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ABT1-Associated Protein (ABTAP), a Novel Nuclear Protein Conserved From Yeast to Mammals, Represses Transcriptional Activation by ABT1

Tsukasa Oda,[1] Aya Fukuda,[2] Hiroko Hagiwara,[3] Yasuhiko Masuho,[4] Masa-aki Muramatsu,[5] Koji Hisatake,[2] and Takayuki Yamashita[1]*

[1]Division of Genetic Diagnosis, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[2]Department of Molecular Biology, Saitama Medical School, 38 Morohongo, Moroyama, Iruma-gun, Saitama 350-0495, Japan
[3]National Institute of Advanced Industrial Science and Technology (AIST) Central 6, 1-1, Higashi, Tsukuba, Ibaraki 305-8566, Japan
[4]Laboratory of Drug Discovery Genomics, Faculty of Pharmaceutical Science, Tokyo University of Science, Hunagawara 12, Shinjuku, Tokyo 162-0826, Japan
[5]Department of Molecular Epidemiology, Medical Research Institute, Tokyo Medical Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan

Abstract Various TATA-binding protein (TBP)-associated proteins are involved in the regulation of gene expression through control of basal transcription directed by RNA polymerase (Pol) II. We recently identified a novel nuclear protein, activator of basal transcription 1 (ABT1), which binds TBP and DNA, and enhances Pol II-directed basal transcription. To better understand regulatory mechanisms for ABT1, we searched for ABT1-binding proteins using a yeast two-hybrid screening and isolated a cDNA clone encoding a novel protein termed ABT1-associated protein (ABTAP). ABTAP formed a complex with ABT1 and suppressed the ABT1-induced activation of Pol II-directed transcription in mammalian cells. Furthermore, ABTAP directly bound to ABT1, disrupted the interaction between ABT1 and TBP, and suppressed the ABT1-induced activation of Pol II-directed basal transcription in vitro. These two proteins colocalized in the nucleolus and nucleoplasm and were concomitantly relocalized into discrete nuclear bodies at higher expression of ABTAP. Taken together, these results suggest that ABTAP binds and negatively regulates ABT1. The ABT1/ABTAP complex is evolutionarily conserved and may constitute a novel regulatory system for basal transcription. J. Cell. Biochem. 93: 788–806, 2004.

Key words: TBP; ABT1; RNA polymerase II; transcription; yeast two-hybrid screening

In eukaryotes, the control of transcription of protein-coding genes by RNA polymerase (Pol) II involves sequence-specific DNA binding proteins which bind cis-acting regulatory elements, a number of coactivators and corepressors, and chromatin remodeling proteins which alter the local chromatin structure. Pol II and general transcription factors (GTFs) such as TFIIA, -B, -D, -E, -F, and -H assemble on the core promoter, located at the beginning of a gene, and form the basal transcription machinery. This machinery integrates the signals from various sequence-specific DNA binding proteins and coregulators and drives Pol II-directed transcription [Roeder, 1996; Sauer and Tjian, 1997; Hampsey, 1998].

Recruitment of TFIID to the core promoter is a crucial step in the initiation of transcription by Pol II. TFIID is composed of TATA-binding protein (TBP), which recognizes the TATA box

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*Correspondence to: Takayuki Yamashita, Division of Genetic Diagnosis, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. E-mail: y-taka@ims.u-tokyo.ac.jp

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of the core promoter [Horikoshi et al., 1989; Gasch et al., 1990; Hoey et al., 1990; Hoffmann et al., 1990a,b; Kao et al., 1990], and 10–14 TBP-associated factors (TAF_{II}s) [Roeder, 1996; Sauer and Tjian, 1997; Hampsey, 1998]. TAF_{II}s serve as either core promoter recognition factors, which orient and stabilize TFIID or as general coactivators which couple activator-mediated signals to the basal transcription machinery [Albright and Tjian, 2000]. In addition, TBP interacts with a variety of transcriptional regulatory factors, including sequence-specific transcriptional activators such as c-Fos [Ransone et al., 1993; Metz et al., 1994], c-Myc [Hateboer et al., 1993; Maheswaran et al., 1994] and p53 [Seto et al., 1992; Truant et al., 1993], and nuclear complexes such as CCR4-NOT [Lee et al., 1998], SAGA [Eisenmann et al., 1992; Barlev et al., 1995; Saleh et al., 1997], and RSC [Sanders et al., 2002]. The CCR4-NOT complex is implicated in repression of Pol II transcription [Hampsey, 1998], whereas SAGA and RSC complex are chromatin-remodeling complexes [Cairns et al., 1996; Hampsey, 1998; Roth et al., 2001].

Recently, we identified a novel TBP, an activator of basal transcription 1 (ABT1) [Oda et al., 2000]. ABT1 is conserved from yeast to mammals, and a yeast ABT1 homolog is essential for generation of viable spores. ABT1 stimulates Pol II-directed basal transcription in vitro and in mammalian cells. However, ABT1 apparently has no functional motifs seen in other transcriptional factors and DNA-binding proteins. To better understand regulatory mechanisms governing functions of ABT1, we searched for ABT1-associated proteins (ABTAPs) using a yeast two-hybrid screening and we identified a novel nuclear protein, termed ABTAP. We here provide evidence indicating that ABTAP binds and negatively regulates ABT1.

MATERIALS AND METHODS

Molecular Cloning of Mammalian ABTAP cDNA

Yeast two-hybrid screening was performed as previously described [Oda et al., 2001], using the mouse ABT1 cDNA. pGAD10 I62, which contains the partial rat ABTAP cDNA, was isolated from the rat brain MATCHMAKER cDNA Library (Clontech Laboratories, Inc., Palo Alto, CA). Both strands of the ABTAP cDNA were sequenced using an ABI 377 DNA sequencer (Perkin Elmer, Inc., Boston, MA). Since the I62 clone encoded partial ABTAP protein, 5′-upstream sequences and 3′-downstream sequences of the ABTAP cDNA were cloned from rat brain. 5′-Upstream sequences of the ABTAP were cloned from rat mRNA by rapid amplification of 5′ cDNA ends (5′-RACE) (Invitrogen Corp., Carlsbad, CA), according to the supplier's instructions. The primers (5′-ATC TAA TTC TCT CCA AGC ATG-3′), (5′-ATC TGC CAA ATC ATC TTC ATC-3′), and (5′-TTC AGA ACT AGT TTC TAC ATT-3′) were used for first strand cDNA synthesis, polymerase chain reaction (PCR), and nested PCR, respectively. 3′-Downstream sequence was cloned by PCR from rat brain cDNA library (Gibco BRL) with sense primer (5′-TAC TGA GTA TTC CTG AGG ATG-3′) and anti-sense primer (5′-TGC TCT AGA TTA TAA GTT AGT TCT ACA AGC-3′). The sequence of the anti-sense primer was obtained from the expressed sequence tags database (dbEST). The PCR product was subcloned into a pT7Blue T-vector (Novagen, Madison, WI) and sequenced. The full-length rat ABTAP cDNA was obtained by combining PCR products of 5′-upstream sequences and 3′-downstream sequences with I62 cDNA. Mouse ABTAP cDNA was cloned from NIH3T3 cells with Super-Script™ One-Step™ RT-PCR System (Invitrogen). The sequences of sense primer (5′-GAGA GAA TTC ATG TCA TCC AAA CAA GAA ATA ATG-3′) and anti-sense primer (5′-GAGA CTC GAG AGG CGA GAG CAG TTA TTT GAC CCT-3′) used for the RT-PCR were obtained from the dbEST.

Northern Blot Analysis

Rat multiple tissue Northern blot (Clontech) was prehybridized and hybridized in ExpressHyb hybridization solution (Clontech) at 65°C with a ^{32}P-labeled rat ABTAP cDNA probe. The blot was washed twice for 1 h at room temperature in solution containing 0.3 M NaCl, 0.03 M sodium citrate, 0.1 % sodium dodecyl sulfate (SDS), and then washed for 1 h at 65°C in solution containing 15 mM NaCl, 1.5 mM sodium citrate, 0.1 % SDS, and thereafter subjected to autoradiography.

Plasmids

pcDNA3-myc-ABTAP was constructed as follow: *Spe*I-*Sal*I fragment of pGAD10-I62 was subcloned into *Spe*I-*Sal*I sites of pT7Blue T-vector (Novagen) (termed pT7Blue-I62). Then, the *Spe*I fragment of the 5′-RACE product was

subcloned into the *Spe*I site of the pT7Blue-I62 (termed pT7Blue-I62 1.9K). *Eco*RV-*Xho*I fragment of pT7Blue-I62 1.9K was subcloned into *Eco*RV-*Xho*I sites of pcDNA3-myc [Oda et al., 2000] (termed pcDNA3-myc-I62 1.9K). *Xho*I-*Xba*I fragment of the PCR product containing 3′-downstream sequences of ABTAP was subcloned into pcDNA3-myc-I62 1.9K (termed pcDNA3-myc-ABTAP o/f). Then, the 5′-untranslated region of pcDNA3-myc-ABTAP o/f was replaced with the *Hind*III fragment corresponding to the N-terminus of ABTAP that was amplified with primers (5′-GCG CAA GCT TGG TAC CAT GTC CTC CAA ACA AGA A-3′) and (5′-GCT CAT CAT CAA AGG-3′) from pcDNA3-myc-ABTAP o/f (termed pcDNA3-myc-ABTAP). pIRES FLAG-ABT1 was constructed by subcloning the *Eco*RI-*Stu*I fragment of pcDNA3-myc-ABT1 between *Eco*RI site and blunt-ended *Bam*HI site of pIRES FLAG. pEGFP-ABTAP was constructed by subcloning the *Kpn*I-*Apa*I fragment of pcDNA3-myc-ABTAP into *Kpn*I-*Apa*I sites of pEGFP-C1 (Clontech). pDsRed1-ABT1 was constructed by subcloning the *Bam*HI-*Xho*I fragment of pcDNA3-myc-ABT1 into *Bgl*II-*Sal*I sites of pDsRed1 (Clontech). pGEX-6P-ABTAP was constructed by inserting the *Kpn*I-*Xba*I ABTAP cDNA fragment from pcDNA3-myc-ABTAP into pGEX-6P-1 (Amersham Biosciences, Piscataway, NJ) based vector, in which *Kpn*I and *Xba*I sites were created by inserting the annealed oligonucleotides. pRSET-ABT1 was constructed by subcloning the *Bam*HI-*Xho*I fragment from pcDNA-myc-mABT1 into *Bam*HI-*Xho*I sites of pRSET vector (Invitrogen). pBTM-ABT1 was constructed by subcloning the *Eco*RI-*Xho*I fragment from pcDNA-myc-mABT1 into *Eco*RI-*Sal*I sites of pBTM116 [Oda et al., 2001]. Several pGAD10-ABTAP plasmids for yeast two hybrid assay were constructed by subcloning rat ABTAP cDNA fragments into *Bam*HI site of pGAD10 (Clontech).

