLTYDGHPASSPLL 109
PIAS3.    .......R.TLG.S...LLS----------LPPG-TS.VG..GPLAPIP------PTL.T 102
PIASxβ.   .....Y.RTLEGLC...TIKSSVFSLDGSSSPVE.DL.VAGIHSL.STSITPHSP...VG 110
PIASy.    ..ET.YAK.SAE.GPQAPRP----------------LDP.ALHSMPRTP-----L.G.TV 99

PIAS1.    PVSLLGPKHELELPHLTSALHPVHPDIKLQKLPFYDLLDELIKPTSLASDNSQRFRETCF 169
PIAS3.    .GT.....R.VDMH--PPLPQ.VH..VTMKP....EVYG...R..T...TS....E.AH. 160
PIASxβ.   S.L.QDT.PTF.MQQPSPPIP.....VQ.KN........V.......VQSSI...Q.KF. 170
PIASy.    DYPV.YG.YLNG.G--RLPTKTLK.EVR.V....FNM....L...ELVPQSAEKLQ.SPC 157

PIAS1.    AFALTPQQVQQISSSMDIS-GTKCDFTVQVQLRFCLSETSCPQEDHFPPNLCVKVNTKPC 228
PIAS3.    T.......L...LT.REVLP.A...Y.I........C........Y.....F....G.L. 220
PIASxβ.   I........RE.CI.R.FLP.GRR.Y.......L..A........NY.NS..I...G.LF 230
PIASy.    I.....R..EM.RN.RELQP.V.A---...V..I.Y.D.......QY...IA....HSY. 214

PIAS1.    SLPGYLPPTKNGVEPKRPSRPINITSLVRLSTTVPNTIVVSWTAEIGRTYSMAVYLVKQL 288
PIAS3.    P...........A.............P.A...A........N.SS.F..N..LS....R.. 280
PIASxβ.   P....A..P...I.Q...G..L.........SA...Q.SI..AS...KN...S....R.. 290
PIASy.    .V...Y.SN.P....N..C....L.H.MY..SAT-.R.T.T.G-NY.KS..V.L...R.. 272

PIAS1.    SSTVLLQRLRAKGIRNPDHSRALIKEKLTADPDSEIATTSLRVSLLCPLGKMRLTIPCRA 348
PIAS3.    TAGT...K.........................EV.........M.........V.... 340
PIASxβ.   T.AM.....KM.................................M.............. 350
PIASy.    T.SD.....KTI.VKH.ELCK..V....RL.........GV....I...V....SV.... 332

PIAS1.    LTCSHLQCFDATLYIQMNEKKPTWVCPVCDKKAPYEHLIIDGLFMEILKYCTDCDEIQFK 408
PIAS3.    ...A...S...A.............T...........S............NS.S........ 400
PIASxβ.   V..T.......A..L.........I.........A..S..L.........ND.S.V...K.Q 410
PIASy.    E..A.......VF.L.........M......P.A.DQ......LSK..SE.EGA...E.L 392
                          N ◄──► C
PIAS1.    EDGSWAPMRSKKEVQEVTASYN-GV----------------------DGCLSSTLEHQVAS 446
PIAS3.    .....C..KP...AS..CPPPGY.L----------------------..LQY.AVQEGIQP 439
PIASxβ.   .....C...P...AMK..SQPCTK.----------------------ESSSVFSKPCS.TV 449
PIASy.    AE...R.I.AE..PS--CSPQGPILVHGTSDANGLAPASSTPGIGSGLSGPGSAGSGAGA 450

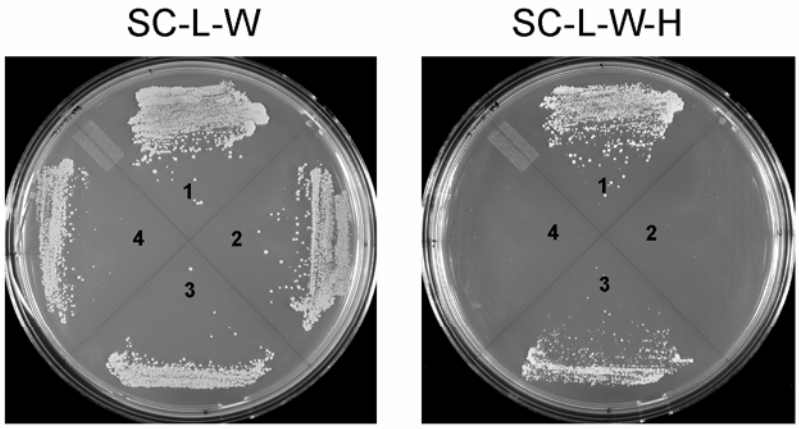
PIAS1.    HNQSSN-KNKKVEVIDLTIDSSS------------DEEEEEPPAKRTCPSLSPTSPLS-- 491
PIAS3.    ESK------.R........E...-------------...D-L..T.KH..VT.AAI.ALPG 480
PIASxβ.   ASDASK---..ID......E...------------....-D.....K.IFM.E.QSSP-- 491
PIASy.    AGSLE.G.T-GAD.V...L....SSEDEDEDEDDDED.D.G.RPN.R..FQKGLV.AC   507

PIAS1.    NKGILSLPHQASPVSRTPSLPAVDTSYINTSLIQDYRHPFHMTPMP---YDLQGLDFFPF 548
PIAS3.    S..A.TSG..P.S.L.S.AMGTLGSDFLSSLPLHE.PPA.PLG------A.I....L.S. 534
PIASxβ.   T..V.MY--.P.S.R-V..VTS..PAA.PP.-LT..SV...H..VSSMSS..P....LSL 547

PIAS1.    LSGDNQHYNTSLLAAAAAAVSDDQDLLHSSRFFPYT-SSQMFLDQLS-AGGSTSLPATNG 606
PIAS3.    .QTES...GP.VITS-----L.E..T.G--H..Q.RGTPSHF.GP.APTL.SSHRSS.PA 587
PIASxβ.   IPV.P.YCPP-------------------------------.F..S.TSPLTAS.TSV.TT 577

PIAS1.    SSSGSNSSLVSS-NSLRESHGHGVASRSSADTASIFGIIPDIISLD 651
PIAS3.    PPP.RV..I.APGS....G..GPLP.GP-----.LT.CRS.V.... 628
PIASxβ.   .PHE.-.TH...-S.S.SET.VITS.GR---------N........ 612
```

Figure 2.7: Alignment of mouse PIAS1, PIAS3, PIASxβ, and PIASy proteins. At top is indicated the full amino acid sequence of mouse PIAS1. For other family members, dots represent amino acids that are identical to PIAS1. Comparison of the PIAS protein sequences revealed that the deduced TBP-interacting domain of PIAS1 (denoted in bold) is highly conserved in PIAS3 and PIASxβ, with the greatest amino acid identity occurring within the Ser/Ac domain. PIASy contains a similar, albeit more extended Ser/Ac domain (183). Shaded amino acids represent the first amino acid encoded by the shortest 5'-truncated PIAS1 clone ($PIAS1_{453-651}$) that interacted with TBP and the single PIAS3 clone ($PIAS3_{325-628}$) that was isolated in yeast two-hybrid screens described above. The boxed amino acid in PIAS1 represents the last amino acid encoded by the most 3'-truncated mutant ($PIAS1_{6-491}$) that interacted with TBP and defined the C-terminal boundary of the deduced TBP interaction domain of PIAS1.



sector	bait	prey
1	TBP-FL	PIASx
2	empty	PIASx
3	TBP-FL	PIASy
4	empty	PIASy

Figure 2.8: Interaction of PIASx and PIASy with TBP. Two-hybrid assays were performed as in previous Figs. PIASx$\beta_{1\text{-}612}$ and PIASy$_{1\text{-}507}$ interacted with TBP-FL by yeast two-hybrid (sectors 1 and 3, respectively) and neither was auto-active when co-expressed with the empty bait plasmid (sectors 2 and 4, respectively).

Discussion

TBP is a central player in transcription initiation whose activity is controlled by interactions with activators, repressors, and other transcriptional regulatory proteins. In agreement with the recently reported interaction between TBP and human ZNF76 (238), we show that mouse ZFP523, which shares 92% amino acid identity with ZNF76, interacts with TBP (Figs 2.2, 2.3B). The clone of ZFP523 we isolated in our screen was a partial cDNA that encoded the last 366 amino acids of the 568 amino acid protein. This clone contained the entire GARD domain (amino acids 362-444), which is the region of the human protein shown to interact with TBP (238).

Most of the TBP interacting clones isolated in our two-hybrid screens encoded PIAS1 or PIAS3 proteins (Fig. 2.2). Previously, regulation of gene transcription by the PIAS family proteins has been shown to occur via interactions with factors that are upstream of TBP in the transcriptional initiation process, some of which have also been shown to directly interact with TBP. These include the general co-activator CBP/p300 (208), the transcriptional factors MSX2 (232), p53 (81, 188), p73 (145), and others. To our knowledge, this is the first report of a direct interaction between PIAS proteins and a component of the basal transcription machinery.

The interaction between TBP and PIAS proteins involves the conserved C-terminal core of TBP and a C-terminal 39 amino acid region found in common between PIAS1, PIAS3, PIASx, and PIASy proteins. Although the functions of the more highly conserved N-terminal region of PIAS proteins, including its SUMO E3-ligase activity, are well characterized, the C-terminal region is less well understood (78). The C-terminal regions of individual PIAS proteins have been shown to mediate protein-protein interactions with some regulators (78). However, alignment shows that only a region within the TBP interacting domain described here (Fig. 2.4B) is shared among all seven PIAS proteins (Fig. 2.7). Our interpretation is that all PIAS proteins share the property of interacting directly with TBP; the remainder of the PIAS C-terminal domains determine the interactions that distinguish the activities of each family member from the others.

