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TATA-Binding Protein and Nuclear Differentiation in *Tetrahymena thermophila*

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Unambiguous TATA boxes have not been identified in upstream sequences of *Tetrahymena thermophila* genes analyzed to date. To begin a characterization of the promoter requirements for RNA polymerase II, the gene encoding TATA-binding protein (TBP) was cloned from this species. The derived amino acid sequence for the conserved C-terminal domain of *Tetrahymena* TBP is one of the most divergent described and includes a unique 20-amino-acid C-terminal extension. Polyclonal antibodies generated against a fragment of *Tetrahymena* TBP recognize a 36-kDa protein in macronuclear preparations and also cross-react with yeast and human TBPs. Immunocytochemistry was used to examine the nuclear localization of TBP during growth, starvation, and conjugation (the sexual phase of the life cycle). The transcriptionally active macronuclei stained at all stages of the life cycle. The transcriptionally inert micronuclei did not stain during growth or starvation but surprisingly stained with anti-TBP throughout early stages of conjugation. Anti-TBP staining disappeared from developing micronuclei late in conjugation, corresponding to the onset of transcription in developing macronuclei. Since micronuclei do not enlarge or divide at this time, loss of TBP appears to be an active process. Thus, the transcriptional differences between macro- and micronuclei that arise during conjugation are associated with the loss of a major component of the basal transcription apparatus from developing micronuclei rather than its appearance in developing macronuclei.

TATA-binding protein (TBP) is required for transcription by all three nuclear RNA polymerases. TBP, along with an assortment of distinct TBP-associated factors, has been found to make up the RNA polymerase I selectivity factor I (9, 36), the polymerase II general transcription factor IID (14, 73, 83), and the RNA polymerase III general transcription factor IIIB (38, 43, 62, 72, 75). Given the need for TBP to function in these different complexes in such fundamental cellular processes, it is not surprising that TBP is highly conserved throughout eukaryotes. Yeast and human TBP are functionally interchangeable in basal transcription reactions reconstituted with yeast or human components (5, 17, 39) and have nearly identical TATA element binding specificities (78). The C-terminal 180 residues of TBP from a variety of organisms are typically 80% identical in amino acid sequence, whereas the N-terminal regions are divergent both in length and in amino acid sequence (6, 16, 20, 26, 28–30, 33, 37, 49, 55, 61). The C-terminal domain is necessary and sufficient for TATA element binding and basal transcription in vitro (28, 41, 55) and for the essential functions of yeast TBP in yeast cells (10, 21, 57, 84). This region forms an independent structural domain within the context of the intact protein (41). It contains an interrupted repeat of 67 amino acids, a short basic repeat, and a region weakly homologous with bacterial sigma factors (10, 80). In addition to its role in the basal transcription apparatus, TBP also has been shown genetically (27, 31, 63, 69, 70) and biochemically (32, 35, 60, 68) to be a likely target for polymerase II upstream activators.

Virtually nothing is known about RNA polymerases, promoters, or the factors that bind to them in ciliated protozoa. Evolutionary analyses of ribosomal RNAs (7, 65, 79) and of sequences encoding histones (74a) and tubulins (19) indicate that protozoans, plants, animals, and fungi represent four major branches of the eukaryotes. Analysis of the transcription apparatus in protozoans should, therefore, provide evolutionary information about the process and the components involved.

The study of transcription in *Tetrahymena thermophila* is made more interesting by the fact that, like most ciliates, *T. thermophila* possesses two functionally distinct nuclei (22, 23). The macronucleus is transcriptionally active throughout the life cycle and thereby controls the cell's phenotype. It divides amitotically during vegetative growth and is eliminated during the sexual process of conjugation. In contrast, the micronucleus is the germline nucleus and thus maintains the genetic continuity from one generation to the next. It is transcriptionally inert during vegetative growth and divides mitotically. The micronucleus is transcriptionally active only for a brief period early in conjugation (47, 71). Since micronuclei give rise to new macro- and micronuclei during conjugation, this system offers an opportunity to study the behavior of a ubiquitous transcription factor during nuclear differentiation. A final intriguing feature pertaining to transcription in *T. thermophila* is that neither canonical TATA boxes nor any other conserved sequence located a fixed distance upstream of the start sites of transcription has been identified. Thus, either ciliates have a TBP-binding site which differs from other eukaryotes and it has a somewhat flexible sequence or they employ an RNA polymerase II promoter recognition mechanism which does not involve TBP binding to DNA in a sequence-specific manner.

To initiate studies aimed at determining whether *T. thermophila* uses a typical RNA polymerase II transcription mechanism, the *Tetrahymena* TBP gene was cloned. *Tetrahymena* TBP was found to be encoded by a message of 1,300 bases from a single copy gene. The derived amino acid

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sequence for the carboxy-terminal 180 amino acids is only 70% identical to the yeast sequence, while the majority of TBPs show 80 to 93% identity within this region. In addition, *Tetrahymena* TBP has a unique 20-amino-acid carboxy-terminal extension. A polyclonal antiserum was generated to a 55-amino-acid domain within the conserved carboxy terminus fused to the bacterial TrpE′ protein. This serum recognizes a 36-kDa protein which is enriched in *Tetrahymena* macronuclear fractions and also cross-reacts with *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and human TBPs. The antiserum was used for immunofluorescence analyses, and the distribution of TBP was found to be macronuclear specific during growth and starvation. Interestingly, micronuclei stained with anti-TBP at the onset of conjugation just prior to the brief period during which they are transcriptionally active. Anti-TBP staining persisted even after micronuclear transcription stopped and was lost from developing micronuclei very late in conjugation, approximately coincident with the time developing macronuclei become transcriptionally active. Therefore, the transcriptional differences observed between macro- and micronuclei which are generated during conjugation are coupled to the loss of a major component of the basal transcription apparatus from developing micronuclei rather than its appearance in developing macronuclei.

MATERIALS AND METHODS

Cells and culture conditions. *Tetrahymena thermophila* (strains CU427, mating type VI, and CU428, mating type VII) were grown axenically in enriched proteose peptone at 28°C as described previously (24). Cells were starved at a density of 2×10^5 to 3×10^5 cells per ml in 10 mM Tris-HCl (pH 7.4) for 18 to 22 h, without shaking. Conjugation was induced by mixing equal numbers of cells of different mating types.