Yeast Two-Hybrid Assay

Yeast two-hybrid assay was performed as described [Oda et al., 2000, 2001]. Yeast lines L40 and AMR70 were transformed with bait plasmid (pBTM-ABT1) and prey plasmids (pGAD10-ABTAP), respectively. Transformed L40 and AMR70 were mated by mixing and incubating for 16 h at 30°C. Diploid yeast clones were selected, then β-galactosidase assay was performed.

Cell Culture and Transfections

COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. The cells were plated approximately 16 h before transfection at a density of 2.0×10^5 cells in a well of 35 mm multiwell plates (Falcon 3046, Becton Dickinson, Franklin Lakes, NJ). Two micrograms of plasmids were pre-incubated with 13 μl of Lipofectamine (Gibco BRL) in 200 μl of serum-free DMEM for 45 min at room temperature. The cells were washed once with DMEM. The pre-incubated mixture was diluted with DMEM to a final volume of 1 ml and added to the cells. The cells were then incubated for 5 h at 37°C and FBS was added to a final concentration of 10%. Alternatively, 1 μg of plasmids was transfected to the cells with 3 μl of FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN), according to the manufacturer's protocol. At 24 h after transfection, the cells were washed once with DMEM then incubated for 24 h in DMEM−10% FBS. At 48 h after transfection, the cells were washed twice with cold phosphate-buffered saline (PBS: 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) and lysed in 150 μl of lysis buffer (25 mM glycylglycine, pH 7.8, 15% glycerol, 8 mM $MgSO_4$, 1 mM ethylenediaminetetraacetate (EDTA), 1% Triton X-100, 1 mM dithiothreitol (DTT)), followed by incubation for an additional 20 min at 4°C. The cell lysates were transferred to a 1.5 ml tube and centrifuged, and 10 μl of the supernatant was used for luciferase assays.

Immunoblot Analysis

Whole cell lysates (WCL) or immunoprecipitate were separated by SDS−polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. These membranes were blocked by overnight incubation at 4°C in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.05% Tween 20 and 5% nonfat milk. The blots were incubated for 4−16 h at 4°C with the indicated primary antibodies. Antibody reactions were detected using anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Biosciences) and visualized using an enhanced Luminol reagent (Perkin Elmer, Inc.). Alternatively, antibody reactions were detected with second antibodies

conjugated to alkaline phosphatase and nitro blue terazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) color development substrate (Promega Corp., Madison, WI). Anti-Myc (9E10; sc-40) and anti-FLAG (M2) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Sigma-Aldrich Inc. (St. Louis, MO), respectively.

Protein Expression and Purification

Glutathione *S*-transferase (GST) fused ABT1 proteins was prepared as described [Oda et al., 2000]. His-ABT1 protein was prepared from *Escherichia coli* (*E. coli*) BL21 (DE3) transformed with pRSET-ABT1. *E. coli* was cultured in Luria-Bertani (LB) medium containing ampicillin (100 μg/ml) overnight at 37°C. The culture was diluted 1:10 in the same medium and cultured until OD_{600} was 0.5, then His-ABT1 protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 4 h at 37°C. His-ABT1 was purified using Ni-NTA agarose (Qiagen Inc., Valencia, CA) according to manufacture's protocol. Recombinant ABTAP protein was prepared from *E. coli* JM109 transformed with pGEX-6P-ABTAP. *E. coli* was cultured in LB medium containing ampicillin (100 μg/ml) overnight at 37°C. The culture was diluted 1:10 in the same medium and cultured for 2 h at 37°C, then GST-ABTAP was induced by adding IPTG to a final concentration of 0.5 mM for 20 h at 25°C. GST-ABTAP protein was purified as described [Oda et al., 2000] and cleaved with PreScission Protease (Amersham Biosciences). Purified His-ABT1 and ABTAP proteins were dialyzed against the *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (25 mM HEPES pH 7.6, 0.1 mM EDTA, 40 mM KCl).

In Vitro Transcription Assay

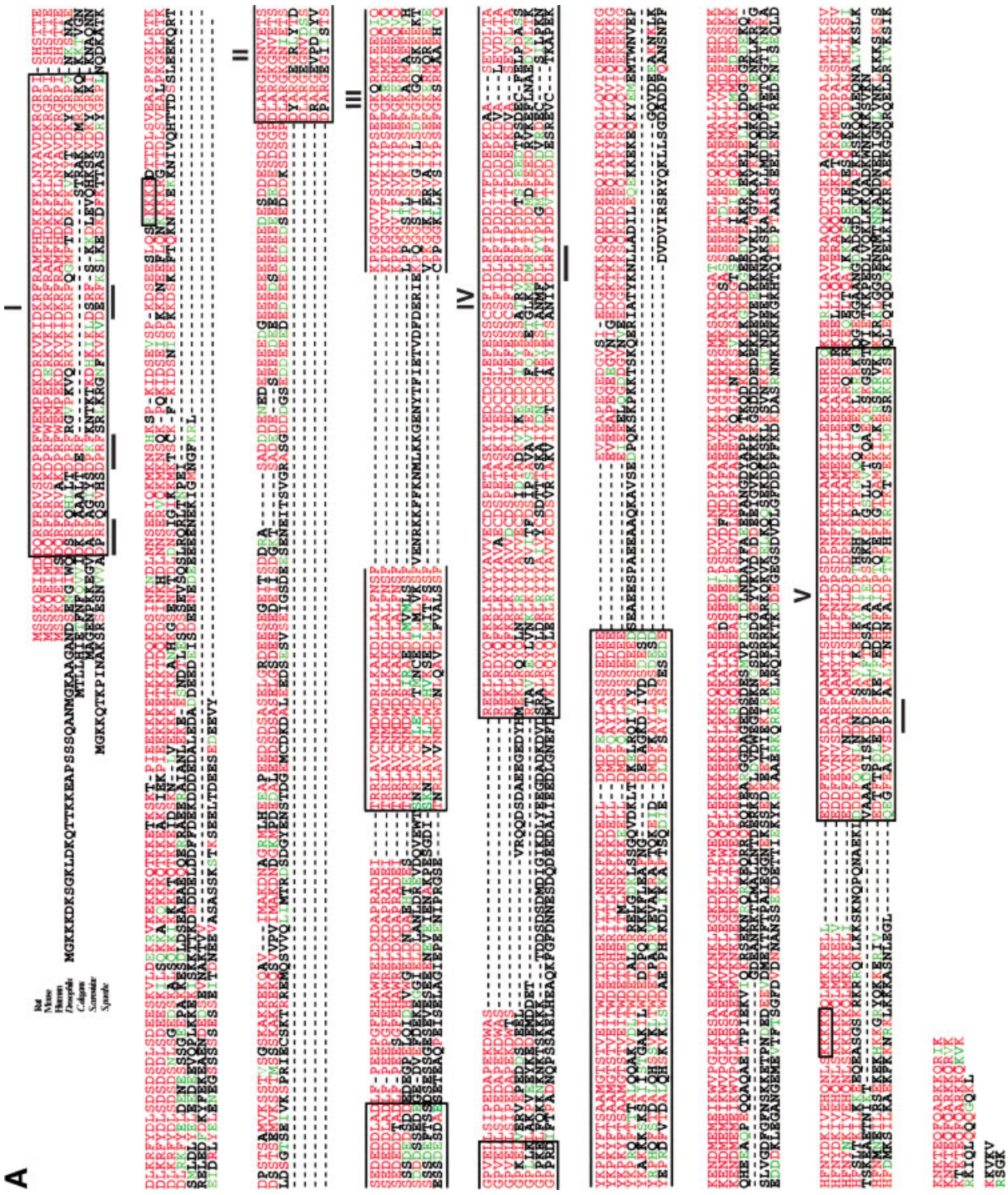
Preparation of transcription factors and in vitro transcription reactions were done essentially as described elsewhere [Oda et al., 2000; Fukuda et al., 2001, 2002]. The reaction mixture contained 50 ng of DNA template (pMLΔC2AT), protein factors including 4 ng of TBP, 10 ng of TFIIB, 20 ng of TFIIF, 100 ng of RNA polymerase II, and indicated amounts of His-ABT1 and ABTAP, nucleotides including 0.2 mM ATP, 0.2 mM UTP, 12.5 μM CTP, 0.1 mM 3′-*O*-methyl GTP, and 10 μCi of [α-^{32}P] CTP, and 20 U RNase inhibitor (Takara Bio Inc., Tokyo, Japan), in a total volume of 25 μl. The template and protein factors were preincubated for 60 min at 30°C before the addition of nucleotides, and the reaction was incubated further for 10 min. Synthesized RNA was extracted and analyzed on a 5% polyacrylamide–urea gel. The levels of transcription were quantified using Fujix Bas 2000.