Another activity shared by all PIAS proteins is that they function as SUMO E3-ligases through amino acids in the conserved N terminus (95, 155, 183, 189). PIAS1-dependent sumoylation of the ZNF76 prevents ZNF76 from interacting with TBP (238). Our data suggest that the interaction of the PIAS1 C terminus with TBP could also

influence the interaction of ZNF76 with TBP. The localization of SUMO E3-ligase activity and TBP binding activity to opposite ends of PIAS proteins suggests that PIAS proteins might "dock" at TBP and sumoylate transcription factors at the promoter. Further investigation of the interactions between TBP, PIAS proteins, and transcription factors like ZNF76 will be required to determine which interactions are cooperative, which are antagonistic, and how these proteins interact at the TBP interface to regulate the expression of specific target genes.

HUNTINGTIN-ASSOCIATED PROTEIN INTERACTS WITH THE C-TERMINAL CORE DOMAIN OF TATA-BINDING PROTEIN

Introduction

Huntingtin-Associated Protein 1 (HAP1) was first identified via its direct interaction with the Huntingtin Protein (Htt) (106, 110). Htt is ubiquitously expressed and is important for development and survival of neurons. Htt, in association with HAP1, was shown to be involved in endocytic trafficking and signal transduction pathways in the cytoplasm (52, 126). Htt is also implicated in transcriptional regulation. To this end, Htt has been shown to interact with numerous nuclear transcription factors (63) and regulate BDNF expression via interactions with REST/NRSF (240). Mice lacking the Htt protein appear to have specific deficiencies in gene expression (231).

In addition to its role in normal cellular processes, Htt is also known for its role in Huntington's disease. Htt contains a polyglutamine (polyQ) region that, upon expansion beyond 36 glutamines, acquires a toxic "gain of function," allowing a fragment of the mutant protein to bind cellular proteins that would not normally be associated with wild-type Htt (187). More specifically, an N-terminal fragment (encoded by exon 1 of the htt gene) is cleaved and able to translocate to the nucleus, where it becomes incorporated into neuronal nuclear aggregates (185, 224). These aggregates were also shown to contain additional polyglutamine-containing proteins, such as TBP, cyclic AMP response element-binding protein, and others (135, 161). Interaction of polyQ-containing transcription factors with mutant Htt causes structural destabilization of the interacting protein, which can result in deactivation of their transcriptional activity (187). It still

remains unclear whether HAP1-Htt interactions contribute to Huntington's disease pathology, since HAP1 expression does not seem to correlate with specific areas that are affected in HD pathology (7). Nevertheless, HAP1 does seem to play a neuroprotective role in HD transgenic mice (108, 241).

Two alternately spliced HAP1 isoforms have been characterized in mice, HAP1-A, a 598 amino acid protein, and HAP1-B, which is the longer isoform encoding a 628 amino acid protein. The difference between HAP1-A and HAP1-B is found in the extreme C terminus, whereby both proteins contain a unique C-terminal polypeptide beyond the end of the 577 amino acid HAP1 common region (112, 131, 151). Analysis of transcripts formed by alternate splicing at the 3' end of the *Hap1* gene revealed a third isoform, Hap1-C. The HAP1-C protein does not differ from HAP1-B since the difference between the two splice isoforms is found in the 3' untranslated region (151).

HAP1 expression is highest in specific neuronal locations in the brain (111), although it is also expressed in tissues outside of the brain (113). The predominant vesicle-associated subtype is HAP1-B (131). Subcellular localization studies showed that HAP1 is mostly cytoplasmic and associated with microtubules and specific membranous organelles, including mitochondria, endoplasmic reticulum, endosomal and lysosomal organelles, and synaptic vesicles (62). Studies conducted within the past seven years have solidified a role for HAP1 in microtubule-based transport via its interaction with dynactin p150 (45, 105). Additional HAP1 protein interaction and localization studies support a role for HAP1 in endosomal trafficking (62, 112, 131, 138). HAP1 also was shown to play a role in neuronal calcium signaling in conjunction with Htt (211). Disruption of the Hap1 gene product in mice resulted in a depressed feeding behavior

(19). In agreement with these findings, Li and colleagues showed that mice lacking HAP1 had a degenerated hypothalamic region of the brain, which is responsible for feeding behavior (108). Later findings showed that litter size reduction allowed some HAP1 mutants to survive to adulthood. Those mice that survived were retarded in growth, although they appeared to have no abnormal brain pathology (40).

In addition to interactions with cytoplasmic proteins, HAP1 was also shown to bind the nuclear transcription factor NeuroD (ND) (126). This study found that, in the cytoplasm of the cell, Htt interacted with ND, but only when HAP1 was present in the complex. This resulting heterotrimeric assembly of proteins then recruits the mixed-lineage kinase 2 (MLK2) protein. MLK2 phosphorylates ND, targeting it to the nucleus. Therefore, in this scenario, HAP1 served as a critical scaffold protein which was required for proper neuronal gene expression (126).

Using the yeast two-hybrid system, we screened mouse cDNA prey libraries in an attempt to identify proteins that interact with TBP. Two of our screens identified the Huntingtin-Associated Protein 1 (HAP1) as a protein that interacted with the pan-eukaryotic C-terminal (CORE) domain of TBP. Co-immunoprecipitation assays showed that HAP1 could associate with TBP in co-transfected h293 cells. Mapping studies identified two distinct regions of HAP1 that independently interacted with the TBP C terminus. These data suggest that HAP1 may associate with TBP in a cellular context, although the function of this interaction remains uncertain.

Materials and Methods

Yeast Plasmids, Bait Clones, Prey Libaries, and Yeast Transformations

TBP baits were generated by polymerase chain reaction (PCR) from a plasmid containing the predominant mouse somatic TBP cDNA (154, 206). Full-length TBP (TBP-FL) was amplified by PCR using TBP-N-start primer and TBP-C-end primer; the TBP N terminus clone (TBP-N), which encoded amino acids 1-136, was amplified using TBP-N-start primer and TBP-N-end primer; the TBP C terminus clone (TBP-C), which encoded amino acids 134-316, was amplified using TBP-C-start primer and TBP-C-end primer (Table 3.1). TBP-FL and TBP-N were cut with *Sal* I and *Not* I and inserted into the pDBLeu bait vector (Gibco BRL ProQuest Two-Hybrid System). TBP-C was digested with *Nco* I and *Not* I and inserted in pDBLeu.

Oligo(dT)-primed cDNA prey libraries were constructed and inserted into the pPC86 vector (Gibco BRL ProQuest) as follows. Total RNA was extracted and CsCl-purified (191) from embryonic day 10.5 (E10.5) wild-type C57Bl/6J whole pregnant uteri or placentas. In each case, either whole pregnant uteri or placentas were obtained from four pregnant dams to generate a pool of RNA. Poly(A+) mRNA from these samples was purified using Oligo$(dT)_{25}$ Dynabeads (Dynal Biotech ASA, Oslo, Norway) following the manufacturer's protocols. Oligo(dT)-primed libraries were constructed from ~3μg of poly(A+) RNA; cDNA inserts for each library had an average size of >1 kb (Fig. 3.1B). The whole pregnant uteri and placental libraries contained ~2.1 x 10^6 and ~2.5 x 10^6 independent recombinants, respectively, each with ~95% bearing inserts (Fig. 3.1B). To access more 5' regions of prey cDNAs, a random hexamer-primed library was

also constructed using 3μg each of whole pregnant uteri and placental poly(A+) RNA. Construction of rando- primed cDNAs was performed using a standard oligo(dT)-primed cDNA synthesis kit (Stratagene), with the following adaptations. First, instead of an oligo(dT) primer, random hexamer oligonucleotides were used to prime the first-strand cDNA. Second, an adapter/coordinator containing a 5' *Eco* RI overhang and a blunt 3' end was constructed by annealing the 5' adapter and 3' coordinator and ligating this to the blunt-ended double-strand cDNA product, according to the Stratagene cDNA synthesis kit manual. When assembled, the 5' and 3' ends of the final cDNA product both contained an *Eco* RI overhang (Table 3.1), which allowed for direct insertion of the library product into an *Eco* RI-digested vector. The adapter/coordinator also encoded both a *Not* I and a *Sal* I site, each internal to the *Eco* RI overhang (Table 1), which can be used for insertion of the cDNA into either a *Not* I- or *Sal* I-digested vector. Two versions of the random-primed library were constructed and subsequently pooled. The first consisted of non-PCR-amplified cDNA that was inserted into the *Eco* RI site of pPC86. This library contained ~6 x 10^5 primary transformants with ~60% bearing inserts. The second library was constructed from random-primed cDNA that had been amplified by PCR using oligos that annealed to the adaptor/coordinator sequences located at the 5' and 3' ends of the final cDNA library product. PCR-amplified cDNA was digested with *Not* I and inserted into the *Not* I site of pPC86. This library contained ~1.8 x 10^6 independent recombinants with ~90% bearing inserts. The two libraries were pooled to achieve a library representing more primary clones. The combined library consisted of ~2.4x10^6 cDNA clones and had an average insert size of ~1.0 kb with ~90% bearing inserts (Fig. 3.1B).

Table 3.1. Oligonucleotide primer sequences for construction of bait and prey cDNAs.

name	sequence[1]
HAP1-157-forward	5'-tat*gtcgac***agaacgggacctgaacacagc**-3'
HAP1-238-forward	5'-tat*gtcg**ac***acagagggatcaagaccagca**-3'
HAP1-300-forward	5'-tat*gtcgac***gatgctcattctggaatgtgtg**-3'
HAP1-380-forward	5'-tat*gtcgac***ctcctacatgcaggattatggg**-3'
HAP1-473-forward	5'-tat*gtcgac***agatctcaagccacctgaagat**-3'
HAP1-261-reverse	5'-tat*gcggc**cgc***tgtctcagccttagggtgt-3'
HAP1-306-reverse	5'-tat*gcggccg**cacacattccagaatgagcatc**-3'
HAP1-386-reverse	5'-tat*gcggccg**cccataatcctgcatgtaggag**-3'
HAP1-478-reverse	5'-tat*gcggccgc***tcttcaggtggcttgagatctt**-3'
HAP1β-581-reverse	5'-tat*gcggccgc***gttggggagtcttttgggacca**-3'
TBP-N-start	5'-atc*gtcgact***atggaccagaacaacagccttcca**-3'
TBP-N-end	5'-ata*gcggccgc*ttaa**gagctctcagaagctggtgtggca**-3'
TBP-C-start	5'-tatggatcca*ccatgg*accagagc**tctggaattgtaccgcagcttca**-3'
TBP-C-end	5'-gcta*gcggccgc*ccaagtagcagcacagagc-3'
5' adapter	5'-aattc*gcggccgcgtcgac*-3'
3' coordinator	5'-phos-*gtcgacgcggccgcg*-3'

[1]Endogenous protein-coding sequences are in bold and engineered restriction sites are in italics.