Cloning of the *Tetrahymena* TBP gene by using a size-selected library. The PCR was used to amplify a fragment of the *Tetrahymena* TBP gene from a random-primed cDNA library. The 5′ oligonucleotide (o-TBP) (see Fig. 2) corresponds to amino acids 89 through 108 of the *S. cerevisiae* protein sequence and was constructed by using *Tetrahymena* codon preferences (45). The 3′ oligonucleotides, BK11 and BK12 (kindly provided by Arnold Berk), were previously described, along with the PCR conditions employed (37). The amplified products were cloned and sequenced. Clone pPCR12 (derived from a reaction with BK12) was used to probe genomic Southern blots, for which DNA was prepared from isolated macronuclei (67), as described previously (2), except that proteinase K (20 μg/ml) was substituted for pronase and incubation was done at 65°C. A 3-kb *Bgl*II fragment was identified, and a size-selected genomic library was constructed by digesting 100 μg of genomic DNA with *Bgl*II. The fragments were separated on a 1% agarose–1× Tris-acetate-EDTA (TAE) gel, and the region of the gel from 2.5 to 3.5 kb was excised. This region was further divided into 16 slices, and DNA was eluted from the slices by 2 cycles of freeze-thawing in an equal volume of water. Approximately 10% of this liquid was denatured in 0.1 N NaOH and slot blotted onto a Magnagraph (MSI) membrane. Hybridization was done according to the manufacturer's instructions, with a random-primed probe generated from the pPCR12 insert. Eluted DNA fragments from slices which were enriched in the TBP gene were pooled, isolated, and ligated into *Bam*HI-digested, calf alkaline phosphatase (GIBCO)-treated Bluescript vector (Stratagene). The ligation mix was electroporated into DH5α cells, and colony lifts were performed. Positive clones were identified by probing with random-primed pPCR12 insert. Approximately 1 in 400 clones was positive.

DNA sequence analysis. The 3-kb *Bgl*II insert was subcloned into Bluescript after digestion with *Hin*dIII, which produced an ~1.5-kb fragment (containing mostly 3′ untranscribed sequence), a 700-bp fragment, and a 750-bp fragment (containing over 90% of the coding sequence). Both strands of DNA were sequenced by the Sanger method (59), with Sequenase (U.S. Biochemical Corp.), according to manufacturer's instructions.

RNA blots, primer extension, and 3′-end mapping. RNA was isolated essentially as described previously (8). Cells were resuspended in buffered 4 M guanidine isothiocyanate and passed through an 18-gauge needle 10 times. RNA was pelleted through a 6.7 M CsCl cushion by centrifugation in a Beckman 50Ti rotor for 16 h at 110,000 × *g*, resuspended in water, and precipitated in ethanol. For RNA blots, 10 μg of total RNA was electrophoresed on a 2.2 M formaldehyde–0.7% agarose gel. Gels were blotted onto Magnagraph nylon membranes according to manufacturer's instructions. An α-^{32}P-labelled random-primed probe was made from the 750-bp *Hin*dIII fragment containing most of the coding region of TBP. Primer extension reactions were performed by standard techniques (1) with two oligonucleotides which corresponded to positions −174 to −158 and −223 to −249 with respect to the ATG codon of the TBP gene. For 3′-end mapping, a DNA adapter was ligated directly to the ends of total RNA (42). Nested PCR was performed with oligonucleotides corresponding to positions +418 to +443 and +604 to +619. After amplification, PCR products were gel purified, the ends were phosphorylated and blunted with T4 polynucleotide kinase (New England Biolabs) and T4 DNA polymerase (New England Biolabs) according to the manufacturer's instructions, and the fragments were cloned into *Sma*I-cut, calf alkaline phosphatase-treated Bluescript vector.

TBP antibody generation. The insert from pPCR12 was fused to the TrpE′ gene on an indolacrylic acid-inducible, tryptophan-repressible *Escherichia coli* vector (13). A directed cloning was performed into *Eco*RI-*Xba*I-cut Path1 vector (a gift from David Hinkle), with an *Eco*RI-*Xba*I-cut insert, producing a fusion protein of 393 amino acids: 55 amino acids from TBP sequences and 4 amino acids (PAAV) derived from the Bluescript vector. Induction and partial purification of fusion protein were done as described previously (66). To isolate pure fusion protein for antibody generation, preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. After electrophoresis, protein was visualized by staining for 5 min at 4°C with ice-cold 250 mM KCl–1 mM dithiothreitol. Bands were excised, and gel slices were rinsed three times for 10 min each in 1 mM dithiothreitol (cold) and frozen at −70°C. A total of 2.5 mg of fusion protein (in acrylamide) was isolated in this manner, and this material was used for all intradermal and subcutaneous injections. Fusion protein for intravenous injection was eluted from the gel pieces with a Little Blue Tank (ISCO). Gels were fragmented into 0.5-cm pieces, and each chamber was loaded with five or six fragments. Elution was done for 2 h in 1× Laemmli running buffer at 4 to 5 mA per chamber. Samples were then dialyzed against 100 volumes of phosphate-buffered saline (PBS) (150 mM NaCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4 [pH 7.4]) at room temperature for 1 h. Dialysis was continued at 4°C for

a total of 2 h with two changes (for 1 h each). A total of 500 μg of material was purified in this manner.

Polyclonal antibodies were generated at the Pocono Rabbit Farm and Laboratory (Canadensis, Pa.). Fusion protein in acrylamide was mixed 1:1 with Freund's complete adjuvant for subcutaneous and intradermal injections, of which 720 μg was injected per rabbit. One intravenous injection of 25 μg was also performed.

