A yeast protein has been identified that stimulates basal transcription by RNA polymerase II, binds both single- and double-stranded DNA, and interacts with both a general transcription factor and a transcriptional activator. Phosphorylation appears to regulate these interactions. The gene for the transcriptional stimulatory protein, termed TSP1, was cloned and found to be dispensable for yeast cell viability. The deduced amino acid sequence is similar to that of mammalian coactivator protein PC4.

A set of general transcription factors required for initiation at most RNA polymerase II promoters has been isolated from yeast and mammalian cells (reviewed in Refs. 1 and 2). All 15 polypeptides of this minimal set of protein factors exhibit significant homology between yeast and human systems. Functional attributes of the protein factors so far determined are similar in the two systems as well.

Additional proteins are required for positive regulation (activation) of transcription by enhancer-binding proteins (reviewed in Refs. 3 and 4). TAF complexes from several systems and characterization of this protein and the cloning and expression of TBP in vitro (reviewed in Ref. 5). Mediator complex from yeast enables a response to activator proteins and also stimulates basal (unregulated) transcription (6, 7). A human cofactor of activated transcription, termed TF, transcription factor.

In the course of purifying one of the general transcription factors (TF), TFII F, from yeast, we resolved a polypeptide stimulatory to basal transcription. We report here on the isolation and characterization of this protein and the cloning and expression of the gene that encodes it. Functional and sequence similarities to PC4 and other mammalian proteins are noted.

**EXPERIMENTAL PROCEDURES**

Buffers—Buffer A contained 20 mM Hepes, pH 7.6, 20% glycerol, 0.25 mM EDTA, 0.01% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitors (Sigma; Ref. 10). Buffer B contained 50 mM Tris, pH 8.0, 0.15 M NaCl, 10% glycerol, 1% Triton X-100, 2 mM imidazole, 0.01% Nonidet P-40, 5 mM β-mercaptoethanol, and protease inhibitors. Buffer C contained 50 mM Tris, pH 8.0, 0.5 M NaCl, 10% glycerol, 2 mM imidazole, 5 mM β-mercaptoethanol, and protease inhibitors. Buffer D was the same as buffer C except with 0.1 M NaCl and varying amounts of imidazole.

Buffer E contained 25 mM Hepes, pH 7.6, 2 mM imidazole, 5 mM β-mercaptoethanol, and protease inhibitors. Buffer F contained 20 mM Hepes, pH 7.6, 2 mM imidazole, 0.5 mM NaCl, 0.2% Tween-20, 5 mM β-mercaptoethanol, and protease inhibitors. Buffer G was the same as buffer F except with 0.1 M NaCl, with 0.01% Nonidet P-40 instead of Tween-20, and with varying amounts of imidazole. Buffer H contained 20 mM Hepes, pH 7.6, 20% glycerol, 0.01% Nonidet P-40, 5 mM β-mercaptoethanol, and protease inhibitors.

Purification and Sequence Determination of p43—Yeast strain BJ 926 (48 liters) was grown in YPD (1% yeast extract, 2% peptone, 3% glucose) to an A600 of 6. Cells were disrupted, and whole cell extract was prepared and fractionated as described (10), with chromatography on Bio-Rex 70 (Bio-Rad), DEAE-Sephadex (Pharmacia Biotech Inc.), Bio-Gel HTP hydroxyapatite (Bio-Rad), Bio-Gel DEAE-5-PW (Bio-Rad), Mono S HR5/5 (Pharmacia), and TSK-heparin-5-PW (Superco). SDS-polyacrylamide gel electrophoresis revealed p43 after the Mono S step. The pool of fractions from TSK-heparin-5-PW containing the peak of TF1IF and p43 was diluted to the conductivity of buffer A-0.2 (buffer A containing 0.2 M potassium acetate) and applied at 0.5 ml/min to an SP-5-PW column (75 x 7.5 mm; Bio-Rad) equilibrated in buffer A-0.2. The column was developed with a linear gradient (30 ml) to buffer A-0.7. TF1IF eluted at 425 mM potassium acetate, and p43 eluted in a sharp peak at 600 mM potassium acetate. Purified p43 was transferred to polyvinylidene difluoride membrane (Bio-Rad), trypsin digestion and sequencing were performed (Harvard Microchemistry Facility, Cambridge, MA), and two sequences were obtained.