All interactions were tested in *Saccharomyces cerevisiae* strain MaV203 (Gibco BRL), which contained the HIS3, URA3, and LacZ reporters, each under the control of a different promoter. For library transformations, a culture of AH109 that contained the TBP bait construct was grown at 30ºC for 48 hours in liquid synthetic complete medium (SC) lacking leucine (SC-L) (Q-BIOgene, Irvine, CA). This culture was used to seed 300 ml of 2X yeast extract/peptone/adenine/dextrose (YPAD) at 5 x 10^6 yeast/ml, which was determined by counting on a hemacytometer. The culture was grown at 30ºC for ~5 hours to a density of 2 x 10^7 yeast/ml. Yeast were collected by centrifugation, washed once with water and once with 100mM LiAc, and transformed with 14.4 ml 50% PEG (average m.w. 3350), 2.16 ml 1.0 M LiAc, 0.3 ml 10 mg/ml sheared denatured salmon sperm DNA, and 120 μg of the cDNA library in pPC86. Single- or two-component transformations of bait and/or prey plasmids into yeast used a standard PEG/LiAc protocol.

Two-hybrid screens were performed on SC medium lacking leucine, tryptophan, histidine, and uracil (SC-L-W-H-U). Both two-hybrid screens used TBP-FL as bait to screen either a combination of 50% whole pregnant uteri and 50% placental (screen 1) or random-primed (screen 2) cDNA prey libraries. Primary transformants were transferred onto a new SC-L-W-H-U plate and clones that grew well on these plates were transferred to either a SC-L-W-H + 80 mM 3-AT plate or were grown on SC-L-W and tested for LacZ expression following the X-Gal Assay protocol detailed in the ProQuest Two-Hybrid system manual (Gibco BRL cat. series 10835). Prey plasmids from those clones that grew under all selection conditions and scored positive for LacZ expression (turned blue) were isolated from yeast by glass bead lysis. Recovered plasmids were transformed into bacteria, clones were selected, and inserts were sequenced to determine the cDNA identity. Isolated prey plasmids were re-transformed into MaV203 with the bait or with the empty pDBLeu plasmid (autoactivation test) and grown on SC-L-W-H + 50 mM 3-AT, SC-L-W-H + 75 mM 3-AT, and SC-L-W-H-U plates to verify the interaction.

To identify the domain of TBP that interacted with HAP1, TBP bait constructs in combination with the empty pGADT7 prey plasmid (auto-activation test) or with either the HAP1 random-primed or the HAP1 oligo-dT-primed clone were co-transformed into MaV203 and plated onto SC-L-W. Individual colonies that grew on SC-L-W plates (containing both the bait and prey plasmid) were suspended in water and streaked onto SC-L-W, SC-L-W-H + 50 mM 3-AT, SC-L-W-H + 75 mM 3-AT, and SC-L-W-H-U plates to verify the interaction.

HAP1 Deletion Series PCRs and Analysis of HAP1 Truncated Protein Interactions Using the Yeast Two-Hybrid System

Plasmid DNA, which encoded amino acids 155-582 of the mouse HAP1-B cDNA was used as the template for PCR amplification. PCR primer sets for amplification of each truncated cDNA were: $HAP1_{157-261}$, HAP1-157-forward primer and HAP1 261-reverse primer; $HAP1_{157-306}$, HAP1-157-forward primer and HAP1-306-reverse primer; $HAP1_{157-386}$, HAP1-157-forward primer and HAP1-386-reverse primer; $HAP1_{157-479}$, HAP1-157-forward primer and HAP1-479-reverse primer; $HAP1_{157-581}$, HAP1-157-forward primer and HAP1-581-reverse primer; $HAP1_{238-386}$, HAP1-238-forward primer and HAP1-386-reverse primer; $HAP1_{238-479}$, HAP1-238-forward primer and HAP1-479-reverse primer; $HAP1_{238-581}$, HAP1-238-forward primer and HAP1-581-reverse primer; $HAP1_{300-386}$, HAP1-300-forward primer and HAP1-386-reverse primer; $HAP1_{300-479}$, HAP1-300-forward primer and HAP1-479-reverse primer; $HAP1_{300-581}$, HAP1-300-forward primer and HAP1-581-reverse primer; $HAP1_{380-581}$, HAP1-380-forward primer and HAP1-581-reverse primer; $HAP1_{473-581}$, HAP1-473-forward primer and HAP1-581-reverse primer (Table 3.1). PCR products were digested with *Sal* I and *N*ot I and were inserted into the pPC86 prey vector.

Three plasmid clones of each truncated HAP1 construct were isolated and separately co-transformed with pDBLeu-mTBP-N, pDBLeu-mTBP-C, or empty pDBLeu bait plasmid into yeast as above. Yeast were plated onto SC-L-W agar plates and grown at 30ºC. Resultant colonies were suspended in water and streaked onto SC-L-W, SC-L-W-H + 50mM 3AT, SC-L-W-H + 75mM 3AT, and SC-L-W-H-U to test each protein interaction pair.

Co-Transfection and Co-Immunoprecipitation

h293 cells were plated at ~30% confluence on 60 mm dishes 16 hours prior to transfection. Transfection mixes consisting of 1 µg each of pCMV-MYC-HAP1$_{155-582}$ and pCMV-HA-TBP-FL and 10 µl of Novafector in 300 µl Dulbecco's Modified Eagle's Medium (DMEM) were mixed and added to a single plate of cells in 2ml DMEM. Two plates were transfected for each antibody co-immunoprecipitation experiment. Cells were incubated with transfection mixes for 5 hours at 37°C, 7.5% CO_2, after which 2 ml of DMEM containing 20% NCS, 4% FBS, and 2X antibiotic/antimycotic (Ab/Am) (Mediatech) solution was added to plates and incubated overnight. The next morning, the medium was replaced with DMEM containing 10% newborn calf serum, 2% fetal bovine serum, and 1X Ab/Am.

Forty-eight hours after transfection, cells were rinsed twice in ice-cold phosphate buffered saline and 500 µl ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% TX-100, 0.1% SDS, 1 mM EDTA, 0.8% deoxycholic acid, 10% glycerol, 0.2 mM NaVO3, 1 mM phenymethylsulphonylfluoride (PMSF), and 0.005 mg/ml each of leupeptin, pepstatin, and aprotinin) was added to each dish. Dishes were rocked at 4°C for 30 min and then transferred to 1.5 ml tubes and incubated on ice for an additional 30 min with occasional vortexing. Lysed cells were cleared by centrifugation at 10,000 x g for 20 min at 4°C. Whole cell lysates were pre-cleared with 10 µl of non-specific rabbit polyclonal antibody and 20 µl of Protein G plus/Protein A agarose (Calbiochem) for 2 hours, rotating at 4°C, after which the agarose was pelleted and 0.9 ml of supernatant was transferred to a new tube containing 12.5 µl of either anti-TBP Nc rabbit polyclonal antibody or a non-specific control antibody and rotated at 4°C for 16 hours, followed by

one hour with 30 µl of Protein G plus/Protein A agarose added. Beads were washed once with lysis buffer, once with wash buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2% TX-100, 10% glycerol, and 1 mM PMSF), three times with wash buffer that contained 500 mM NaCl, and a final time with wash buffer that contained 100mM NaCl. Proteins bound to agarose beads were released by boiling for 5 minutes in 1X SDS-PAGE sample buffer, separated by polyacrylamide gel electrophoresis, and analyzed by Western blotting with the designated antibodies.

Results

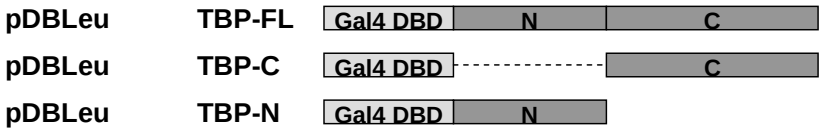
Identification of HAP1 as a TBP Interacting Protein by Yeast Two-Hybrid

To identify proteins that interacted with TBP, we screened a mixture of oligo(dT)-primed placental and whole pregnant uteri cDNA libraries (50% of each library was used) (screen #1) and a random-primed library which also consisted of 50% placental and 50% whole pregnant mouse uteri cDNAs (screen #2). Both screens were performed using TBP-FL as the bait protein, which was expressed from the pDBLeu plasmid (Fig. 3.1). In screen #1, ~ 6 x 10^6 primary transformants (yeast containing both a bait and prey plasmid) were plated onto SC-L-W-H-U. From these plates, 82 colonies grew and were transferred to fresh SC-L-W-H-U plates. Those clones that re-grew were further tested for activation of the LacZ reporter gene. Those that tested positive for an interaction under all conditions were grown in liquid SC-L-W medium and individual prey plasmids were isolated from each clone. Each prey plasmid was re-transformed into yeast in combination with the TBP bait plasmid or with an empty bait plasmid to verify the interaction in a new host cell, as well as to test for auto-activation in the absence of the

interacting TBP bait protein. In these assays, seven of the clones grew on SC-L-W-H+50 mM 3-AT, SC-L-W-H + 75 mM 3-AT, SC-L-W-H-U, and also activated the LacZ reporter gene (turned blue in X-gal filter assays) (data not shown). Each of the inserts was sequenced to attain the identity of the interacting protein. Three clones encoded a partial cDNA of B'-related factor 1 (BRF1), a known TBP interacting protein (196). A fourth clone encoded a portion of the HAP1 protein, beginning at amino acid 10. The remaining three clones encoded the following proteins: an unknown protein with similarity to human SR-rich proteins (accession number BAB30779) (68), which are components of the splicing machinery; an unknown protein product containing similarity to chromosome segregation ATPases (accession number BAE22251); and Rho-associated coiled-coil forming kinase 1 (accession number NM_009071), a serine-threonine protein kinase.