In some cases, TBP antibodies were affinity purified essentially as described previously (52), with the following modifications. Bacterially expressed pET-TBP fusion protein was blotted to nitrocellulose (MSI). This construct contained the C-terminal domain of *Tetrahymena* TBP subcloned into pET-15b by PCR according to the manufacturer's instructions (Novagen). The TAA and TAG glutamine codons had been altered by using the Muta-Gene kit (Bio-Rad) following the manufacturer's directions, except that the *dut ung* mutant strain RZ1032 (40) was used. In all buffers, 3% bovine serum albumin (BSA) replaced the gelatin. After incubation with crude anti-TBP serum, blots were washed extensively before bound antibodies were released by three successive treatments with 5 M sodium iodide for 5 min each. Pooled, eluted material was diluted with 1 volume of PBS, and BSA was added to a final concentration of 1 mg/ml. The affinity-purified antibodies were concentrated in a Centricon-30 (Amicon) to 200 μl (the original volume of crude serum used) and washed three times with 2 ml of PBS each, with reconcentration each time to 200 μl.

PAGE and immunoblotting. SDS-PAGE and immunodetections were performed by standard techniques (1). All *Tetrahymena* samples were derived from logarithmically growing cultures of the CU428 strain. Macronuclei were prepared as described previously (67). For whole-cell samples, cells were harvested as described previously, (67) washed one time in 10 mM Tris-HCl (pH 7.4), and extracted extraction as described previously (44) or immediately suspended in 1× SDS sample buffer (5% β-mercaptoethanol [BME], 10% glycerol, 2% SDS, 60 mM Tris-HCl [pH 6.8]). Human TBP was a gift from Robert Roeder, and *S. cerevisiae* and *S. pombe* TBP samples were gifts from Roger Kornberg.

Indirect immunofluorescence. Cells were harvested, paraformaldehyde fixed, and incubated with antisera as described previously (18). For TBP and histone detections, 50 μl of cell suspension was spotted and allowed to dry onto a poly-L-lysine-pretreated coverslip. Affinity-purified anti-TBP serum was diluted 1:5 in 3% BSA–10% normal goat serum (GIBCO) in PBS; crude anti-H2A serum was diluted 1:500.

Phylogenetic tree construction. The derived amino acid sequences were obtained from DNA sequences in GenBank (release 73.1, December 1992) or from the literature (see figure legends). Sequence alignments were made by a simplified form of the progressive method (12, 15). Evolutionary distances (d) were calculated from the proportion of amino acid identity (S) with a Poisson correction, $d = -\ln S$. These values were used to construct a phylogenetic tree by the neighbor-joining method (58).

Nucleotide sequence accession number. The *Tetrahymena* TBP has been assigned GenBank accession no. L16957.

RESULTS

Cloning the *Tetrahymena* TBP gene. PCR was used to generate a fragment of the *Tetrahymena* TBP gene from a random-primed cDNA library. Nested PCR performed with a single 5′ oligonucleotide (BK10) and two 3′ oligonucleotides (BK11 and BK12), which had been successfully used to amplify human TBP (37), failed to produce specific products. Therefore, published sequence information from the conserved carboxy terminus was used to generate a 5′ oligonucleotide (o-TBP) which corresponded to amino acids 89 through 108 of the *S. cerevisiae* TBP amino acid sequence and was constructed by using *Tetrahymena*-preferred codons (45). This region is the most conserved in the cloned TBP sequences and was used with both BK11 and BK12 to generate fragments of the predicted sizes, which were cloned and sequenced (data not shown). With the PCR clones as probes, *Tetrahymena* TBP was found to be encoded by a 1,300-base polyadenylated message from a single copy gene (Fig. 1). Subsequently, a size-selected (3,000-bp ± 500-bp) *Bgl*II-cut, genomic library was constructed and screened with the PCR clones. A 3-kb TBP gene-containing clone was identified by hybridization and isolated, and both strands were sequenced by a combination of double-stranded and single-stranded sequencing (Fig. 2).

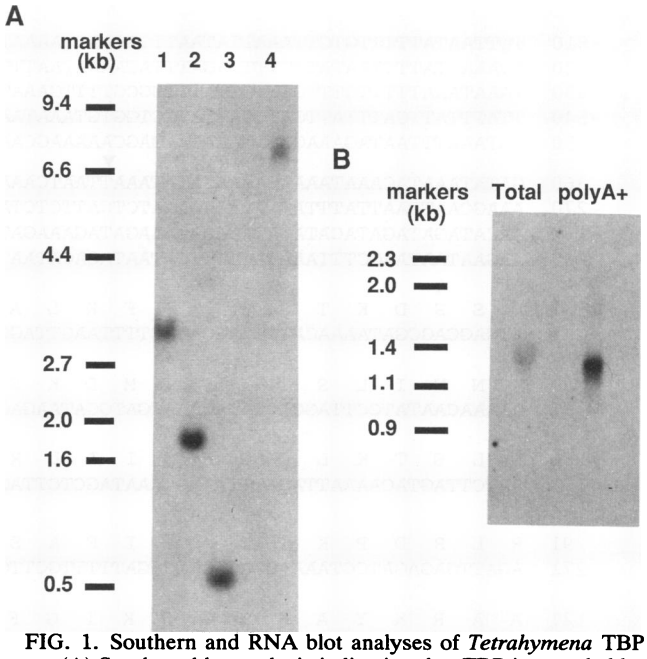

FIG. 1. Southern and RNA blot analyses of *Tetrahymena* TBP gene. (A) Southern blot analysis indicating that TBP is encoded by a single copy gene. Macronuclear DNA (10 μg) was digested with *Bgl*II (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), or *Pst*I (lane 4), electrophoresed in a 0.7% agarose gel, transferred to a nylon membrane and hybridized with a random-primed probe generated from the insert of the pPCR12 clone (see the text). The *Bgl*II fragment was subsequently targeted for the generation of the size-selected library. (B) RNA blot analysis of 15 μg of total RNA or 2 μg of poly(A)$^+$ RNA electrophoresed in a formaldehyde-agarose gel, transferred to a nylon membrane, and probed with the PCR12 insert demonstrates a single message of 1,300 bases.