Protein Binding Assays

GST proteins immobilized to glutathione sepharose beads were incubated with recombinant ABTAP protein and His-TBP [Makino et al., 1999]. After incubation for 16 h at 4°C, the beads were washed four times in ice-cold PBS containing 0.1% Triton X-100 and boiled in SDS-sample buffer. Proteins were separated by SDS–PAGE and detected by protein staining or immunoblotting.

ABT1 and ABTAP Localization

pEGFP-ABTAP and pDsRed1-ABT1 plasmids were co-transfected in COS7 cells with Fugene. At 24 h after transfection, localization of enhanced green fluorescent protein (EGFP) fused ABTAP proteins and red fluorescent protein (DsRed) fused ABT1 proteins were observed under an Olympus IX70 microscope. EGFP-transfected cells and DsRed-transfected cells were visualized with an Olympus U-MNIBA and U-MWIG filter blocks, respectively. Images were taken with a SenSys CCD camera system (Roper Scientific, Inc., Tuscon, AZ) and captured with Meta-Vue software (Universal Imaging Corporation, Westchester, PA). The digital images were processed to produce figures using Adobe Photoshop software. Localization of phosphorylated Pol II was detected using an anti-Pol II antibody (H5, BabCO, Richmond, CA). Transfected cells grown on chambered coverglass (Nalge Nunc International, Naperville, IL) were briefly washed with PBS, fixed in 2% paraformaldehyde PBS for 20 min at room temperature, permeabilized in 0.3% Triton X-100 PBS for 20 min at room temperature. Cells were blocked in PBS containing 3% BSA for 1 h at room temperature, then incubated with a primary antibody for 2 h followed by incubation with a secondary antibody (FITC-conjugated anti-mouse IgM antibody, ZYMED Laboratories, San Francisco, CA) for 1 h. Antibodies were diluted in PBS–3% BSA and all incubations were done at room temperature.


A
I
II
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V
Rat
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Caligus
Scorpion
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Fig. 1.

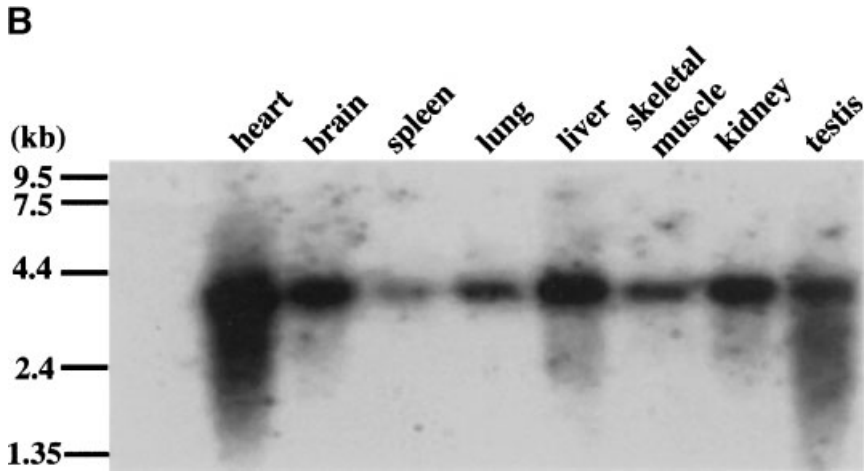


Fig. 1. (*Continued*)

Accession Number

The sequence data for rat ABTAP has been submitted to the DDBJ/EMBL/GenBank databases under accession number AB038233.

RESULTS

Identification of Rat ABTAP and its Homologs

To search for proteins that bind to ABT1, we carried out a yeast two-hybrid screening with full-length mouse ABT1 cDNA and we obtained several positive cDNA clones from a rat brain cDNA library. One of the clones, I62, was sequenced and the deduced amino acid sequences of I62 were subjected to a sequence similarity search in GenBank. As a result, I62 encoded a novel protein with similarity to Ydr365cp (GenBank accession no. AAB64801) of *Saccharomyces cerevisiae*, SPBC337.17c (accession no. T40270) of *Schizosaccharomyces pombe*, F58B3.4 (accession no. T22897) of *Caenorhabditis elegans*, bA526K24.1 (accession no. AL161659.17) of *Homo sapiens*, and EG:8D8.4 (accession no. NP_Q569906) of *Drosophila melanogaster*, that are hypothetical proteins and have not been characterized. A protein encoded by the I62 was designated ABTAP. Since I62 did not seem to be a full-length ABTAP cDNA, compared with ABTAPs of other species, we used 5′-RACE and PCR to clone 5′- and 3′-cDNA fragments of ABTAP, respectively, from the rat brain. Then, 5′- and 3′-fragments of ABTAP were combined with I62 cDNA to obtain a full-length rat ABTAP cDNA (hereafter ABTAP indicates rat ABTAP, and ABT1 indicates mouse ABT1). During the course of our characterization of rat ABTAP, an amino acid sequence of mouse ABTAP (RIKEN cDNA 261010J03; accession no. XP_Q130548) was submitted to GenBank. Subsequently, we cloned mouse ABTAP cDNA and confirmed that mouse ABTAP had similar actions to rat ABTAP in our analyzes (data not shown).

The alignment of several species of ABTAP is shown in Figure 1A. Five separate regions of the proteins (segments I–V) were highly conserved through the above species. Interestingly, all ABTAP homologs contained large amounts of charged amino acids (rat 45.6%, mouse 45.2%, human 44.3%, *Drosophila* 41.3%, *C. elegans* 44.1%, *S. cerevisiae* 43.8%, *S. pombe* 40.2%). No functional motifs, except for putative nuclear localization signals, were found in rat ABTAP. Although functions are unknown, several DXRF motifs were conserved in all ABTAP homologs (Fig. 1A). Northern blot analysis of poly(A)$^+$ RNA from various rat tissues showed that the mRNA of ABTAP was ubiquitously

Fig. 1. Structure of activator of basal transcription 1-associated protein (ABTAP) and expression in various tissues. **A**: Alignment of ABTAP homologs of different species. The hypothetical protein sequences of *Mus musculus* (RIKEN cDNA 261010J03), *Homo sapiens* (bA526K24.1), *D. melanogaster* (EG:8D8.4), *C. elegans* (F58B3.4), *S. cerevisiae* (Ydr365cp), and *S. pombe* (SPBC337.17c) ABTAP show high similarities to the rat ABTAP sequence. Identical and similar amino acids are shown in red and green, respectively. Conserved segments (I ∼ V), and DXRF motifs are boxed and underlined, respectively. Putative nuclear localization signals in rat ABTAP are boxed. **B**: Northern blot analysis showing the ubiquitous tissue expression of ABTAP mRNA in rat. The rat ABTAP mRNA is expressed as an approximately 4.0 kb transcript in all tissues examined.

expressed as a transcript of approximately 4.0 kb (Fig. 1B).

Interaction Between ABTAP and ABT1

To confirm the physical interaction between ABT1 and ABTAP, we first tested whether ABTAP binds to ABT1 in vitro. As shown in Figure 2A, a small fraction of recombinant ABTAP (approximately 10% of the input protein) bound to immobilized GST-ABT1, but not GST. The direct interaction between these two recombinant proteins was weak, under this condition. Other components may stabilize the interaction in vivo.

To examine the interaction between ABT1 and ABTAP in mammalian cells, coimmunoprecipitation assay was done. FLAG-tagged ABT1 and Myc-tagged ABTAP were coexpressed in COS7 cells. The cell lysates were immunoprecipitated (IP) with an anti-FLAG antibody and immunoblotted (IB) with an anti-myc antibody. The results showed that ABTAP associated with ABT1 (Fig. 2B). The association was confirmed by reciprocal immunoprecipitation followed by immunoblotting.