**Homology Searches**—The protein data bases Swiss-Prot (release 31 plus updates), PIR (release 45), and Genpept (release 91 plus updates), PIR (release 45), and Genpept (release 91 plus updates) were searched with the amino acid sequence of Tsp1 using the BLAST plus updates), PIR (release 45), and Genpept (release 91 plus updates) at 0.1. Multiple sequence alignments were performed using the CLUSTAL V computer application (17).

**Null Mutation**—A fragment of TSP1 containing the coding region and 757 base pairs of upstream sequence was amplified by polymerase chain reaction from genomic DNA. The fragment was cloned into pBlueScript SK + (Strategene), which lacked the region from EcoRV to XbaI (plasmid pPL293) with the use of XhoI to create plasmid pPL297. This plasmid was digested with EcoRV and BamHI, end-filled with Klenow enzyme, and the AatI-NdeI fragment from pRS303 bearing the HIS3 gene, end-filled with Klenow enzyme, was inserted to create pPL301.

The plasmid was digested with XhoI, which cut in the polylinker but not in the coding region, and transformed into the Saccharomyces cerevisiae strain CRY3 (MAT a leu2-3,112 his3-1 ade2-1 can1-100 trp1-1 ura3-1 trp1-1 ura3-1; Ref. 18). Yeast genomic DNA was isolated, and Southern blot analysis was performed to verify that the HIS3 gene had recombined correctly into the gene. The strain harboring the null mutation was induced to undergo meiosis. Tetrad were dissected by micromanipulation on YPD agar (19) and replica plated to synthetic medium lacking histidine.

**Antibody Production**—For expression of a C-terminal fragment of TSP1 (amino acids 106–292) in bacteria, the coding region of the gene was amplified by polymerase chain reaction using the primers 5'—ATATCATATGGGATCCGAAGACACCA-3' and 5'-ATATGAATTCCTCGAGTCTTTCTACATTAGT-3' (NdeII and XhoI sites underlined). The
The expression plasmid was cotransfected with BaculoGold-linearized plasmid pPL299, 20, and peak fractions, indicated by numbers above the lanes. Positions of molecular mass markers (sizes in kDa) are indicated at the left. B, transcription assay of reactions containing no p43 (far left) and p43 in amounts indicated above the lanes. The ratio of level of transcription in the presence of p43 to that in its absence (fold stimulation) is indicated below the lanes.

resulting fragment was cloned into the pET20b expression plasmid (Novagen), which incorporated a six-histidine tag at the C terminus of the protein to create pPL299.

Escherichia coli BL21 (DE3) pLysS strain (Stratagene) was transformed with pPL299, grown in Luria broth (2 liters) supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol at 30 °C to an A600 of 0.4, and induced with 0.1 mM isopropyl-β-D-thio-galactopyranoside for 2 h. The cells were harvested by centrifugation, suspended in 40 ml of buffer, and centrifuged for removal of cellular debris. The soluble fraction was incubated for 2 h at 4 °C with 5 ml of Ni²⁺-NTA-agarose resin (Qiagen) equilibrated in buffer B, followed by washing in a column with buffer B, buffer C, buffer D-0.02 (buffer D containing 0.02 M imidazole), and elution with buffer D-0.1. The peak fraction was injected into a rabbit (Berkeley Antibody Co.), and polyclonal serum was collected 10–14 days after immunization.