In screen #2 of the random-primed prey library, ~1.7 x 10^7 primary transformants were plated and tested as in screen #1. Out of 183 colonies that grew on SC-L-W-H-U, five yeast clones activated all three reporter genes (HIS3, ADE2, and LacZ), were not auto-active (data not shown), and thus appeared to express a TBP interacting prey protein. One of these final clones encoded amino acids 155-582 of the HAP1-B ($HAP1_{155-582}$) protein. The other four encoded identical clones of protein kinase, cyclic AMP-dependent, regulatory protein alpha (accession number AAH03461), a regulatory subunit of cyclic AMP-dependent protein kinases (16). The interaction of the random-primed HAP1 clone with TBP-FL is shown in Figure 3.2, sector 1. The HAP1-TBP-FL interaction and control protein interaction pairs which were supplied with the ProQuest Two-Hybrid System are shown in Figure 3.2, sectors 1 and 7-8, respectively.

A



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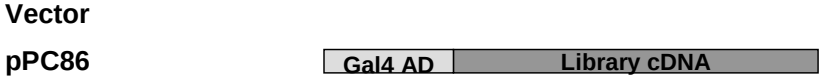
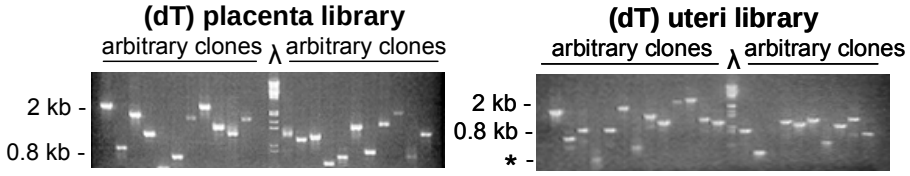


Library characteristics

library	RNA source	complexity	% inserts	insert size
oligo(dT) placenta	E10.5 C57Bl/6 placentas	~2.5 x 10^6	95	>1 kb
oligo(dT) uteri	E10.5 C57Bl/6 pregnant uteri	~2.1 x 10^6	95	>1 kb
random p+u	E10.5 C57Bl/6 p+u	~2.4 x 10^6	90	~1 kb

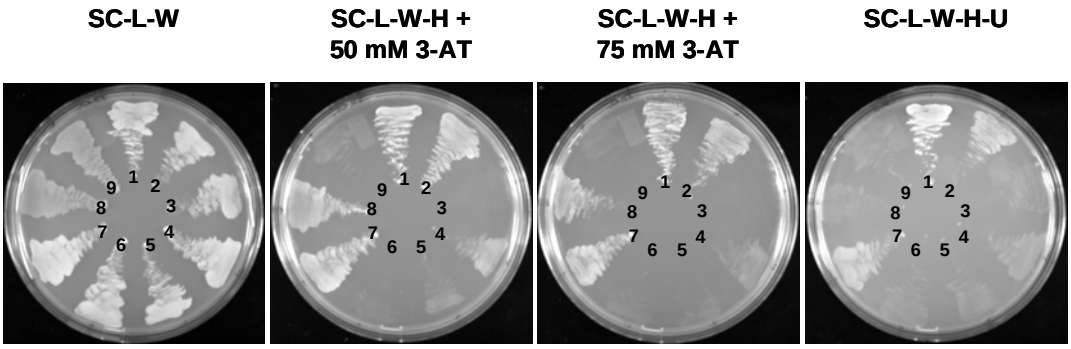


C

Yeast two-hybrid screens

screen	bait	prey library	transformants	interacting clones
1	TBP-FL	oligo(dT)-primed placenta + uteri	6.0 x 10^6	7
2	TBP-FL	random-primed placenta + uteri	1.7 x 10^7	5

Figure 3.1: Yeast two-hybrid bait constructs, prey libraries, and screens. **(A)** Bait constructs. TBP-FL, TBP-C, and TBP-N were expressed from the pDBLeu plasmid, which fused the Gal4 DBD upstream of TBP. N and C designate the vertebrate-specific N terminus and the pan-eukaryotic C terminus of TBP (TBP_{CORE}), respectively. **(B)** Three prey libraries were constructed and inserted into $pGADT7^{SN}$, which fused prey cDNAs downstream of the Gal4 AD. Characteristics of each library are indicated. Below is shown PCR analysis of arbitrary clones from both of the oligo-dT-primed libraries using a primer pair that spans the multiple cloning site of the vector. Lane "λ" contained *Hind* III/*Eco* RI-cut λ-phage DNA markers. Landmark band sizes are indicated at the left of each gel; the asterisk denotes the size of the PCR product arising from empty prey vector. **(C)** The results of the two yeast two-hybrid screens performed are shown. In both screens, TBP-FL was used as bait to screen either the oligo(dT)-primed or random-primed placenta + uteri (p+u) prey libraries for interacting proteins. TBP-interacting prey library clones were subsequently identified by sequencing.



sector	bait	prey
1	TBP-FL	$HAP1_{155-582}$
2	TBP-C	$HAP1_{155-582}$
3	TBP-N	$HAP1_{155-582}$
4	TBP-FL	empty vector
5	TBP-C	empty vector
6	TBP-N	empty vector
7	rat cFos	mouse cJun
8	human RB	human E2F1
9	empty vector	empty vector

Figure 3.2: Protein interactions in the yeast two-hybrid system. Bait and prey plasmid combinations that were contained within each individual yeast clone are indicated below. Sector designations correspond to those on plates. Growth of yeast on SC-L-W indicated that all clones contained both the bait and prey plasmid. Sectors 1-3 show the interaction of $HAP1_{155-582}$ with TBP-FL, TBP-N, or TBP-C, respectively. The interaction of HAP1 with TBP-FL and TBP-C, but not TBP-N resulted in growth under all conditions tested. Sectors 4-6 are auto-activation controls for TBP baits. Although TBP-C appeared to be slightly auto-active on SC-L-W-H + 50 mM 3-AT, none of our TBP bait constructs were auto-active under conditions of greater stringency, as shown in right two panels. Sectors 7-8 are strong and weak positive interaction controls, respectively, that were supplied with the ProQuest system. As expected, the strong interaction control grows well under all conditions tested. The weak interaction control did not grow on SC-L-W-H-U, suggesting that this condition allows growth of yeast containing only strong bait-prey interactions. Sector 9 is a negative "auto-activation" control showing that yeast bearing only the empty bait vector and an empty prey plasmid (no inserts) and did not grow on SC-L-W-H + 50 mM 3-AT, SC-L-W-H +75 mM 3-AT, or SC-L-W-H-U.

Interaction of HAP1 with TBP Domains

The Htt protein is known to be associated with TBP in Huntington's disease patients (135, 161). Since HAP1 was also shown to interact with the Htt protein (106, 110), and it interacted with TBP in our two-hybrid assays, we decided to further

characterize the HAP1-TBP interaction. To determine whether the $HAP1_{155-582}$ clone interacted with the conserved C-terminal TBP_{CORE} or the vertebrate-specific TBP N terminus, yeast were co-transformed with the following baits and preys: TBP-N/ $HAP1_{155-582}$; TBP-C/ $HAP1_{155-582}$. Growth of all clones on SC-L-W verified the presence of both the bait and the prey plasmid (Fig. 3.2, left panel). The TBP-FL clone appeared to be slightly auto-active on SC-L-W-H +50 mM 3-AT, although it fails to grow on SC-L-W-H-U or onSC-L-W-H medium containing 3-AT above a final concentration of 50 mM (Fig. 3.2, sector 4). TBP-C and TBP-N were not auto-active and did not grow under any protein interaction selection conditions (Fig. 3.2, sectors 5-6). Similarly, yeast that contained both the empty bait and empty prey plasmids (negative control) did not grow on plates that selected for interaction of bait and prey proteins (Fig. 3.2, sector 9, panels 2-4), as expected. Finally, we found that the $HAP1_{155-582}$ clone interacted with the C-terminal TBP_{CORE} (Fig. 3.2, sector 2, panels 2-4), but not with the TBP N terminus (Fig. 3.2, sector 3, panels 2-4).

Interaction of TBP with HAP1 in Co-Transfected Cells

For verification, the interactions between TBP and HAP1 identified by two-hybrid screens were tested by co-immunoprecipitation in mammalian cell culture. HAP1 co-precipitated with TBP when expressed in h293 cells (Fig. 3.3, lane 3), but was not detected using a species-matched, non-specific antibody (Fig. 3.3, lane 4).