Next, we determined the ABT1-binding region of ABTAPA series of several different regions of ABTAP were constructed and ABT1-binding ability of these regions were examined by yeast two-hybrid assay. A relatively extensive region of rat ABTAP (aa 284–aa 585) containing three conserved segments II, III,

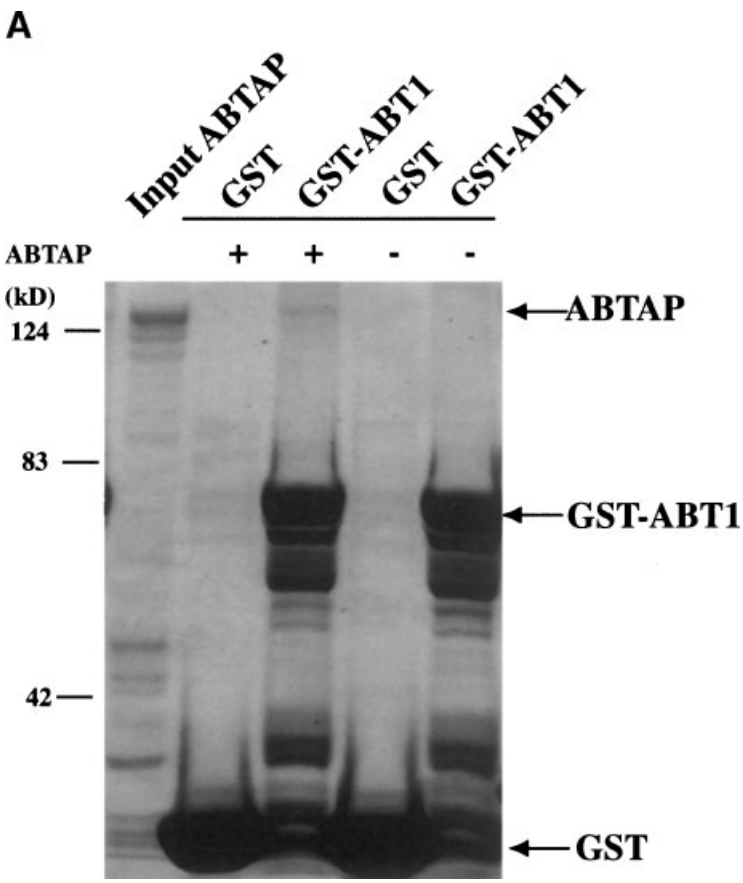
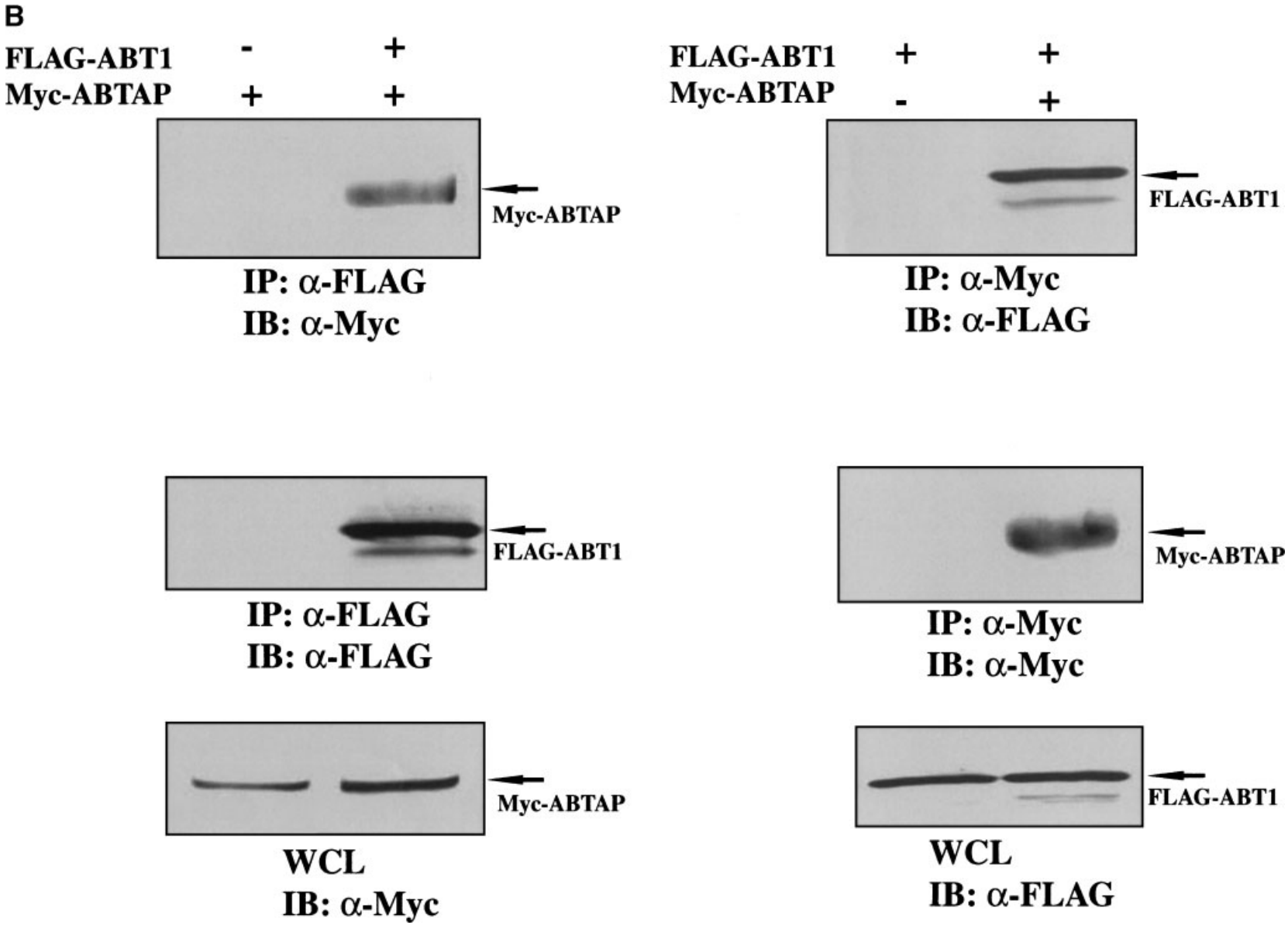


Fig. 2. Interaction between ABT1 and ABTAP. **A**: Direct interaction of ABTAP with ABT1. Glutathione *S*-transferase (GST) or GST-ABT1 (5 μg) immobilized to glutathione sepharose beads was incubated with recombinant ABTAP protein (0.5 μg) for 16 h at 4°C. Proteins bound to the beads were separated on an sodium dodecyl sulfate (SDS)–polyacrylamide gel and stained with Coomassie stain solution. **B**: Interaction of ABTAP with ABT1 in mammalian cells. pcDNA3-myc-ABTAP (0.5 μg) and pIRES-FLAG-ABT1 (0.5 μg) were cotransfected into COS7 cells. At 48 h after transfection, cell lysates were immunoprecipitated (IP) with an anti-Myc or an anti-FLAG antibody and immunoblotted (IB) with anti-Myc and anti-FLAG antibodies. Whole cell lysates (WCL) served as control. **C**: Central conserved region of ABTAP binds to ABT1. Several truncated mutants of ABTAP were constructed and examined for the binding ability to ABT1 by yeast two hybrid analysis. Interaction between ABT1 and ABTAP was estimated by β-galactosidase assay (+, blue; −, white). Rectangles indicate the conserved region of ABTAP from yeast to mammalian cells (see Fig. 1A). Numbers show the sequence position of amino acids in ABTAP.



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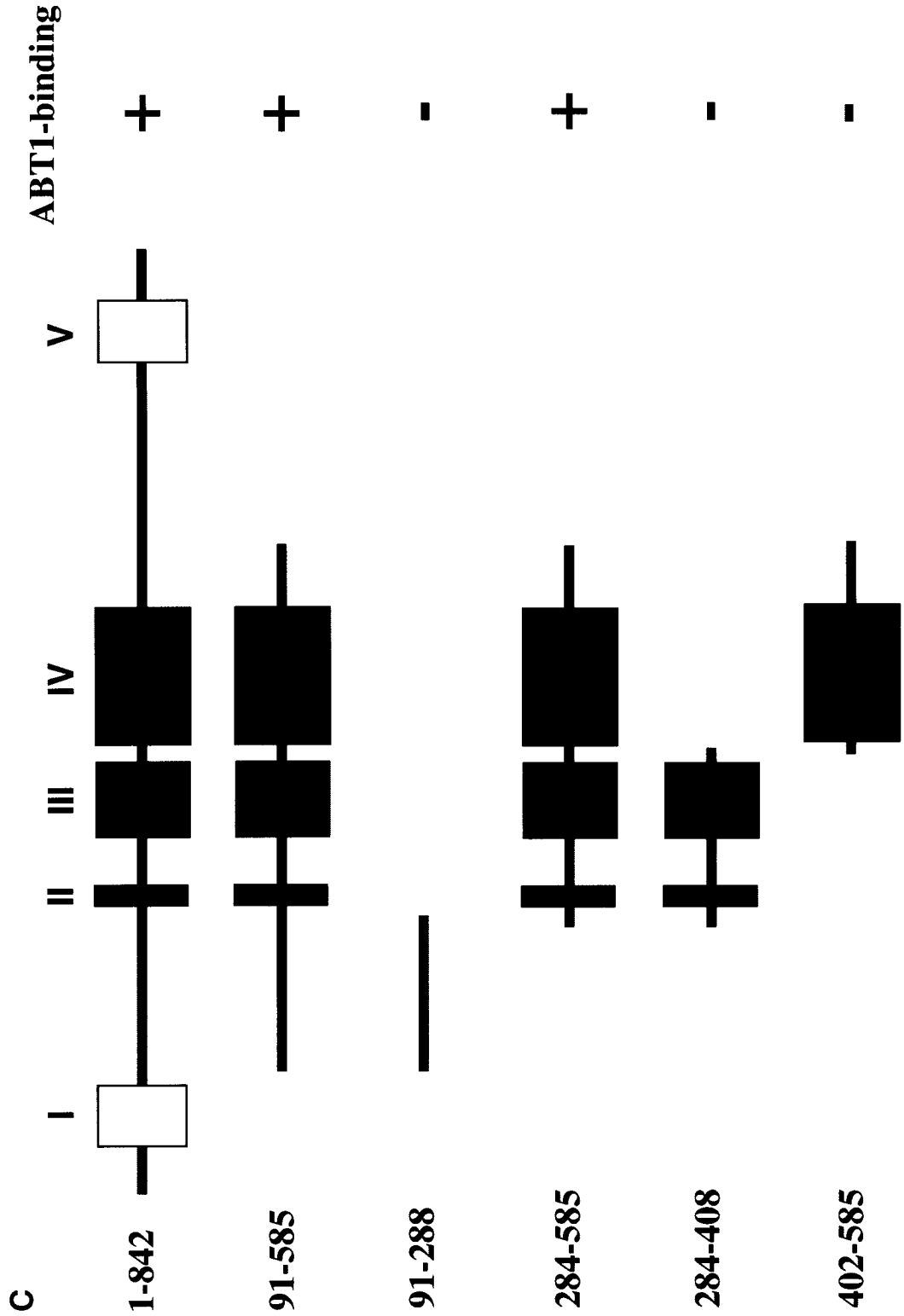


Fig. 2. (*Continued*)

and IV (see Fig. 1A) was shown to be sufficient for ABT1-binding (Fig. 2C).