The C-terminal fragment of Tsp1 was also coupled to cyanogen bromide-activated Sepharose (Sigma) and used to affinity purify anti-Tsp1 polyclonal sera as described (20). Western blots were performed according to Chasman and Kornberg (21). The dilutions of the affinity-purified anti-Tsp1 antibody and the goat anti-rabbit IgG (conjugated to alkaline phosphatase; Bio-Rad) secondary antibody were both 1/1,000.

Insect cells and Baculovirus—Spodoptera frugiperda Sf9 cells grown attached in tissue culture flasks were propagated at 27 °C in Grace's medium supplemented as described. The cells were grown in suspension at 27 °C for 39 h and harvested by centrifugation, and all subsequent manipulations were performed at 4 °C. Following resuspension of the cells (10.5 g) in buffer E (2 ml/g of cells), they were lysed with 15 strokes of a tight fitting pestle in a Dounce homogenizer. The lysates were treated with calf intestine alkaline phosphatase (Boehringer Mannheim) in 0.1 mM Tris, pH 7.6, 2 mM MgCl2, and 0.1 mM ZnCl2 at 37 °C for 30 min. Imidazole was added to 2 mM, and Tween 20 was added to 0.2%. The mixture was incubated with 400 μl of TALON resin (Clontech) equilibrated in buffer F and washing in a column at 60 ml/h with 60 ml of buffer F, 60 ml of buffer G-0.01 (buffer G containing 0.01 M imidazole), and 60 ml of buffer G-0.02. Some Tsp1 eluted with buffer G-0.02, but a majority of the protein was recovered by elution with buffer G-0.1.

Peptide sequences derived from yeast p43 are underlined.

E. coli strain BL21 (DE3) pLysS was transformed with expression plasmid pLYS3 carrying the 5' promoter of the TSP1 gene. Recombinant E. coli cells were grown in suspension in 200 ml of Sf9 cells (Life Technologies, Inc.) at a concentration of 2 × 10⁶ Sf9 cells/ml grown in Sf900-II medium (1 liter) supplemented as described. The cells were grown in suspension at 27 °C for 3 h and harvested by centrifugation, and all subsequent manipulations were performed at 4 °C. Following resuspension of the cells (10.5 g) in buffer E (2 ml/g of cells), they were lysed with 15 strokes of a tight fitting pestle in a Dounce homogenizer. Sucrose was added to a final concentration of 10% and sodium chloride to a concentration of 0.5 M. The extract was rotated slowly for 30 min and centrifuged in a 70Ti rotor (Beckman) for 90 min at 31,000 rpm. Tween 20 was added to the supernatant to a concentration of 2.5%, followed by incubation for 2 h. Washed with 4 ml of TALON resin (Clontech) equilibrated in buffer F and washing in a column at 60 ml/h with 60 ml of buffer F, 60 ml of buffer G-0.01 (buffer G containing 0.01 M imidazole), and 60 ml of buffer G-0.02. Some Tsp1 eluted with buffer G-0.02, but a majority of the protein was recovered by elution with buffer G-0.1. A fraction of the buffer G-0.1 eluate was applied at 0.5 ml/min to a Bio-Gel SP-5-PW high performance liquid chromatography column (75 × 7.5 mm; Bio-Rad) equilibrated in buffer H-0.2 (buffer H containing 0.2 M potassium acetate). The column was washed with buffer H-0.4, and developed with a linear gradient (24 ml) to buffer H-0.8. Tsp1 eluted in two peaks, a minor one at 350 mM potassium acetate and a major one at 600 mM potassium acetate.

Dephosphorylated Tsp1—Tsp1 (300 μg), which eluted from the SP-5-PW column, was treated with calf intestine alkaline phosphatase (Boehringer Mannheim) in 0.1 M Tris, pH 7.6, 2 mM MgCl2, and 0.1 M ZnCl2 at 37 °C for 30 min. Imidazole was added to 2 mM, and Tween 20 was added to 0.2%. The mixture was incubated with 400 μl of TALON resin equilibrated in buffer F at 4 °C for 2 h, washed in a
Yeast Transcriptional Stimulatory Protein

column with 8 ml of buffer F and 8 ml of buffer G-0.01, and eluted with buffer G-0.25.