***Tetrahymena* TBP message characterization by mapping the 5′ and 3′ ends.** Primer extension analysis of the TBP message revealed one strong start site at position −328 relative to the ATG, with two weaker ones at −333 and −340 (data not shown). To our knowledge, this is the longest leader sequence described for a *Tetrahymena* gene (which typically has leaders between 50 and 80 bases). PCR was used to map the 3′ ends of the message. cDNA template was prepared by ligating an adapter directly onto the 3′ end of all RNAs and

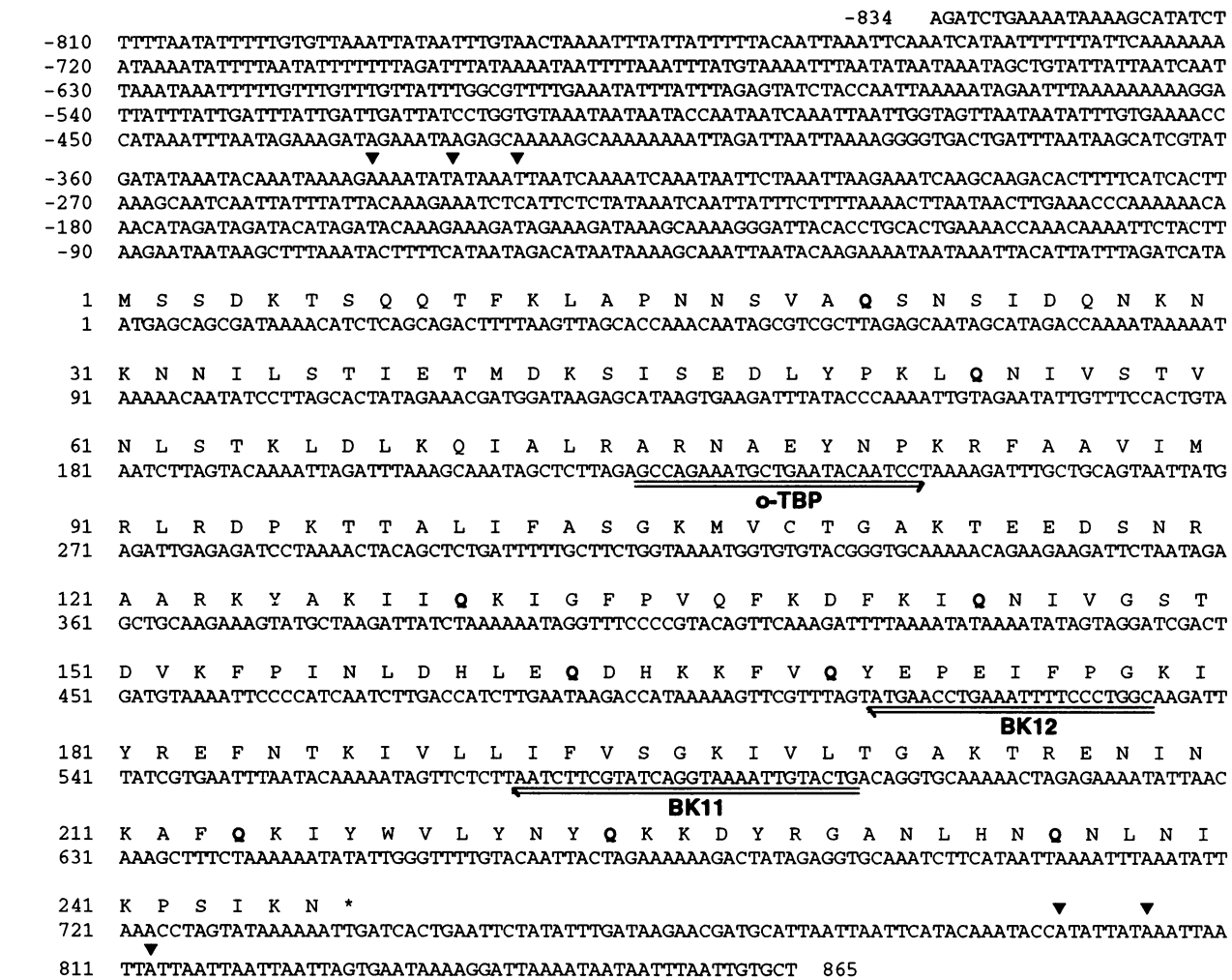

FIG. 2. TBP gene of *T. thermophila*. The sequence of the TBP gene was obtained by a combination of double- and single-stranded sequencing of both strands of DNA (GenBank accession no. L16957). The 5′ and 3′ message ends are indicated by triangles. The nine glutamines (Q) encoded by TAA or TAG are shown. An asterisk indicates the first nucleotide of the stop codon. The regions homologous to the oligomers used to obtain the initial clones by PCR (o-TBP, BK11, and BK12) are underlined and labelled (arrows indicate orientation).

using the compliment of the adapter in the reverse transcription reaction (42). This technique has advantages over using the oligo(dT) primer for preparing the cDNA because all RNAs are represented [poly(A)$^+$ and poly(A)$^-$] and, once cloned, the length of the poly(A) tail can be determined. Specific products were observed after using two nested 5′ oligonucleotides and the adapter. Four clones from the pool of products were sequenced. Two of the clones ended at position 803; the other two were at position 796 or 812 (Fig. 2). For all four clones, the site of poly(A) addition is ambiguous because each is at an A base. Thus, the 3′ ends spanned a range of 20 bases. A range of poly(A) tail lengths was also observed: two clones had 47 A's, one clone had 60 A's, and one had 95 A's. The predicted message length of approximately 1,130 ± 30 bases (due to the range of 5′- and 3′-end sites), plus a poly(A) tail of 60 to 100 bases, is consistent with the message length of approximately 1,300 bases observed on RNA blots (Fig. 1).

Derived amino acid sequence of *Tetrahymena* TBP. With the first methionine present on the message as the start codon, *T. thermophila* TBP contains 246 amino acids and has a predicted molecular weight of 28,180. The GC content of the coding region is only 30.4%, which is considerably lower than the 39.5% GC content observed for 35 other *Tetrahymena* coding regions (74a) and is a reflection of the preference for AT-rich codons in this DNA sequence. Besides GC-poor coding sequences, *T. thermophila* also uses a nonstandard genetic code in which TAA and TAG code for glutamine (Q) (34). Of a total of 14 glutamine residues in *Tetrahymena* TBP, 9 are encoded by either TAA or TAG (Fig. 2), making expression of the entire protein in a heterologous system impossible without modifications. *Tetrahymena* TBP possesses a short N-terminal sequence of 47 amino acids, the length of which is characteristic of lower eukaryotes and plants (Fig. 3). The 180-amino-acid C-terminal domain has a typical 67-amino-acid interrupted repeat, but it is one of the most divergent described with 70% identity to the *S. cerevisiae* sequence; most TBPs (14 of 17) are 80 to 93% identical within this region. *Tetrahymena* TBP also possesses a unique hydrophilic, positively charged C-terminal extension of 20 amino acids.