ABTAP Represses the ABT1-Stimulated Transcription in Mammalian Cells

To assess functions of ABTAP, we first examined effects of ABTAP on ABT1-stimulated basal transcription in mammalian cells. Consistent with previous results [Oda et al., 2000], ABT1 stimulated transcription from pTATA-Luc, which includes a TATA box and a luciferase coding sequence but no cis-regulatory elements, in a dose-dependent manner. When ABTAP was coexpressed with ABT1, transcription from the pTATA-Luc was repressed (Fig. 3A). We confirmed the repression by using another reporter gene *pSRE-Luc*, which contains five-serum responsive elements (SRE), the TATA box, and the luciferase coding sequence (Fig. 3B). ABT1 enhanced transcription from the SRE reporter gene, as reported previously [Oda et al., 2000], whereas coexpression of ABTAP repressed the ABT1-stimulated transcription in a dose-dependent manner (Fig. 3B,C). Transfection with ABT1 was performed in submaximal doses [Oda et al., 2000], because of dose limitations of transfectable plasmid DNAs. Under these conditions, ABTAP significantly repressed the ABT1-induced transcription. ABTAP had no effects on GAL4-VP16-induced stimulation of expression of a reporter gene, pGAL4-Luc, which contains five-GAL4 binding sites, the TATA box, and the luciferase coding sequence (Fig. 3D), thus indicating that the action of ABTAP depends on ABT1.

Antagonistic Effects of ABTAP on ABT1 In Vitro

To gain insight into the molecular mechanisms by which ABTAP represses ABT1-stimulated transcription in vivo, we carried out two in vitro analyzes. Since ABT1 stimulates Pol II-directed basal transcription in a reconstituted cell-free system [Oda et al., 2000], we first examined effects of ABTAP in this assay. ABT1 significantly enhanced transcription as previously reported, whereas ABTAP dose-dependently suppressed the ABT1-induced stimulation of basal transcription (Fig. 4A).

ABT1 directly binds to TBP in vitro, suggesting that ABT1 stimulates transcription through the interaction with TBP [Oda et al., 2000]. We next examined effects of ABTAP on the interaction between ABT1 and TBP. His-TBP bound to immobilized GST-ABT1, but not GST, whereas addition of ABTAP inhibited this binding in a dose-dependent manner (Fig. 4B).

Relocalization of the ABT1/ABTAP Complex Among Different Subnuclear Compartments

To examine the subnuclear localization of ABT1 and ABTAP, we transiently expressed DsRed-ABT1 and EGFP-ABTAP in COS7 cells. When DsRed-ABT1 and EGFP-ABTAP were independently expressed, they showed a different localization. DsRed-ABT1 was localized in the nucleolus and nucleoplasm (Fig. 5A), as previously reported [Oda et al., 2000]. Notably, nucleoplasmic Red-ABT1 colocalized with the hyperphosphorylated form of the large subunit of Pol II (Fig. 5B), which is an active form of Pol II and colocalizes with sites of transcription [Iborra et al., 1996; Grande et al., 1997]. This is consistent with the observation that ABT1 stimulates Pol II-directed transcription. On the other hand, EGFP-ABTAP was primarily localized in discrete nuclear bodies (Fig. 5C). However, when DsRed-ABT1 and EGFP-ABTAP were coexpressed, these two proteins showed almost complete colocalization (Fig. 5D). Interestingly, the subnuclear distribution of ABT1 and ABTAP dramatically changed when different amounts of plasmid DNAs were used for transfection. Both proteins were localized primarily in the nucleolus and nucleoplasm when pDsRed1-ABT1 was dominant (Fig. 5D,

Fig. 3. (*Overleaf*) ABTAP represses ABT1-induced gene expression in vivo. pcDNA3-myc-ABT1 (0–1 μg) and reporter genes (0.2 μg), pTATA-Luc (**A**) and pSRE-Luc (**B**), were cotransfected with or without pcDNA3-myc-ABTAP (1 μg) in COS7 cells, using Lipofectamine. The total amount of plasmids used for each transfection was normalized with the pcDNA3-myc plasmid. The structures of the reporter plasmids are shown at the top. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–10% fetal bovine serum (FBS). At 48 h after transfection, cell lysates were prepared and subjected to luciferase assays and immunoblotting. Protein expression of Myc-ABT1 and Myc-ABTAP in each sample was detected by immunoblotting using an anti-Myc antibody. **C**: pcDNA3-myc-ABT1 (0.3 μg) and pSRE-Luc (0.1 μg) were cotransfected with pcDNA3-myc-ABTAP (0–0.7 μg), using FuGENE 6. Luciferase assay and immunoblotting were performed as described above. **D**: pGAL4-VP16 (0–10 ng) and pGAL4-Luc reporter gene (0.2 μg) were cotransfected with or without pcDNA3-myc-ABTAP (1 μg), using Lipofectamine. Luciferase assay and immunoblotting were performed as described above. The values are the mean of triplicated sample and the standard deviation is shown as error bars. Similar results were obtained in two or three independent transfections. Suppressive effects of ABTAP were significant ($P < 0.05$), under a simple ANOVA analysis with standard post-hoc tests.

left), whereas both were localized in nuclear bodies when pEGFP-ABTAP was dominant (Fig. 5D, right). Similar observations were obtained in murine fibroblasts, NIH3T3 (data not shown). These results suggest that subnuclear localization of the ABT1/ABTAP complex is regulated by the relative amounts of these proteins.