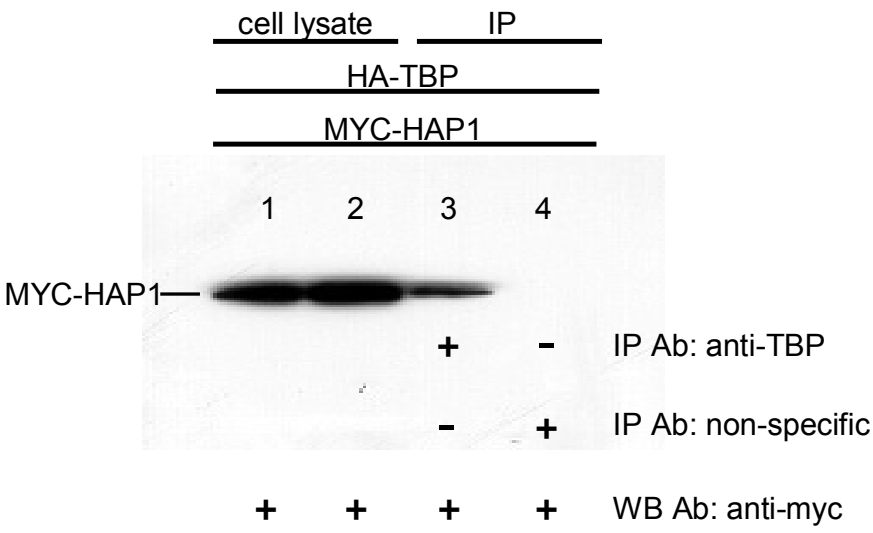
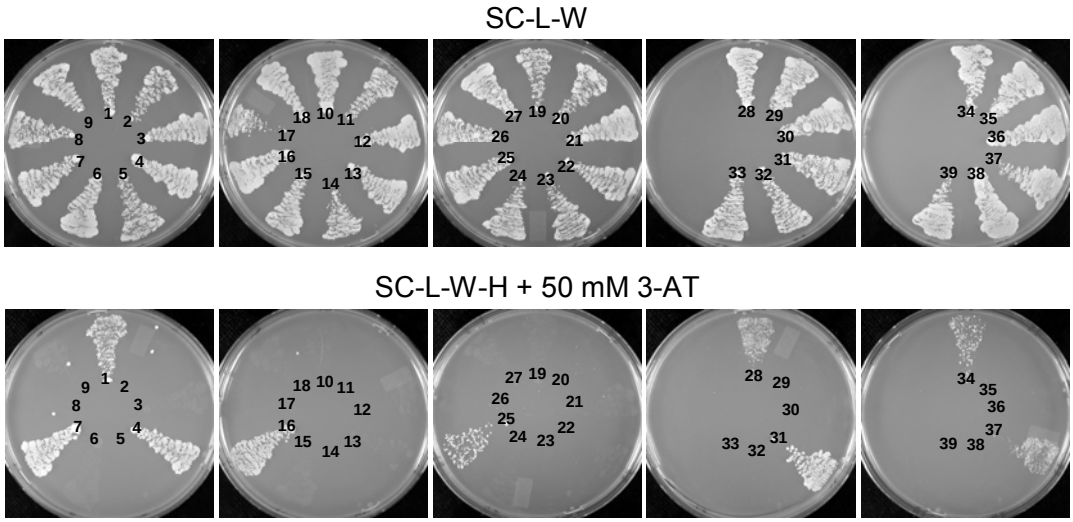


Figure 3.3: Co-immunoprecipitation assay. TBP-HAP1 interactions in co-transfected cells. h293 cells were co-transfected with pCMV-HA-TBP-FL and pCMV-MYC-HAP1. Whole cell lysates (lanes 1-2, HAP1) or immunoprecipitated samples (lanes 3-4, HAP1) were assayed using western blots and anti-myc antibody. Myc-tagged HAP1 co-precipitated with TBP in the presence of the anti-TBP antibody (lane 3), but not with the non-specific antibody (lane 4).

Mapping the TBP Interaction Domains on HAP1

To further define the domain of HAP1 that interacted with TBP, we constructed truncated versions of HAP1 in the pDBLeu bait vector. Truncated clones encoded the following HAP1-B amino acids: 157-386, 157-479, 157-581, 238-386, 238-479, 238-581, 300-386, 300-479, 300-581, 157-261, 157-306, 380-581, and 473-581. Each of the HAP1 truncated clones was tested for interaction with TBP-C and TBP-N in the two-hybrid system (Fig. 3.4, bottom panel). We found that all clones that began at amino acid 157 interacted with TBP-C (Fig. 3.4, sectors 1, 4, 7, 28, and 31; Fig 3.5), but not with TBP-N (Fig. 3.4, sectors 2, 5, 8, 29, and 32). The shortest fragment of HAP1 that interacted with TBP encoded amino acids 157-261, which suggested that a TBP interacting region was between amino acids 157-261 of the HAP1 protein. The linear



sector	bait	prey	sector	bait	prey	sector	bait	prey
1	TBP-C	$HAP1_{157-386}$	14	TBP-N	$HAP1_{238-479}$	27	empty vector	$HAP1_{300-581}$
2	TBP-N	$HAP1_{157-386}$	15	empty vector	$HAP1_{238-479}$	28	TBP-C	$HAP1_{157-261}$
3	empty vector	$HAP1_{157-386}$	16	TBP-C	$HAP1_{238-581}$	29	TBP-N	$HAP1_{157-261}$
4	TBP-C	$HAP1_{157-479}$	17	TBP-N	$HAP1_{238-581}$	30	empty vector	$HAP1_{157-261}$
5	TBP-N	$HAP1_{157-479}$	18	empty vector	$HAP1_{238-581}$	31	TBP-C	$HAP1_{157-306}$
6	empty vector	$HAP1_{157-479}$	19	TBP-C	$HAP1_{300-386}$	32	TBP-N	$HAP1_{157-306}$
7	TBP-C	$HAP1_{157-581}$	20	TBP-N	$HAP1_{300-386}$	33	empty vector	$HAP1_{157-306}$
8	TBP-N	$HAP1_{157-581}$	21	empty vector	$HAP1_{300-386}$	34	TBP-C	$HAP1_{380-581}$
9	empty vector	$HAP1_{157-581}$	22	TBP-C	$HAP1_{300-479}$	35	TBP-N	$HAP1_{380-581}$
10	TBP-C	$HAP1_{238-386}$	23	TBP-N	$HAP1_{300-479}$	36	empty vector	$HAP1_{380-581}$
11	TBP-N	$HAP1_{238-386}$	24	empty vector	$HAP1_{300-479}$	37	TBP-C	$HAP1_{473-581}$
12	empty vector	$HAP1_{238-386}$	25	TBP-C	$HAP1_{300-581}$	38	TBP-N	$HAP1_{473-581}$
13	TBP-C	$HAP1_{238-479}$	26	TBP-N	$HAP1_{300-581}$	39	empty vector	$HAP1_{473-581}$

Figure 3.4: Identification of two domains within HAP1 that interact with TBP. The interaction of truncated HAP1 clones with TBP-C, TBP-N, and auto-activation controls are shown. Yeast containing the indicated bait and prey plasmids (listed below) were grown on SC-L-W (top panel), which selects for the presence of the bait and prey plasmids, and SC-L-W-H + 50 mM 3-AT (bottom panel), which selects for the interaction between bait and prey. All baits were expressed from the pDBLeu plasmid and all truncated HAP1 preys expressing the indicated amino acids were expressed from the pPC86 plasmid. Sectors 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, and 39 were controls confirming that truncated HAP1 clones were not auto-active. Sectors 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, and 38 confirmed that none of the HAP1 clones interacted with TBP-N. Clones 10, 13, 19, and 22 did not grow on SC-L-W-H + 50 mM 3-AT, which suggested that these clones lacked the TBP interaction domain, whereas growth of clones 1, 4, 7, 16, 25, 28, 31, 34, and 37 on SC-L-W-H + 50 mM 3-AT suggested that these clones did contain a TBP interaction domain. Three independent clones of all PIAS1 truncations were tested and each gave the same result; one representative set is shown.

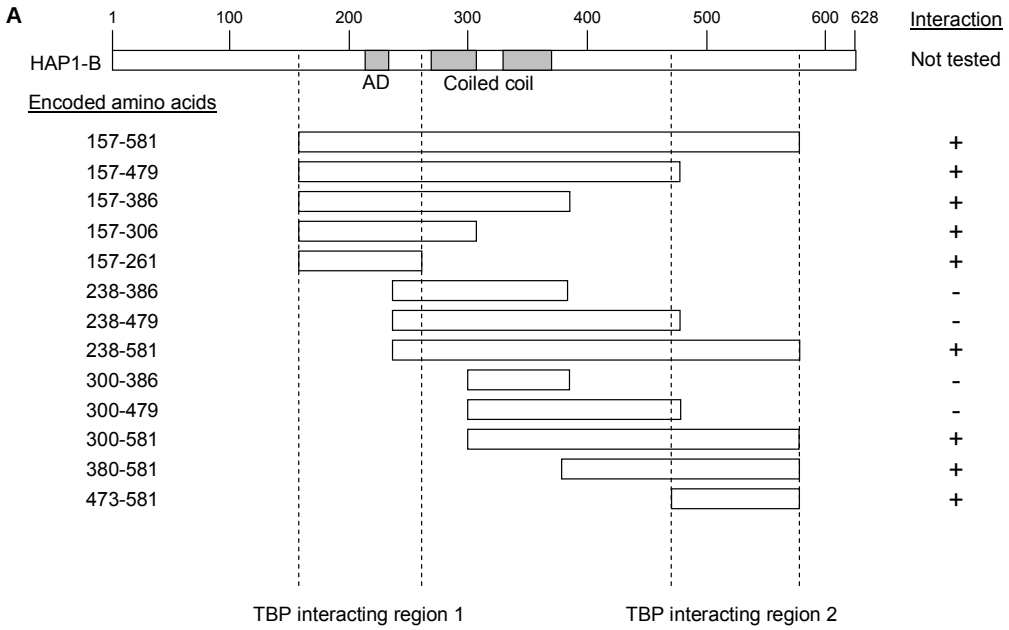


Figure 3.5: Deletion analysis of the TBP interacting regions on HAP1. The full-length HAP1-B protein is represented at top, including the location of the known acidic domain (AD) and the two coiled coil domains. HAP1 amino acids encoded by each cDNA clone in pPC86 are shown at left. The presence (+) or absence (-) of interaction with TBP-C is shown at right. Results suggested that the TBP interacting regions on HAP1 were between amino acids 157 and 261, and between amino acids 473 and 581.

sequence of amino acids within TBP interacting region 1 is shown in figure 3.5. In addition, the lack of interaction between TBP-C and $HAP1_{238\text{-}479}$ (Fig. 3.4, sector 13) suggested that this TBP interacting region on HAP1 could be narrowed to within amino acids 157-237, although a clone containing only these amino acids was not tested for interaction with TBP.