Evolutionary analysis of the C-terminal domains of the

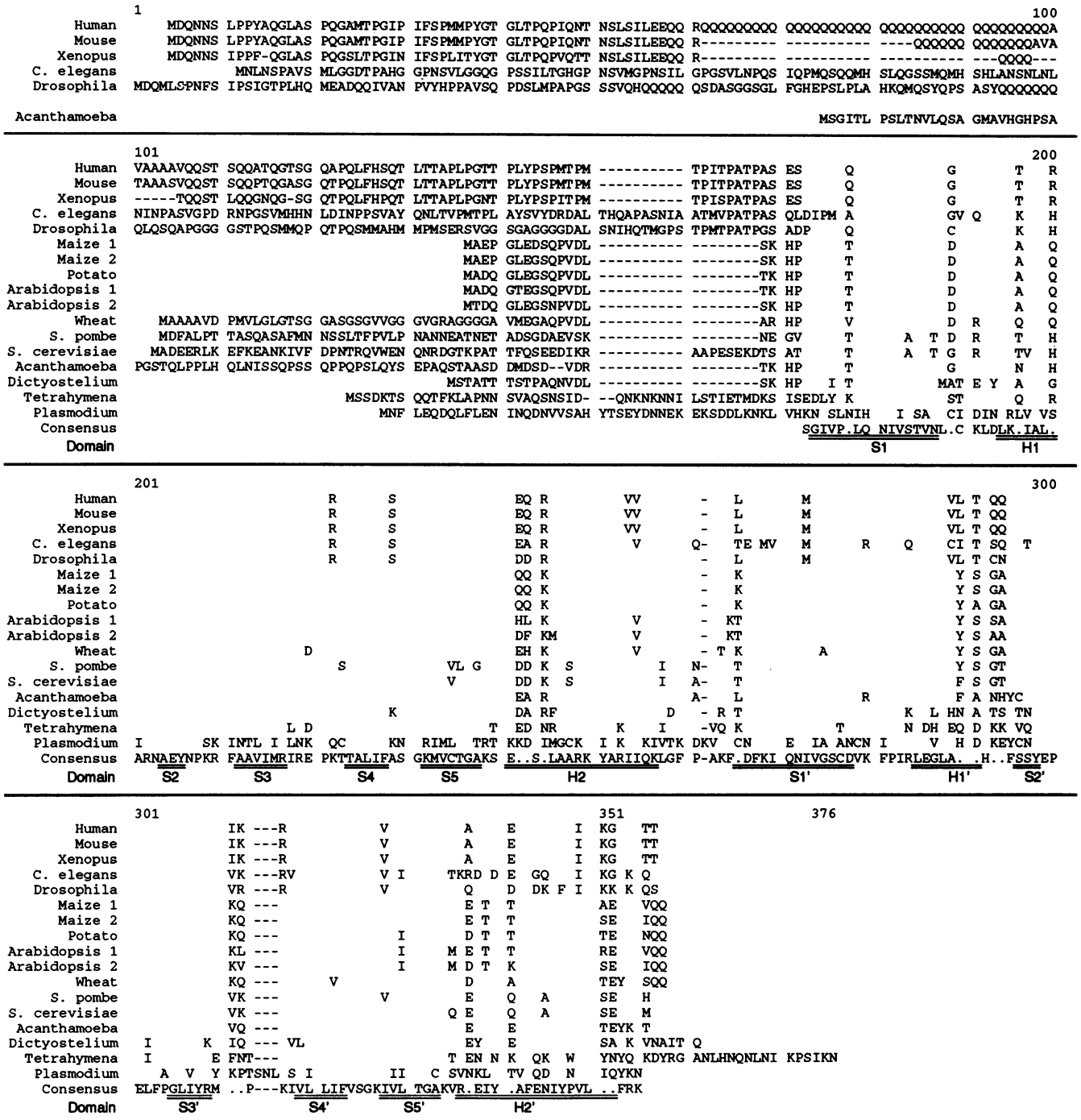

FIG. 3. Amino acid sequence alignment of 17 TBPs from 15 different organisms. TBP sequences were conceptually translated and aligned. After the initiator methionine, a space indicates identity with the consensus, while a dash indicates a gap introduced into the alignment to maximize homology. Also noted are the domains defined by the crystal structure analysis of *Arabidopsis* TBP (51). S denotes beta strands; H denotes alpha helices. The *Tetrahymena* sequence is also shown in Fig. 2. The sequences for human TBP (37), the two *Arabidopsis* (20) and maize genes (25), and *Plasmodium* TBP (48) were obtained from the literature. All other sequences were obtained from the GenBank and EMBL data banks, and their accession numbers are M27135, *S. cerevisiae*; L07754, *C. elegans*; X53415, *S. pombe*; M87495, *Acanthamoeba castellanii*; M64861, *Dictyostelium discoideum*; M38082, *D. melanogaster*; X59874, *T. aestivum* (wheat); D01034, mouse; X66033, *Xenopus* sp.; and X62494, potato.