Surface Plasmon Resonance—Measurements of protein-protein interactions were performed with a Biacore Biosensor (Pharmacia Biotech Inc.). All measurements were performed at 25 °C in running buffer (40 mM Hepes, pH 7.6, 7.5 mM MgCl₂, 120 mM potassium acetate, 0.005% Surfactant P-20) with a flow rate of 15 μl/min. Proteins were immobilized on CM5 research grade sensor chips using the amine coupling kit (Pharmacia). TFIIB was coupled in 100 mM sodium acetate to pH 3.0 at 0.11 mg/ml. All injected proteins were diluted in running buffer from concentrated stocks prior to injection. After the injection, the chip was washed with running buffer containing 1 mM potassium acetate to remove the bound protein.

Protein-DNA interactions were tested on SA5 research grade sensor chips (Pharmacia) with surface-bound streptavidin. To test binding to single-stranded DNA, a 30-mer random oligonucleotide was synthesized and was labeled at the 5'-end. To test binding to double-stranded DNA, a single-stranded oligonucleotide was annealed to form double-stranded DNA, which was immobilized to the chip by flowing over the surface at a rate of 1 m/min. The single-stranded oligonucleotides were annealed to form double-stranded DNA, which was immobilized to the chip by flowing over the surface at a rate of 1 m/min at a concentration of 0.1 mg/ml.

Other Methods—Protein concentrations were determined by the method of Bradford (24) using bovine serum albumin as standard. Proteins were concentrated for SDS-polyacrylamide gel electrophoresis (25) by precipitation with 10% trichloroacetic acid using sodium deoxycholate as carrier and were visualized with Coomassie Blue R-250. Proteins were concentrated for SDS-polyacrylamide gel electrophoresis (24) using bovine serum albumin as standard. Proteins were concentrated for SDS-polyacrylamide gel electrophoresis (25) by precipitation with 10% trichloroacetic acid using sodium deoxycholate as carrier and were visualized with Coomassie Blue R-250.

RESULTS

Isolation of Transcriptional Stimulatory Protein—After six steps of fractionation from a crude yeast extract, general transcription factor TFIIIF was nearly homogeneous, with a single major contaminant of apparent molecular mass 43 kDa (10). Resolution of these proteins was achieved by further chromatography on SP-5-PW, with TFIIIF and the contaminant (p43) eluting at potassium acetate concentrations of 425 and 600 mM, respectively. The specific activity of TFIIIF was diminished upon separation from p43, and addition of pure p43 (Fig. 1A) to transcription mixtures reconstituted with homogeneous TFIIIF gave a 3-5-fold stimulation of the reaction (Fig. 1B). The presence of mediator complex and the activator protein Gal4-VP16, a further stimulation was observed (data not shown). By contrast, addition of TFIIA, stimulatory in some systems, was without effect.

Cloning and Characterization of Transcriptional Stimulatory Protein Gene—Amino acid sequences from two tryptic peptides of p43 were present in an open reading frame on chromosome XIII, which codes for a protein of 292 amino acids, with a molecular mass of 33.1 kDa and pI of 5.31 (Fig. 2). Half of the amino acids are charged, divided almost equally between acidic and basic residues. Major sequence motifs include a single-stranded DNA binding domain and a number of potential phosphorylation sites for casein kinase II.
and protein kinase C. Blot hybridization of total yeast DNA with a fragment of the coding region was consistent with a single copy in the yeast genome (data not shown). In view of the effect of p43 upon transcription in vitro, we refer to the gene as TSP1 (for transcriptional stimulatory protein 1).

BLAST searches of National Institutes of Health data bases identified homologies with three mammalian proteins, a rat pancreatic B-cell protein (28), mouse p9 (29), and human PC4...
The three mammalian proteins were 61% similar and 38% identical to the amino-terminal region of Tsp1 (Fig. 3) and contained single-stranded DNA binding motifs at their carboxyl termini (31) but were considerably smaller than the yeast protein (9–15 kDa, compared with 33 kDa for Tsp1).