Some HAP1 clones that encoded amino acids outside of TBP interacting region 1 also interacted with TBP-C (Fig. 3.4, sectors 16, 25, 34, and 37), but not with TBP-N

(Fig. 3.4, sectors 17, 26, 35, and 38). The shortest C-terminal HAP1 clone that interacted with TBP-C encoded amino acids 473-581, which suggested that a second TBP interaction domain resided between amino acids 473-581 of HAP1. This region is represented as the TBP interacting region 2 in figure 3.5.

Discussion

HAP1 contributes to many processes, including endocytosis, endosomal trafficking, microtubule-dependent transport, survival of neurons in the brain, and transcriptional regulation (107). Since HAP1 has little similarity with other known proteins (112), much of what is known about its function has been discovered through identification of those proteins that interact with HAP1. Some of the protein binding sites on HAP1 have been mapped to specific locations within the rat HAP1-A protein and are as follows: HAP1 amino acids (AAs) 278-370 for interaction with Htt; AAs 246-425 for interaction with hepatocyte growth factor-regulated tyrosine kinase substrate protein; AAs 1-313 for Rho-GEF Kalirin-7; 220-520 for $GABA_A$ receptor; AAs 273-599 for $IP_3$1 receptor; and AAs 247-446 for NeuroD (ND) (109). Here we show that a clone encoding AAs 155-582 of HAP1-B interacts with the TBP_{CORE} domain by yeast two-hybrid (Fig. 3.2). We also show that TBP co-precipitates with HAP1 in transient co-transfection assays in h293 cells (Fig. 3.3). Further, two unrelated, TBP interacting regions within HAP1 were shown to independently interact with TBP-C. Specifically, the domains of HAP that interacted with TBP are found between AAs 157-261 and between amino acids 473-581 of the mouse HAP1-B protein (Figs. 3.4 and 3.5).

The role of HAP1 in transcriptional regulation is mostly unknown. In one case, HAP1 was shown to function as a scaffold protein that mediates the interaction of ND with MLK2 and Htt while those proteins are still in the cytoplasm (126). Considering the proposed role for Htt in transcriptional regulation both through interactions with transcriptional regulators and through misregulated gene expression in mice lacking Htt (63, 231), and the growing body of literature that consistently finds HAP1 associated with Htt for many processes in the cell, it is possible that HAP1 could function to assemble TBP into a complex with Htt for transcription of Htt-regulated genes.

IDENTIFICATION OF PROTEINS THAT INTERACT WITH THE VERTEBRATE-SPECIFIC N TERMINUS OF TBP

Introduction

TBP can be divided roughly into two halves. The C-terminal core (TBP_{CORE}) of the protein is conserved from archaea to humans (10, 66) and confers nearly all known properties of TBP, including interactions with the TATA box and assembly of the pre-initiation complex (PIC) (171). The N terminus of TBP found in mammals is conserved only within the vertebrate lineages and is suspected to function as a signaling port for transcriptional processes that are unique to the vertebrate species (10). In general, the N terminus of TBP is phylum-specific. For example, flies, round-worms, cephalochords, yeast, plants, tunicates, and wasps all have N termini that are unrelated to each other, yet conserved within each lineage (10, 66, 114). As such, in each lineage, the TBP N-terminus is suspected to be required for gene expression events that are unique to those organisms (10, 66, 101).

The N terminus of tetrapod (amphibians, birds, reptiles, and mammals) TBP consists of four subdomains, N_N, a glutamine repeat central region , N_C, and a PXT repeat region (where, in most cases, X represents M, A, or I) (10). All of the four subdomains of TBP are dissimilar to one another in their primary amino acid sequence (10). Homozygous mutant mice that lack all but the first 24 amino acids of the TBP N terminus ($TBP^{\Delta N/\Delta N}$) die from a defect that is manifested in the mid-gestational placenta (69). This defect can be rescued by supplying homozygous mutant mice with a wild-type placenta. In addition, $TBP^{\Delta N/\Delta N}$ fetuses survive past the mid-gestational crisis when

reared in mothers that lack mature T and B lymphocytes. This suggests that the mother's adaptive immune system has a role in rejection of the $TBP^{\Delta N/\Delta N}$ placenta (69). Since the placenta is found only in vertebrates (17), this data supports the notion that the N terminus of vertebrate TBP regulates gene expression events that are required for vertebrate-specific processes.

Fossil evidence suggests that the vertebrate lineage arose approximately 514 million years ago (MYA) (98). The vertebrate lineage is comprised of the jawless lampreys and hagfishes (agnathans), and all jawed vertebrates (gnathostomes), including frogs, reptiles, fish, and mammals (5, 96, 98). Specializations that are specific to the vertebrates and the first organism that they appeared in are as follows: thymus gland (lamprey) (93); calcification of both the skull (lamprey) and the vertebral column (bony fishes) (8); cardiac specializations, including separate venous and systemic chambers (lamprey) and a four-chambered heart (reptiles) (8, 20, 46); pulmonary circulation (amphibians) (8); tetrapod body plan (all vertebrates with four limbs) (34); the choana or true "internal nostrils," (arose at the transition of tetrapods from bony fishes) (239); placenta (shark) (17); neuromeres (lamprey) (147); adaptive immunity (lamprey) (159); and neural crest cell-derived tissues and ectodermal placodes (lamprey) (136, 137, 218).

To gain insights into mechanisms that may be regulated by the vertebrate-specific TBP N terminus, we used the yeast two-hybrid system to search for proteins that interact with the mouse N terminus. Searches of mid-gestational pregnancy-associated cDNA prey libraries revealed three proteins, Coactivator Associated Arginine Methyltransferase 1 (CARM1), PAX Transactivation Domain-Interacting Protein (PTIP/PAXIP), and Lipopolysaccharide-Induced Tumor Necrosis Factor Alpha Factor (LITAF), that

interacted specifically with the vertebrate-specific TBP N terminus by yeast two-hybrid. One of these proteins, PTIP, was also shown to co-precipitate with TBP-FL and with TBP-N in co-transfection assays in h293 cells. Our results are the first to suggest that the TBP N-terminus makes multiple contacts with nuclear transcription factors. At least two of these transcription factors are known to regulate vertebrate-specific processes, although the specific roles of these interactions with TBP in transcriptional regulation are not yet known.

Materials and Methods

Yeast Plasmids, Prey Libraries, and Yeast Two-Hybrid Screens

The MP34-TBP-N bait plasmid was used to screen the whole pregnant uteri library (Chapter 2) in two separate screens. All interactions were tested in *S. cerevisiae* strain AH109 (Chapter 2). Library transformations and two-hybrid screens were performed as detailed in Chapter 2, with the following changes. First, in both screens, bulk transformed yeast were plated on SC-L-W-H agar plates. The combined number of primary transformants (containing both a bait and prey plasmid) in both screens was ~9 x 10^5 colony forming units, which was determined by dilution plating of transformed yeast onto SC-L-W agar plates. In both screens, those clones that grew (primary interactors) were transferred to SC-L-W-H + X-alpha-gal and grown for approximately four days. Clones that continued to grow and expressed the LacZ reporter gene product (turned blue) were further tested for growth on SC-L-W-H-A medium. From the two screens, twenty-five clones, eleven from screen #1, and fourteen from screen #2 activated all three reporter genes (HIS3, ADE2, LacZ). Plasmids from all twenty-five clones were isolated,

grown in bacteria, and re-transformed into fresh yeast with MP34-TBP-N (Chapter 2). Of the twenty-five original clones, eleven re-grew on SC-L-W-H and SC-L-W-H-A. These clones were re-transformed into yeast with MP34-TBP-FL and with the empty MP34 bait plasmid (auto-activation test) (Chapter 2). Those that grew on SC-L-W-H with the full-length TBP protein and were not auto-active (did not grow with the empty bait plasmid) were sequenced with a T7 primer (Chapter 2) to determine the identity of the interacting prey protein.

Co-Transfection and Co-Immunoprecipitation

Transfections and immunoprecipitations were performed as in Chapter 2, with the following modifications. h293 cells were lysed in 500 µl lysis buffer that contained 50 mM Tris, pH 7.5, 150 mM NaCl, 1mM EDTA, 0.1 % SDS, 0.5% deoxycholic acid, 1% TX-100, 10% glycerol, 5µg/ml LPA, 1mM PMSF, and 0.2 mM $NaVO_3$. Cleared lysate (450 µl) was added to 900 µl of buffer that contained the same components as the lysis buffer, except that it lacked SDS and Deoxycholic Acid. Binding reactions used either 4 µg of mouse monoclonal anti-flag M2 antibody (Sigma), or 12.5 µl of rabbit anti-TBP polyclonal antibody (defined in figure legends) and 25 µl of Protein G plus/Protein A agarose (Calbiochem). Non-specific mouse and rabbit antibodies were the same as in Chapter 2. Agarose was pelleted by centrifugation for 30 seconds at 500 x g, washed five times with 1 ml of wash buffer that contained 50 mM Tris, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% TX-100, 10% glycerol, 5µg/ml LPA, 1mM PMSF, and 0.2 mM $NaVO_3$. Bound proteins were released from the agarose by boiling for five minutes in SDS/PAGE

sample buffer. Polyacrylamide gel electrophoresis and immunoblotting were performed as in Chapter 2.

Results

CARM1, PTIP, and LITAF Interact with the TBP N Terminus

To identify proteins that interacted with the TBP N terminus, we screened oligo(dT)-primed whole pregnant uteri cDNA libraries in the pGADT7^{SN} prey plasmid (Chapter 2) with the MP34-TBP-N bait plasmid (Chapter 2). Out of two screens, we obtained eleven clones that grew on SC-L-W-H and SC-L-W-H-A. Of these eleven clones, four interacted with TBP-FL and were not auto-active (Figs. 4.1, 4.2). All four clones were sequenced to reveal their identity.