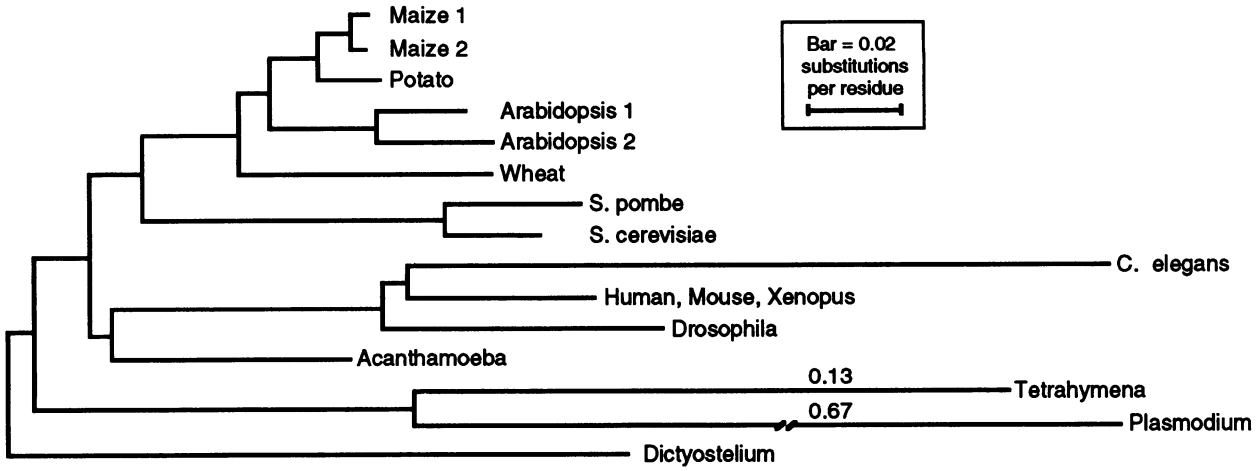

FIG. 4. Phylogenetic tree of carboxy-terminal domains of cloned TBP. Sequence relatedness of the 17 aligned proteins shown in Fig. 3, starting at amino acid 173 and ending at 356, was determined by the neighbor-joining method (see Materials and Methods). The scale bar indicates the branch length corresponding to 0.02 change per amino acid position (total of 183 amino acids). Sequence origins are listed in Fig. 3.

cloned TBPs. The neighbor-joining method was used to generate an evolutionary distance tree comparing the amino acid sequence of the conserved C-terminal domain of 17 cloned TBPs (Fig. 4). The order of the branch points clearly segregates the species into plant, fungal, animal, and protist groups. *Plasmodium* TBP has the longest branch length of the sequences analyzed, suggesting that this organism is ancestral to the other species analyzed. However, evolutionary comparisons of rRNAs and protein encoding sequences indicate that the genus *Dictyostelium* represents the most ancient lineage (65, 79). *Tetrahymena* and *Caenorhabditis elegans* TBPs also possess distinctly longer branch lengths than the remaining 14 TBPs, which may indicate that all three (*Plasmodium*, *Tetrahymena*, and *C. elegans* TBPs) have evolved faster than the other TBPs (see Discussion).

Antibodies generated against *Tetrahymena* TBP recognize a 36-kDa macronuclear protein. Polyclonal antibodies against *Tetrahymena* TBP were generated with a 55-amino-acid domain within the conserved C terminus fused with the TrpE′ protein on an *E. coli* expression vector. This domain, corresponds to amino acids 201 to 255 in the alignment shown in Fig. 3. Antibodies to the hybrid protein cross-react with a 36-kDa protein which is enriched in macronuclear preparations (Fig. 5A). In addition, strong cross-reactivity to a histone is observed. High-pressure liquid chromatography-purified histone samples were assayed by immunoblotting, and it was determined that histone H2B was the reactive species. Antibodies that were affinity purified to a second TBP-fusion protein (pET-TBP) in a different vector lacking TrpE′ sequences reacted only with the 36-kDa protein; antibodies affinity purified to H2B cross-reacted with TrpE′ and not with the 36-kDa protein or the pET-TBP fusion protein (data not shown). Thus, the cross-reactivity with H2B is due to determinants on TrpE′, not on TBP, and can be removed by affinity purification.

The antiserum cross-reacts at a 1:10,000 dilution with purified *S. cerevisiae* and *S. pombe* TBP as well as human TBP (Fig. 5B). To our knowledge, this is the first antiserum which has been shown to cross-react with other TBPs. Previously described polyclonal antisera generated to intact proteins recognize epitopes within the divergent N termini (56, 74).

TBP is localized solely to macronuclei of growing or starved *T. thermophila*. Indirect immunofluorescence with specific antibodies was used to establish the subcellular localization of anti-TBP determinants in growing and starved cells. This approach offers the advantage that specific nuclei can be analyzed in cytologically staged cells. *T. thermophila* has two types of nuclei: a diploid micronucleus that serves as the cell's germline and a polyploid, somatic macronucleus which controls the cell's phenotype and is destroyed during the sexual process of conjugation. The subcellular localization of *Tetrahymena* TBP was ascertained by fluorescence microscopy of growing or starved cells after incubation with affinity-purified anti-TBP antibodies followed by incubation with a fluorescein-labelled secondary antibody. Anti-TBP determinants were found to be localized solely to macronuclei in both growing and starved cells (Fig. 6). This is the predicted pattern for a transcription factor since the micronucleus is transcriptionally inert at these stages.

TBP is localized to both the macronucleus and the micronucleus of mating cells. Despite the significant differences in ploidy, replication mode, and transcriptional activity, macro- and micronuclei are related. During conjugation (46, 53), the sexual phase of the life cycle, cells of different mating types pair and the micronucleus in each undergoes meiosis (Fig. 7). For a brief period during early meiotic prophase, the micronucleus is transcriptionally active (47, 71). After meiosis, three of the four haploid nuclei degenerate. The remaining nucleus divides mitotically to give two identical gametic nuclei. The cells exchange one gametic nucleus which then fertilizes the stationary nucleus of the other cell. Two postzygotic divisions follow, the second of which is oriented to produce two anterior and two posterior nuclei in each cell. Differentiation of the four products of the second postzygotic division into macronuclei or micronuclei is specified by their location in the cytoplasm: the two anterior nuclei develop into macronuclei while the two posterior nuclei become micronuclei. Results obtained by Nanney (50) strongly support a determinative role of localized cytoplasmic regions, since nuclei relocated to the anterior region by centrifugation developed into macronuclei, while those relocated to the posterior region became micronuclei. The differentiation of macronuclei is characterized by striking