A yeast strain was constructed in which a portion of TSP1 was deleted and replaced with HIS3 (see legend for Fig. 2). The consequences of this gene disruption were determined by sporulation and tetrad dissection (data not shown). All four spores of each tetrad formed colonies on rich medium, indicating that TSP1 is not required for yeast cell viability. An additional knockout strain was constructed in which the entire coding region of TSP1 and 370 base pairs upstream was replaced with LEU2, and dissection of this strain confirmed that the TSP1 gene is not essential (data not shown). Wild-type and tsp1 null strains grew at similar rates at 30°C on various carbon sources, including glucose, galactose, and glycerol, and at temperatures up to 37°C (data not shown).

Recombinant Tsp1 with a hexahistidine tag at the amino terminus was expressed in a baculovirus system and purified to homogeneity (Fig. 4A). Two forms of the protein were resolved in the final chromatographic step, one of which was less abundant and phosphorylated to a greater extent than the other (data not shown). The carboxyl-terminal portion of Tsp1 (residues 106–292), which lacks homology with mammalian proteins, was also expressed in bacteria and used for production of polyclonal antibodies. Affinity-purified anti-Tsp1 antibodies reacted both with p43 purified from yeast and with recombinant Tsp1 from baculovirus (Fig. 4B), whereas preimmune sera failed to react with either protein (data not shown). The apparent molecular weight of the recombinant protein was slightly greater than that of p43 from yeast, perhaps due to the addition of the hexahistidine tag or to post-translational modifications. We conclude that p43 is the product of the TSP1 gene.

Tsp1 Phosphorylation and Interaction with Other Transcription Proteins—In view of previous studies of phosphorylation of PC4, we investigated the effects of phosphorylation on Tsp1. Recombinant Tsp1 from baculovirus was treated with alkaline phosphatase and purified by immobilized metal affinity chromatography, resulting in a shift to a faster migrating species in SDS-polyacrylamide gel electrophoresis (Fig. 4C). Dephosphorylation had little effect on the transcriptional stimulatory activity of Tsp1, either in the presence (not shown) or in the absence (Fig. 4D) of mediator and Gal4-VP16.

Interactions of Tsp1 with other transcription proteins and with DNA were investigated by surface plasmon resonance. No interaction with TFIIA was detected (data not shown), in contrast to the behavior of human PC4 (9). No direct interaction between Tsp1 and TFII F was observed (data not shown), indicating that the co-chromatography of the proteins was coincidental. Dephosphorylated recombinant Tsp1 interacted with TFII B and with the activator protein Gal4-VP16, whereas the phosphorylated form of Tsp1 did not bind to TFII B and interacted with Gal4-VP16 almost 10-fold less tightly (Fig. 5, A and B). Conversely, phosphorylated but not dephosphorylated Tsp1 interacted with the Gal4 DNA binding domain of Gal4-VP16 (Fig. 5C). Similar results were obtained with p43 isolated from yeast, which bound Gal4-VP16 about half as well as dephosphorylated recombinant Tsp1, perhaps reflecting partial phosphorylation of Tsp1, because additional partial phospho-

![Fig. 6. Interaction of Tsp1 with DNA.](http://www.jbc.org/)