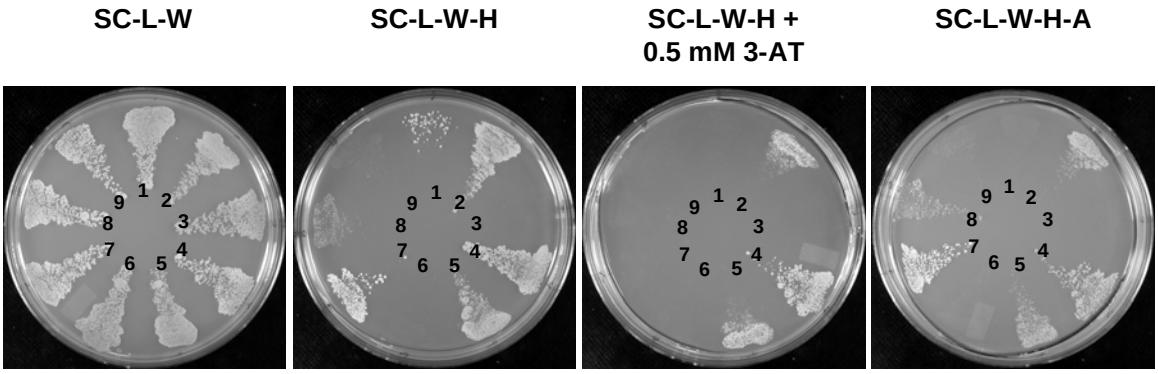
Yeast two-hybrid screens

screen	bait	prey library	transformants	interacting clones
1+2	TBP-N	oligo(dT)-primed whole uteri	9.0 x 10^5	4

Figure 4.1: Yeast two-hybrid screens. By screening approximately 9.0 x 10^5 primary yeast transformants, which contained both the MP34-TBP-N bait plasmid and a pGADT7^{SN} prey plasmid, we identified four interacting prey clones. All clones were eventually sequenced to determine the identity of interacting prey proteins.

The four clones were found to encode the following protein fragments: AAs 350-608 of CARM1; AAs 75-161 of LITAF; and two clones that encoded AAs 429-1056 (long) and AAs 780-1056 (short) of PTIP. The interactions of CARM1, LITAF, and PTIP$_{429\text{-}1056}$ (long clone) with TBP sub-fragments are shown in figure 4.2. All clones interacted with both TBP-N and TBP-FL (Fig. 4.2, sectors 1-2, 4-5, 7-8, second panel

from left), but did not grow in the presence of an empty bait plasmid (Fig. 4.2, sectors 3,6, and 9, right three panels). The interaction between LITAF and TBP did not activate



sector	bait	prey
1	TBP-N	$CARM1_{350-608}$
2	TBP-FL	$CARM1_{350-608}$
3	empty vector	$CARM1_{350-608}$
4	TBP-N	$PTIP_{429-1056}$
5	TBP-FL	$PTIP_{429-1056}$
6	empty vector	$PTIP_{429-1056}$
7	TBP-N	$LITAF_{75-161}$
8	TBP-FL	$LITAF_{75-161}$
9	empty vector	$LITAF_{75-161}$

Figure 4.2: Interaction of CARM1, PTIP, and LITAF with TBP-FL and TBP-N. All yeast clones contained both a bait and prey plasmid, which is indicated by growth on SC-L-W (left panel). CARM1 and PTIP interacted with TBP-FL under all conditions tested (sector 2 and 5, right three panels). The absence of growth in sector 1 in the right two panels suggests that the interaction between CARM1 and TBP-N is not as stable as with TBP-FL (compare sectors 1 and 2, right two panels).

expression of the HIS3 reporter gene as strongly as the interaction between TBP-FL and either CARM1 or PTIP, since growth of the LITAF clone in the presence of either TBP-N or TBP-FL is absent on SC-L-W-H + 0.5 mM 3-AT (Fig. 4.2, sectors 7-8, third panel from left). Also, the interaction of CARM1 with TBP-N did not appear to grow under higher stringency conditions (SC-L-W-H + 0.5 mM 3AT and SC-L-W-H-A) (Fig. 4.2,

sector 1, right two panels), although it did grow with TBP-FL (Fig. 4.2, sector 2, right two panels), suggesting that the interaction of CARM1 is more stable with TBP-FL than with TBP-N. In contrast, PTIP grew with either TBP-FL or TBP-N under all conditions tested (Fig. 4.2, sector 4-5, right three panels).

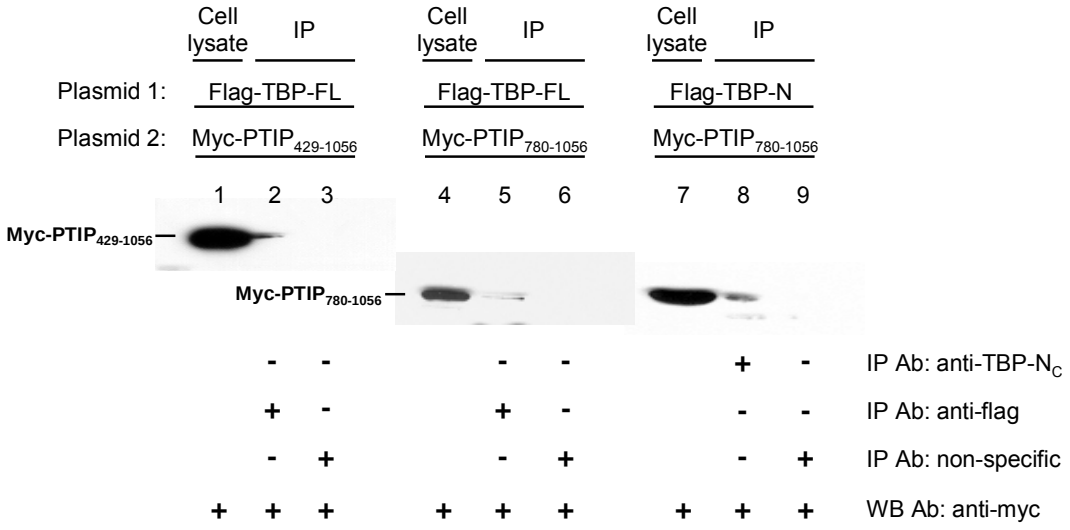


Figure 4.3: Co-immunoprecipitation assay. TBP-PTIP interactions in co-transfected cells. h293 cells were co-transfected with pCMV-MYC-PTIP$_{429-1056}$ (lanes 1-3) or pCMV-MYC-PTIP$_{780-1056}$ (lanes 4-9) and pFLAG-CMV2-TBP-FL (lanes 1-6) or pFLAG-CMV2-TBP-N (lanes 7-9). Whole cell lysates (lanes 1, 4, and 7) or immunoprecipitated samples (lanes 2-3, 5-6, and 8-9) were assayed using western blots and anti-myc antibody. Both the myc-tagged PTIP$_{429-1056}$ and myc-tagged PTIP$_{780-1056}$ proteins co-precipitated with Flag-TBP-FL using anti-flag antibody (lanes 2 and 5, respectively), but not with the non-specific antibody (lanes 3 and 6, respectively). Also, myc-tagged PTIP$_{780-1056}$ co-precipitated with Flag-TBP-N using anti-flag antibody (lane 8), but not with the non-specific antibody (lane 9).

Interaction of PTIP with TBP in Co-Transfected Cells

Since the interaction of PTIP with TBP appeared to be the most robust of our interactions in the two-hybrid system, we pursued this interaction first. To verify the interaction between PTIP and TBP, we co-transfected h293 cells with pCMV-MYC-PTIP and pFLAG-CMV2-TBP-FL or pFLAG-CMV2-TBP-N. Co-immunoprecipitation assays

showed PTIP co-precipitated with TBP-FL and with TBP-N using the anti-flag antibody (Fig. 4.3, lanes 2 and 4), but was not detected with a non-specific antibody (Fig. 4.3, lanes 3 and 6). Additionally, PTIP co-precipitated with TBP-N using the anti-TBP-Nc antibody (Chapter 2) (Fig. 4.3, lane 8), but not with a non-specific antibody (Fig. 4.3, lane 9). These results suggested that PTIP associates with TBP when over-expressed in h293 cells.

Discussion

Protein interactions at the promoter are critical for proper temporal and spatial gene expression. TBP is almost always required for transcription initiation, and its recruitment to the promoter is one of the foremost rate-limiting steps of this process. Because of this, many proteins interact with TBP either to localize it at the promoter, or function as a docking site for assembly of multi-protein complexes (102, 179). This study has identified three proteins, CARM1, PTIP, and LITAF, which interact with the vertebrate-specific TBP N-terminus in the yeast two-hybrid system. PTIP co-precipitated with both TBP-N and TBP-FL in transient co-transfection studies in h293 cells, providing additional evidence for association of PTIP with TBP in mammalian cells.

CARM1 is an S-Adenosyl-1-methionine-dependent protein arginine N-methyltransferase and catalyzes the addition of a methyl group to arginine residues within target proteins (50). CARM1 null mice were shown to be deficient in arginine methylation of p300. They also were found to lack expression of stanniocalcin 2 and complement C3, both of which are known to be regulated by the estrogen receptor signaling (236). This was the first evidence that provided a direct genetic link between

the role of CARM1 in transcriptional regulation and protein arginine methylation. CARM1 is also required for T cell development (85), a vertebrate-specific process (115, 159) and myogenesis (21).

Specific regions of CARM1 have been shown to possess properties inherent to transcriptional activation. Its C-terminal domain (amino acids 461-608) contains a transcriptional activation domain, although the mechanisms by which CARM1 activates transcription via this domain are unknown. The majority of its central domain (amino acids 120-500) is the most highly conserved and conveys the methyltransferase activity of the protein (215). The NCBI conserved domain summary for mouse CARM1 showed the two methyltransferase conserved domains resided within this protein. The first is a homolog of the yeast Skb1 (Shk1 kinase-binding protein 1), a methyltransferase protein. The second is a homolog of the ribosomal protein L11 methyltransferase (PrmA) domain (125).

Importantly, the transactivation domain on CARM1 resides within the portion of our CARM1 clone that interacted with TBP-N, suggesting that a specific interaction between this domain and TBP-N may exist. Mapping studies of the TBP interaction domain on CARM1 will be required to ultimately define the specific amino acid residues that mediate the CARM1-TBP interaction.