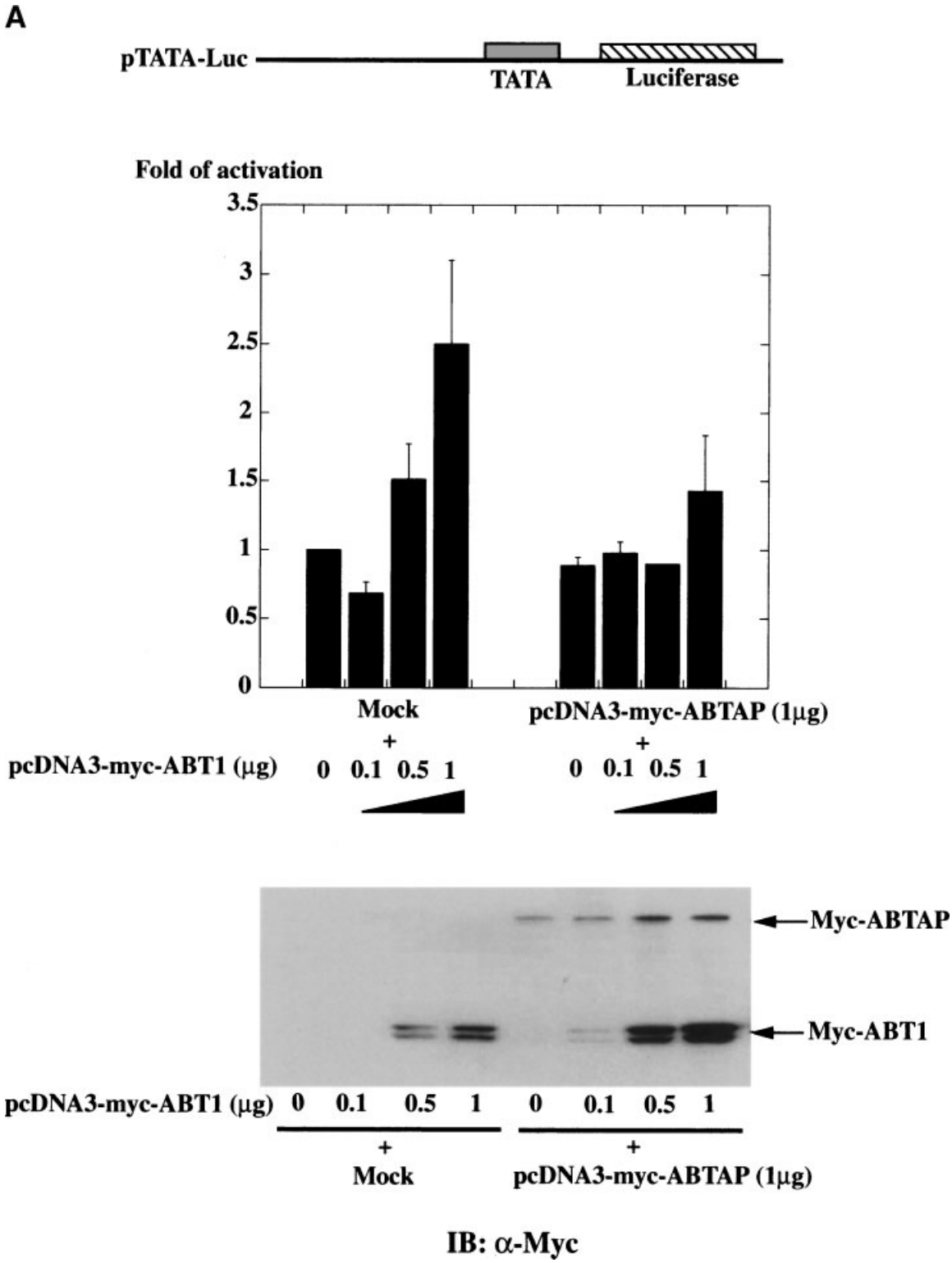
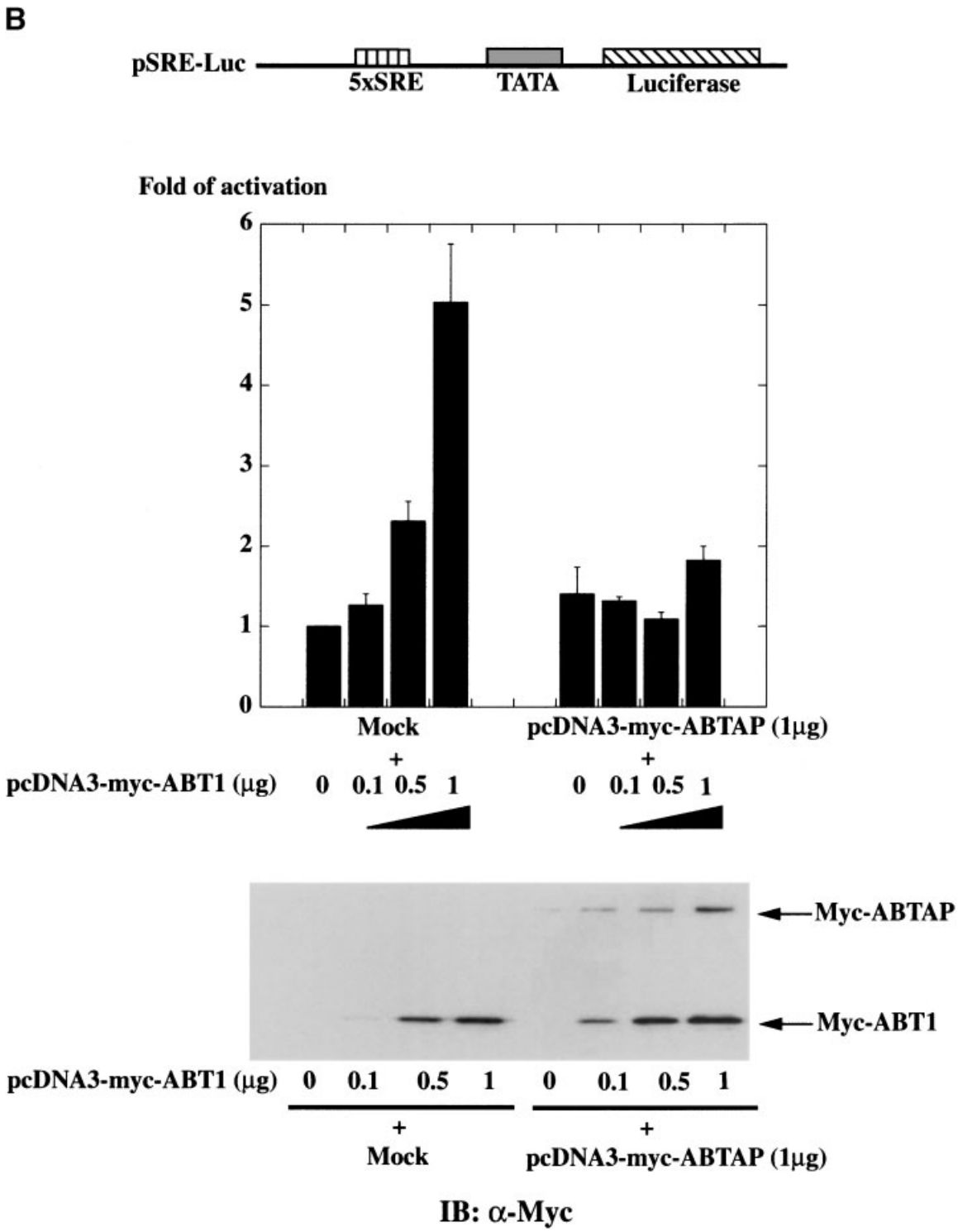


Fig. 3.



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DISCUSSION

To isolate cDNAs of ABTAP as an interactor of ABT1, we used a yeast two-hybrid screening. ABTAP is a novel protein rich in charged amino acids with putative nuclear localizing signals. Like ABT1, ABTAP is conserved from yeast to mammals. Moreover, yeast ABTAP (YDR365C) as well as yeast ABT1 (YNR054C) is essential for generation of viable spores [Oda et al., 2000;

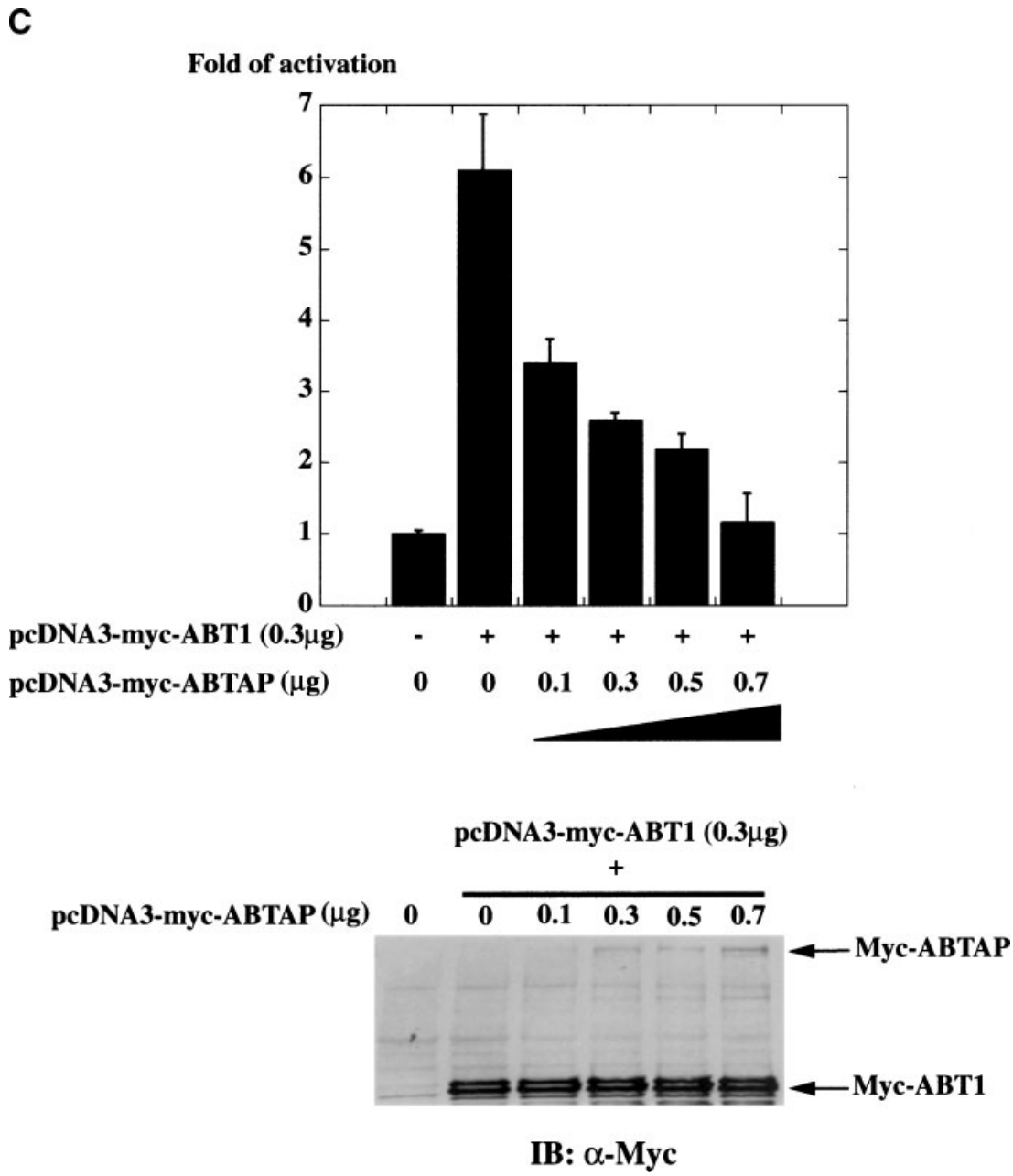
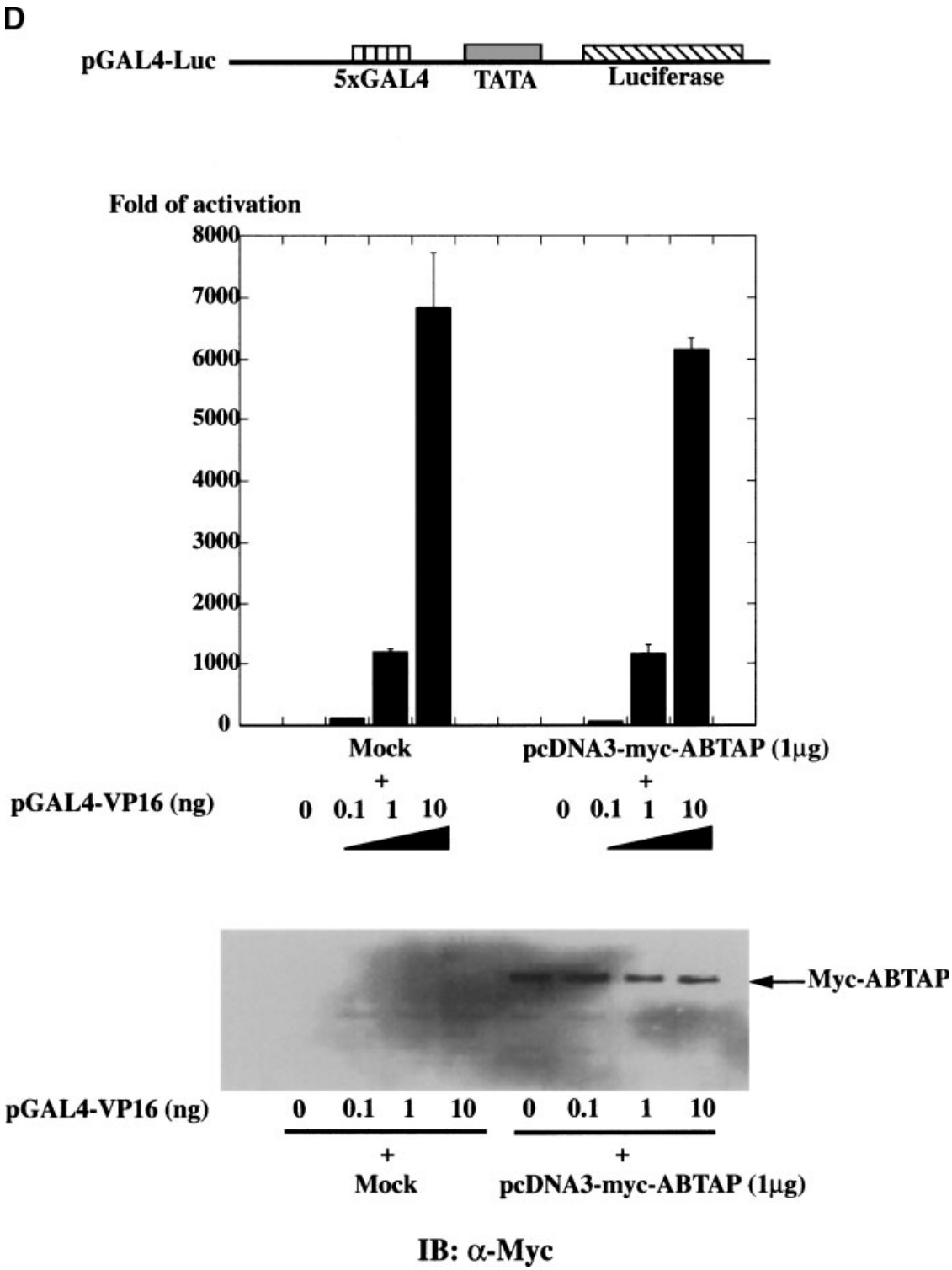