A, dephosphorylated Tsp1 (dotted line) interacts with single-stranded DNA more strongly than does phosphorylated Tsp1 (solid line). Random 30-nucleotide oligonucleotides were bound to the surface, and Tsp1 proteins were injected at a concentration of 20 μg/ml. B, dephosphorylated Tsp1 (dotted line) interacts with double-stranded DNA more strongly than does phosphorylated Tsp1 (solid line). A 33-base pair oligonucleotide corresponding to the TATA region of the adenovirus major late promoter was bound to the surface, and Tsp1 proteins were injected at a concentration of 20 μg/ml.
phorylation, and which failed to bind the Gal4 DNA binding domain (data not shown). While further studies of the ionic strength dependence of these interactions are needed to assess the contribution of electrostatic effects and the question of specificity, binding to TFIIIB is likely to be nonionic, since the positively charged TFIIIB interacted preferentially with the less electronegative, dephosphorylated form of Tsp1. Binding to Gal4-VP16 appears to involve the VP16 activation domain, since the dephosphorylated form of Tsp1 interacted preferentially, and this form did not bind the Gal4 domain. Binding to the VP16 domain could be electrostatic in view of the preference for the less electronegative form of Tsp1 for interaction with the highly negatively charged VP16.

Because Tsp1 encodes a possible single-stranded DNA binding domain, interactions with both single- and double-stranded DNA were investigated. Approximately equal amounts of either a random 30-mer oligodeoxyribonucleotide or a 33-base pair DNA including the adenovirus major late promoter were coupled to a plasmon resonance sensor surface through biotin-avidin interaction. Dephosphorylated recombinant Tsp1 bound strongly to single-stranded DNA and much less well to double-stranded DNA (compare amplitudes of surface plasma resonance signals in Fig. 6, A and B). The phosphorylated form of Tsp1 bound more weakly, and again, p43 isolated from yeast behaved similarly to the dephosphorylated form of the recombinant protein (data not shown).

**DISCUSSION**

Tsp1 exhibits both sequence homology and functional similarities to human PC4. Functional studies indicate a division of PC4 in two parts, an amino-terminal region rich in serine residues, required for transcriptional activity, and a carboxy-terminal region, which includes a DNA binding domain but which is dispensable for function in vitro (31). The amino-terminal region shares sequence homology with viral immediate early proteins involved in transcriptional regulation, such as IE62, ICP4, and IE180 of Varicella-Zoster virus, herpes simplex virus type 1, and pseudorabies virus, respectively (discussed in Ref. 31). Phosphorylation of serine residues in the amino-terminal region negatively regulates transcriptional activity and affects interactions with the VP16 activation domain and with double-stranded DNA (30–32). The sequence similarity between Tsp1 and PC4 includes the entirety of PC4, and Tsp1 shares, in addition, the capacity to stimulate basal transcription severalfold, interaction with the activation domain of Gal4-VP16 and with DNA, and modulation of these interactions by phosphorylation.

Tsp1 differs from PC4 in three regards. First, it is more than twice the size, containing a carboxy-terminal extension of unknown significance, and further differs in containing fewer amino-terminal serine residues (5 serines in 17 residues of the yeast protein, compared with 9 of 16 and 8 of 13 in the two SEAC domains of the human protein; see Ref. 31). Second, Tsp1 fails to bind TFIIA, in contrast with PC4 (9). Finally, Tsp1 had no effect on the level of activated transcription in the yeast system, whereas PC4 enables as much as 90-fold transcriptional enhancement in the human system (9). The difference in regard to transcriptional activation may relate to the use of mediator complex but not TAFs for studies of activation in the yeast system and TAFs but not mediator in the human system. While TAFs are also present in yeast, their actions may be promoter specific, and TAF-dependent promoters remain to be identified, so investigation of Tsp1 in regard to TAF-dependent transcriptional activation is not yet possible.

Following completion of this manuscript, an independent characterization of Tsp1 was reported (33). The protein, termed Sub1, was identified as a suppressor of a cold-sensitive TFIIB mutation, and overexpression of Sub1 was shown to stimulate transcriptional activation in vivo. It was further demonstrated that the protein interacted with TFIIIB in vitro and inhibited the formation of TBP-TFIIIB-promoter complexes. The genetic characterization of Sub1 complements our biochemical analysis and further suggests that this protein may play a role in transcriptional activation.

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N. Lynn Henry, David A. Bushnell and Roger D. Kornberg

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