In this study, we also identified PTIP as a TBP N terminus interacting protein by yeast two-hybrid. The interaction of the shorter PTIP clone with TBP reveals that the a TBP interaction domain resides within the C-terminal 277 amino acids of PTIP. The only known domain within this region is a BRCA domain, which is conserved from

bacteria to humans (76). Whether the interaction of PTIP with TBP is mediated by this domain or other amino acids remains to be discovered.

PTIP was first identified as a Pax2 interacting protein (100). Pax family proteins are DNA binding transcription factors, whose major roles involve developmental aspects of vertebrate organisms (22). Specifically, the Pax2 protein is required for proper development of the kidney, eyes, and ears (180, 217). PTIP was also identified as a protein that is important for proper craniofacial development in mice (49), also a vertebrate-specific process (218).

In light of what is know for regulation of gene expression by PTIP, an interaction between PTIP and TBP could function to co-localize proteins that bind PTIP at the promoter for proper gene regulation. Although PTIP was shown to be associated with active chromatin (100), the exact mechanisms of how PTIP becomes localized at the promoter remain to be known. To date, PTIP has been shown to interact with only two transcription factors, p8 (73) and PAX2 (100). Both of these proteins can bind DNA, and as a result, should localize PTIP at the promoter. Beyond this, specific interactions with PTIP have yet to be identified. Further studies will be required to dissect the transcriptional mechanisms and vertebrate-specific genes that may be regulated by interactions between PTIP and TBP.

The third and final protein indentified as a TBP N-terminal interacting protein in our two hybrid screens was LITAF. Since its discovery several years ago, a role for LITAF in transcription of the TNFα gene has been shown. Initial studies showed that through direct binding of the TNFα promoter, LITAF activated transcription of TNFα (148, 212). In further support of a role in inflammatory cytokine expression, LPS

stimulation of RAW 267.4 mouse monocyte/macrophage cells (9) and human monocytes (148) increased expression of LITAF, which, in-turn, resulted in an increase of TNFα expression in these cells. Moreover, macrophage cells that lacked one copy of LITAF were shown to have reduced expression of TNFα upon stimulation with LPS (9). More recent evidence has identified the STAT family member, STAT6B as a direct interaction partner of LITAF. Interaction of LITAF with STAT6B resulted in increased expression of numerous inflammatory response cytokines, including TNFα, Interferon-gamma, and Interleukin-10 (213), all of which are potent activators of the inflammatory response to bacterial antigens.

Since LITAF was shown to regulate TNF-alpha expression, we looked into other transcription factors that also regulated expression of this gene. To our surprise, most of the transcription factors that are known to regulate TNFα expression, including ATF2, Jun, NFκB, and NFAT have also been found to associate with TBP, either directly or in a TBP-containing complex (88, 119, 158, 176). The identification of LITAF as a TBP interacting protein in our screens, in conjunction with the previous data, suggests that the interaction of TBP or a TBP-containing complex with transcription factors that directly regulate TNFα expression may be a central process to controlled expression of the TNFα gene.

SUMMARY AND CONCLUSIONS

TBP is an essential protein. Mice lacking TBP do not survive once maternally-derived TBP stores become depleted around E3.5 (130). Also, nearly all mice that lack the vertebrate-specific N terminus of TBP die before birth (69), although the specific molecular mechanisms that are disrupted in $TBP^{\Delta N/\Delta N}$ mice remain undiscovered. Studies described here were designed to identify novel protein interactions with both the conserved TBP_{CORE} and the vertebrate-specific N-terminal domain of TBP. These findings may help provide a better understanding of the many roles of TBP in both general and phyla-specific transcription initiation.

TBP is required for nearly all transcriptional events, by all three nuclear RNA polymerases, RNAPI, RNAPII, and RNAPIII (66). TBP is commonly found in multi-protein complexes, such as SL1, TFIID, and TFIIIB, which regulate transcription by RNAPI, RNAPII, and RNAPIII, respectively (171). TBP is also bound by proteins that have distinct functions beyond recruitment of a particular RNA polymerase. Two classes of proteins that commonly interact with TBP are the DNA-binding transcriptional activators and repressors, such as NFκB (61, 158) and ZNF76 (238), respectively. Activators function to localize TBP at the promoter to assure that transcription initiation occurs properly and to allow for enhanced levels of transcription. Repressors typically antagonize recruitment of TBP to the promoter, either by blocking its interaction with DNA or by preventing its association with other general transcription factors (91, 171).

Some promoter-bound activators are also capable of recruiting chromatin remodeling proteins, also known as the cofactors or coactivators of transcription. Unlike

true activators, the coactivators do not specifically bind promoter DNA sequences, although they do promote activated transcription. Typically, they do this by modifying the histone proteins in a manner that facilitates recruitment of the basal transcription machinery and RNA polymerase. Coactivators can also relay signals from DNA-bound activators to the basal transcription machinery, either through direct interactions with the basal transcription machinery, or via other promoter-bound complexes (179).

Coactivator proteins include the CBP/p300 histone acetyltransferase complex (204), CARM1 arginine methyltransferase (2, 30), and the ATP-dependent chromatin remodeling factor SWI/SNF (163). In addition to DNA binding activator/repressor interactions, some coregulatory proteins have been reported to interact with TBP, including p300/CBP (208), the estrogen receptor-associated small ring finger protein, SNURF (144, 186), and the PIAS family proteins (167).

Studies presented in Chapter 2 identified all four PIAS protein family members, PIAS1, PIAS3, PIASx, and PIASy as novel TBP C terminus-interacting proteins by yeast two-hybrid. Screens also identified an interaction between TBP and ZFP523. We further showed that ZFP523, PIAS1, and PIAS3 co-precipitated with TBP in over-expression studies carried out in h293 cells. TBP was also co-precipitated with PIAS1 from mouse liver + spleen nuclear extracts, which suggested that TBP and PIAS1 were associated under these conditions. PIAS1 also co-precipitated with TBP in *in vitro*-translated rabbit reticulocyte lysates, strongly suggesting that the interaction between PIAS1 and TBP does not require other nuclear proteins. Finally, the TBP interaction domain on PIAS1 was mapped to a 39 amino acid region, containing an acidic amino acid stretch that is conserved among all PIAS family proteins. These results suggest that the PIAS family

proteins may localize at the promoter through interactions with TBP to regulate transcription.

Work presented in Chapter 3 identified HAP1 as a protein that interacted with the C terminus of TBP in two-hybrid screens. Due to the association of TBP with the Htt protein, and that many processes within the cell that require Htt also seem to require HAP1, we decided to pursue this interaction further. HAP1 also co-precipitated with TBP from co-transfected h293 cell lysates. Results also showed that two separate domains on HAP1 could independently mediate the interaction with TBP. Previous studies have reported that HAP1 can serve as a scaffold to pre-assemble protein complexes before they translocate to the nucleus. Some HAP1 protein is also found in the nucleus. Since Htt is commonly associated with HAP1, and since Htt participates in transcriptional processes, it is possible that interactions between HAP1, Htt, and TBP could affect transcription. Results presented in Chapter 3 lay the groundwork for functional studies aimed at testing this possibility.

Studies in Chapter 4 identified three proteins that interacted with the vertebrate-specific N terminus of TBP in the yeast two-hybrid system. Importantly, this evidence supports our hypothesis that the N terminus of TBP is a protein interaction module. The three proteins that were identified are CARM1, PTIP, and LITAF. Co-immunoprecipitation analyses are ongoing for the N-terminal interactors, and so far, we have been able to show that TBP and PTIP can be co-precipitated from transfected cell extracts. These results provide clues to the possible mechanisms that may be regulated by the TBP N terminus, and also provide a basis for our future studies.

Together, these data support the hypothesis that the TBP N terminus interacts with specific transcription factors at the promoter to regulate gene expression. In addition to identifying novel protein-protein interactions with the TBP N terminus, we have identified novel protein interactions with the TBP C terminus. These studies expand our understanding of the molecular interactions that signal through TBP to integrate transcriptional control of the vertebrate genome.

FUTURE STUDIES

The studies presented in this dissertation focused on identification of proteins that interact with TBP. Together, these studies provide a basis for further characterization of the molecular mechanisms that may be regulated through each of these interactions. Since the main focus of our lab is to better understand vertebrate-specific gene regulatory processes, the majority of our future studies will focus on interactions involving the vertebrate-specific N terminus. The work presented in Chapter 4 provides a starting point for these studies.

An important first step in these future studies will be to distinguish whether the N-terminal interactors interact directly with TBP or whether they are found in a multi-protein complex that includes TBP. In either case, further characterization of these interactions will be warranted to better understand the signaling mechanisms that are required for expression of genes regulated by all three of the suspected N-terminal interactors. Direct interactions will be first tested as described in Chapter 2, using recombinant proteins from rabbit reticulocyte lysates. As an alternative, we may also begin experiments to produce recombinant GST-tagged proteins in bacteria to test for direct interactions of our proteins, in the absence of additional eukaryotic nuclear proteins. Direct N-terminal interactors will be further characterized as to whether they signal through the N terminus to regulate transcription by using cell lines in our lab that lack either one or both copies of the TBP N terminus. These cells provide a unique resource for studying molecular mechanisms that are controlled by the N terminus of TBP, because they allow us to directly test the hypothesis that removal of the TBP N

terminus will disrupt transcription of genes regulated by CARM1, PTIP, or LITAF. To test this hypothesis, we will first look directly at expression levels of those genes whose expression was reported to be regulated by CARM1, PTIP, or LITAF.

Mapping studies will also be conducted to define the specific domains of those proteins that interact with TBP-N. These studies will provide a greater understanding of the evolution of protein domains and the specific contacts that have arisen to regulate vertebrate-specific gene expression events that require the N terminus of TBP. They also provide a template for ongoing experiments in our lab to look at the other components of the transcription machinery that, like TBP, have acquired a new domain at a time near the vertebrate transition.

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