Fig. 3. (*Continued*)

Giaever et al., 2002]. An ABT1-binding region of ABTAP contains three segments highly conserved through different species. Indeed, the yeast homologs of ABT1 and ABTAP were shown to interact with each other in a yeast two-hybrid system (data not shown). During the course of this research, systematic studies of protein complexes in *S. cerevisiae* revealed that yeast ABT1 (YNR054C) and yeast ABTAP (YDR365C) form a multiprotein complex [Gavin et al., 2002; Ho et al., 2002].

ABT1 is a TBP-associated protein that stimulates Pol II-directed basal transcription in mammalian cells [Oda et al., 2000]. In the present study, we obtained several lines of evidence that ABTAP binds and regulates ABT1 in mammalian cells. First, when ABTAP was coexpressed with ABT1 in mammalian cells, these two proteins formed a complex in a co-immunoprecipitation assay. Second, ABTAP dose-dependently suppressed ABT1-induced activation of Pol II-directed transcription in vivo.



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Third, ABTAP directly bound to ABT1 and inhibited the ABT1-induced transcriptional activation in vitro. The antagonistic effect of ABTAP is, at least in part, explained by disruption of the interaction between ABT1 and TBP. Finally, ABT1 and ABTAP colocalized in subnuclear compartments. Remarkably, increased expression of ABTAP led to

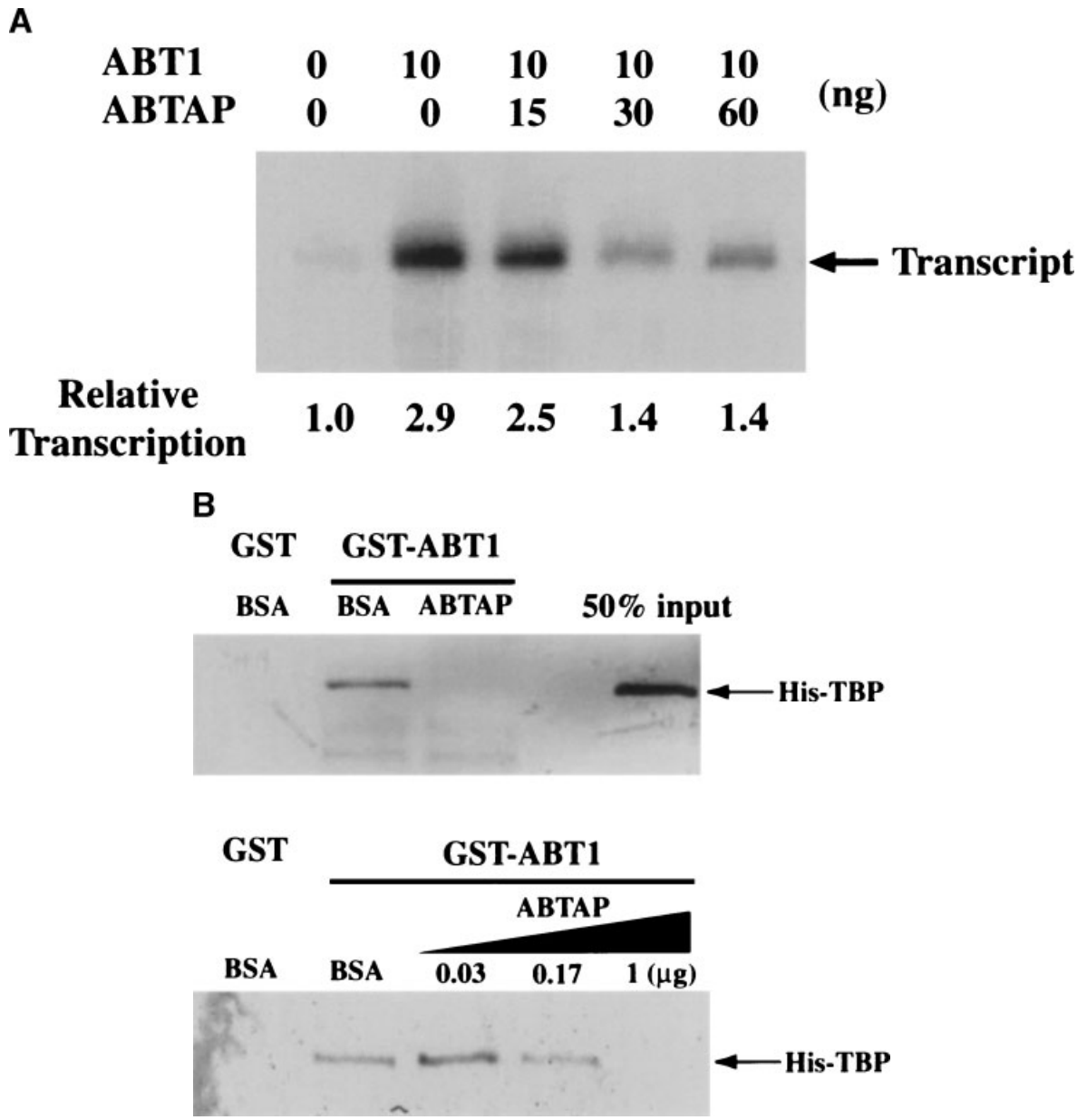

Fig. 4. ABTAP negatively regulates ABT1 in vitro. **A**: In vitro transcription was performed with addition of a constant amount (10 ng) of His-ABT1 and various amounts (0–60 ng) of recombinant ABTAP. Transcription levels were quantified using an image analyzer, and fold activation is indicated at the bottom. **B**: GST or GST-ABT1 (0.5 μg) immobilized to glutathione sepharose beads was incubated with His-TBP (100 ng) in the presence of ABTAP (1 μg) (**upper panel**), or different amounts of ABTAP (0, 0.03, 0.17, 1 μg) (**lower panel**) for 16 h at 4°C. His-TBP bound to GST-ABT1 was detected by immunoblotting with an anti-His antibody. BSA (1 μg) was used as a control.

redistribution of the ABT1/ABTAP complex into nuclear bodies, which may partly account for the suppressive effects of ABTAP on ABT1-induced transcriptional activation. We confirmed that mouse ABTAP behaved exactly like rat ABTAP in vivo (data not shown), as expected from their high homology (Fig. 1A).

Since ABT1 and ABTAP are rich in nucleoli and nuclear bodies, these proteins are likely to have distinct functions in these compartments. A systematic study of protein complexes in *Saccharomyces cerevisiae* revealed that yeast homologs of ABT1 (YNR054C) and ABTAP (YDR365C) form a multiprotein complex including Pwp2 (YCR057C) [Gavin et al., 2002]. Pwp2 (also called as Utp-1) is specifically associated with U3 small nucleolar RNA in a large ribonucleoprotein (RNP) complex and involved

in pre-ribosomal RNA processing [Dragon et al., 2002]. Thus, ABT1 and ABTAP may participate in the pre-ribosomal RNA processing in the nucleolus.