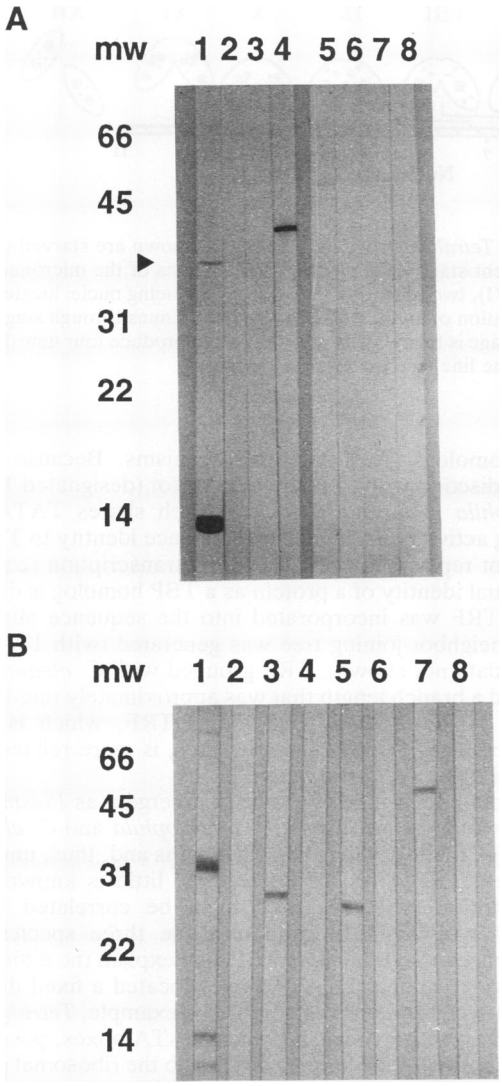

FIG. 5. Immunoblots with anti-TBP serum. (A) A 1:100 dilution of antiserum was used for the samples incubated with immune (lanes 1 to 4) or preimmune (lanes 5 to 8) serum. Lanes: 1 and 5, 10^6 macronuclei; 2 and 6, 10^5 lysed cells; 3 and 7, 10^5 cell equivalents from a whole-cell extract; 4 and 8, 100 ng of TrpE-TBP fusion protein. The arrowhead indicates a 36-kDa protein in lane 1. (B) Cross-reactivity of *Tetrahymena* anti-TBP was assayed at a 1:10,000 dilution against 100 ng of pET-TBP (lanes 1 and 2) or overexpressed *S. cerevisiae* (lanes 3 and 4), *S. pombe* (lanes 5 and 6), or human TBPs (lanes 7 and 8). Odd-numbered strips were incubated with anti-TBP serum; even-numbered strips were incubated with preimmune serum. The cross-reacting material observed in lane 1 is likely due to degradation of TBP in bacterial cells since it is observed only after induction of strains containing TBP fusion constructs. Molecular weight (MW) markers (in thousands) are indicated on the left.

changes in DNA sequence organization and the onset of transcriptional activity (reviewed in references 3, 4, 23, and 81).

The appearance of TBP in nuclei during conjugation was examined by immunofluorescence analyses. We wished to determine precisely when TBP first appears in developing macronuclei and to correlate its acquisition with the onset of gene expression in these nuclei. As expected, it was observed that anti-TBP stains the parental macronucleus

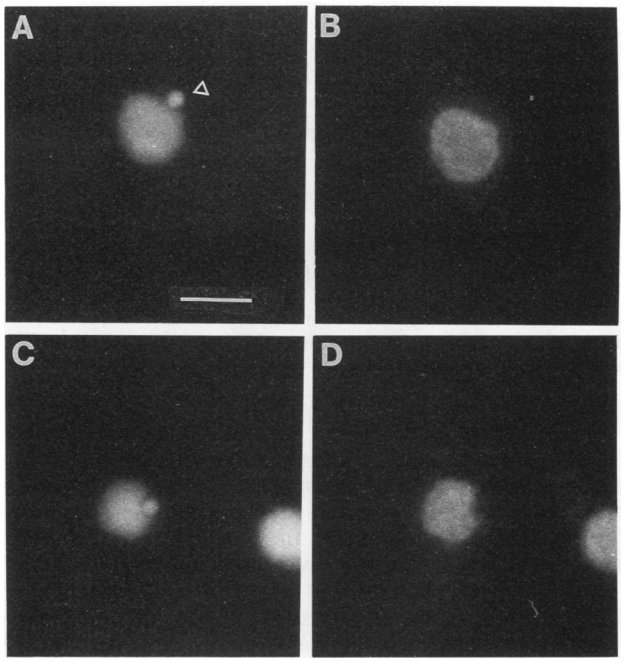

FIG. 6. Macronuclear-specific localization of TBP in growing (A and B) and starved (C and D) *T. thermophila* cells stained with 4′,6-diamidino-2-phenylindole (DAPI) (A and C) or anti-TBP (B and D). The arrowhead in panel A indicates the micronucleus. Bar, 10 μm.

throughout development until these nuclei become pycnotic and begin to degenerate (Fig. 8 and 9). Anti-TBP also stains micronuclei during both gametic and postzygotic stages. This staining appears at early meiosis (~1.5 h after opposite mating types are mixed) just prior to the onset of transcriptional activity (47, 71). Surprisingly, it persists through crescent stage and gametogenesis (Fig. 8). The products of the second postzygotic division also stain, even those nuclei positioned posteriorly which are destined to become micronuclei. Thus, staining remains long after micronuclear transcription has ceased. Staining with anti-TBP is then lost from developing micronuclei and becomes specific to developing macronuclei, coincident with the degradation of the parental macronucleus (Fig. 9) and the onset of transcription from the new macronuclei (47, 71). The transition of developing micronuclei from positive to negative staining occurs in close proximity in the cytoplasm with the still positive macronuclei. Staining with an anti-H2A antibody remains constant throughout this period (Fig. 10), indicating that micronuclear chromatin remains accessible to antichromatin antibodies.