Increasing attention has focused on the regulation of nuclear proteins by movements among different subnuclear compartments. Although the underlying molecular mechanisms are poorly understood, covalent modification such as phosphorylation and SUMOylation is involved in localization of some nuclear proteins to Cajal bodies and PML bodies [Lyon et al., 1997; Muller et al., 1998; Sleeman et al., 1998]. Interestingly, the subnuclear localization of

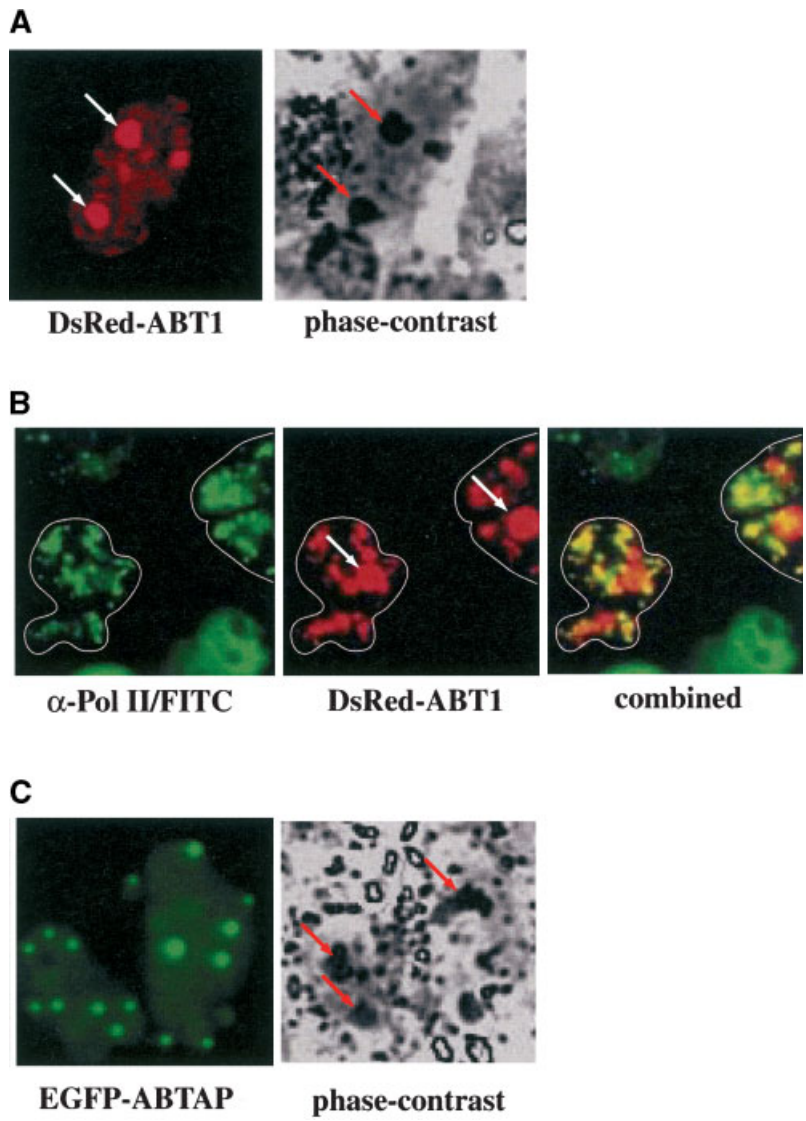

Fig. 5. Subnuclear localization of ABT1 and ABTAP. **A**: Subnuclear localization of ABT1. pDsRed-ABT1 was transfected in COS7 cells. Arrows indicate nucleoli. An image of phase-contrast microscopy in the same field is shown (**right**). **B**: Colocalization of ABT1 with Pol II. The hyperphosphorylated form of the large subunit of Pol II was detected by immunofluorescence staining (**left**). Images of Red-ABT1 (**middle**) and the combination (DsRed-ABT1 and Pol II; **right**) in the same field are shown. Arrows indicate nucleoli. White lines demarcate nuclei. **C**: Subnuclear localization of ABTAP. pEGFP-ABTAP was transfected in COS7 cells. Enhanced green fluorescent protein (EGFP)-ABTAP localized in nuclear bodies. An image of phase-contrast microscopy in the same field is shown (**right**). Arrows indicate nucleoli. **D**: Colocalization of DsRed-ABT1 and ABTAP. Different amounts of cDNAs of pDsRed-ABT1 and pEGFP-ABTAP were used for cotransfection into COS7 cells (**left**, 0.8 μg of pDsRed-ABT1 and 0.2 μg of pEGFP-ABTAP; **right**, 0.2 μg of pDsRed-ABT1 and 0.8 μg of pEGFP-ABTAP). At 24 h after transfection, localization of DsRed-ABT1 and EGFP-ABTAP was examined. Arrows indicate nuclei that Red-ABT1 and EGFP-ABTAP colocalized in nucleoli (left panels), or nuclear bodies (right panels).

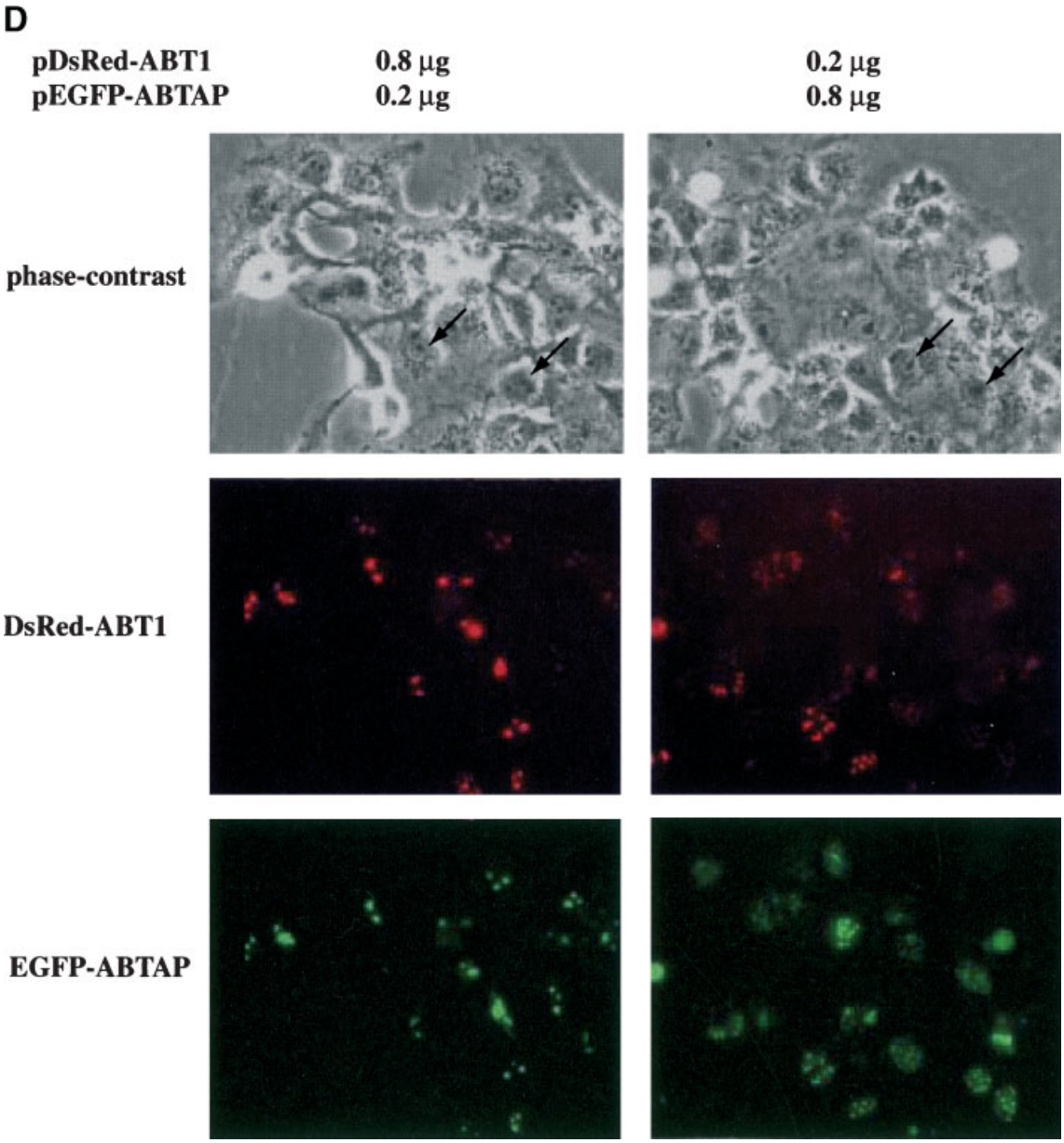

Fig. 5. (*Continued*)

ABT1/ABTAP complex is regulated by relative amounts of the two proteins. One possible explanation is that the ABT1/ABTAP complex has different structures depending on the varying molecular ratios, which would alter interactions with other nuclear proteins. We speculate that the ABT1/ABTAP complex may regulate transcription by shuttling between different subnuclear compartments depending on expression levels. Identification and characterization of other components interacting with the ABT1/ABTAP complex in ongoing studies will aid in clarification of regulatory mechanisms and functions of these proteins.

In summary, we identified ABTAP, a novel protein that directly binds and negatively regulates ABT1, an activator of Pol II-directed transcription. The ABT1/ABTAP complex is evolutionarily conserved and plays an essential role in eukaryotic organisms. Our results suggest that this novel protein complex regulates transcription by mediating TBP and possibly by shuttling among different subnuclear compartments.

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