DISCUSSION

To initiate studies on the interaction between *Tetrahymena* TBP and RNA polymerase II promoters, the TBP gene has been cloned and characterized from this species. We feel this gene represents the *Tetrahymena* TBP homolog since it possesses significant sequence similarity to TBPs of other organisms and is present in a single copy (suggesting that it is not a member of a gene family). Also, antibodies generated to a peptide fragment derived from this gene cross-react with TBPs from distantly related organisms (yeast and human). Furthermore, the protein is localized to the nucleus and its

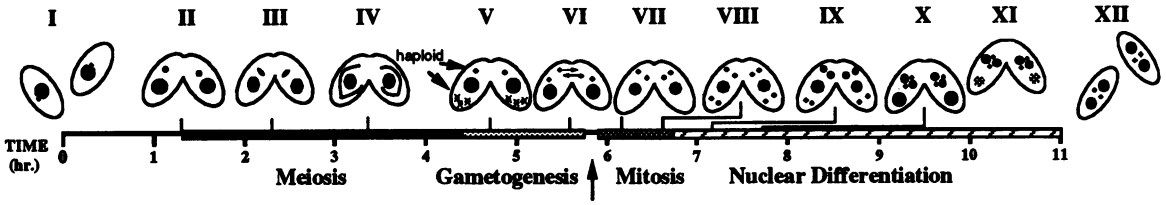

FIG. 7. Illustration of key stages during conjugation, the sexual phase of the *Tetrahymena* life cycle. Stages shown are starved cells (I), pair formation (II), early meiotic prophase (III), late meiotic prophase or crescent stage (IV), postmeiotic divisions of the micronucleus to produce haploid gametic nuclei (V), gametic nuclear exchange and fertilization (VI), two postzygotic divisions producing nuclei located in the posterior and anterior portion of the cell (VII and VIII), macronuclear differentiation of anterior nuclei which continues through stage X (IX and X), loss of parental macronucleus (XI), and cell separation (XII). This final stage is followed by cell division to produce four new daughter cells from the original two cells. Approximate time (in hours) is shown under the line (see the text for details).

detection correlates with changing patterns of transcriptional activity. Because of this strong (albeit circumstantial) evidence, we will refer to this gene as the one encoding *Tetrahymena* TBP. However, the absolute identification of this gene as a TBP homolog awaits functional analysis.

Alignment of the amino acid sequences of 17 cloned TBPs (Fig. 3) reveals several interesting points. Animals possess significantly longer N-terminal domains and, of the five animal species represented, only *C. elegans* lacks a run of glutamine residues in the N-terminal region. Furthermore, although the amino acid sequence is generally not conserved in the N termini, all animal TBPs contain a sequence nearly identical to TP-PATPAS positioned 2 amino acids N-terminal to the start of the conserved C-terminal domain. In contrast, the N-terminal domains of plant TBPs are highly conserved in length and sequence, with the exception of wheat. All plants (including wheat) contain the sequence PVDL near the junction of the N- and C-terminal regions. The N-terminal domains of fungal TBPs are not similar to each other or to those of the plants or animals. The N-termini of protist TBPs are virtually unrelated, consistent with the fact that this classification represents an extremely diverse group of organisms. Interestingly, the N-terminal domain of *Dictyostelium* TBP has both length and sequence characteristics most typical of plants.

A comparison of the amino acid sequence of the conserved C-terminal domains of the cloned TBPs was used to generate an evolutionary distance tree by the neighbor-joining method. This analysis portrays the protists as a series of independent branches preceding the nearly simultaneous separation of plants, animals, and fungi. Of these lineages, only plants contain more than one copy of the TBP gene. The short branch lengths separating the different genes within a plant species indicate either recent duplications or very strict amino acid conservation for functional reasons. These duplications may reflect the polyploidy which is common in the plant kingdom. *Plasmodium* TBP possesses the longest branch length, indicating that it is the most divergent TBP. The simplest interpretation of these data is that *Plasmodium falciparum* is ancestral to the other species analyzed. However, evolutionary comparisons of rRNA sequences and other protein sequences indicate that *Dictyostelium discoideum* diverged from the rest of the eukaryotes earlier than *Plasmodium* spp. (65, 79). Additionally, *Plasmodium* alpha- and beta-tubulins (19) are not unusually divergent, suggesting that *P. falciparum* is not simply a rapidly evolving organism.

When performing evolutionary comparisons with protein sequences, one assumes that the members analyzed represent homologs from different organisms. Because of the recent discovery of a TBP-related factor (designated TRF) in *Drosophila melanogaster* (11), which shares TATA box-binding activity and significant sequence identity to TBP but does not replace TBP in basal level transcription reactions, the actual identity of a protein as a TBP homolog is difficult. When TRF was incorporated into the sequence alignment and a neighbor-joining tree was generated (with 179 amino acids; data not shown), TRF grouped with *C. elegans* TBP and had a branch length that was approximately one-half the length of *Plasmodium* TBP. Thus TRF, which is not a homolog of TBP in *D. melanogaster*, is more related to all the TBPs than is *Plasmodium* TBP.

Although their TBPs are not as divergent as *Plasmodium* TBP, two other organisms, *T. thermophila* and *C. elegans*, also have relatively long branch lengths and, thus, unusually divergent TBPs. To our knowledge, little is known about transcription processes that might be correlated with a rapidly evolving TBP gene in these three species. The divergence in *Tetrahymena* TBP may explain the difficulty in identifying canonical TATA boxes located a fixed distance upstream of *Tetrahymena* genes. For example, *Tetrahymena* genes may entirely lack canonical TATA boxes, possessing instead a novel TBP-binding site, as do the ribosomal protein genes of *S. pombe*, which utilize CAGTCACA boxes instead of TATA (77). Alternatively, the context of the entire promoter region could be critical, and upstream binding sites and their factors (76) as well as initiator sequences and their factors (which bind at the start site of transcription) (54, 64, 82) may be necessary for promoter recognition, placing less constraint on TBP itself. Polyclonal antiserum generated to a fragment within the conserved carboxy terminus of *Tetrahymena* TBP recognizes a 36-kDa protein which is enriched in macronuclear fractions and also shows cross-reactivity with *S. cerevisiae*, *S. pombe*, and human TBPs. By immunofluorescent analyses, the distribution of TBP was found to be macronuclear specific during growth and starvation. This pattern of nuclear distribution is consistent with the transcriptional activity of the two nuclei, since macronuclei are transcriptionally active and micronuclei are transcriptionally inert at these stages of the life cycle.

Immunocytochemistry was also used to determine the distribution of TBP during conjugation, the sexual phase of the life cycle. Conjugation in *T. thermophila* has many similarities to developmental processes in higher eukaryotes, including meiosis, gamete formation, fertilization, determination, and differentiation. However, instead of morphologically and functionally distinct tissues produced as the end product of the developmental pathway in multicellular

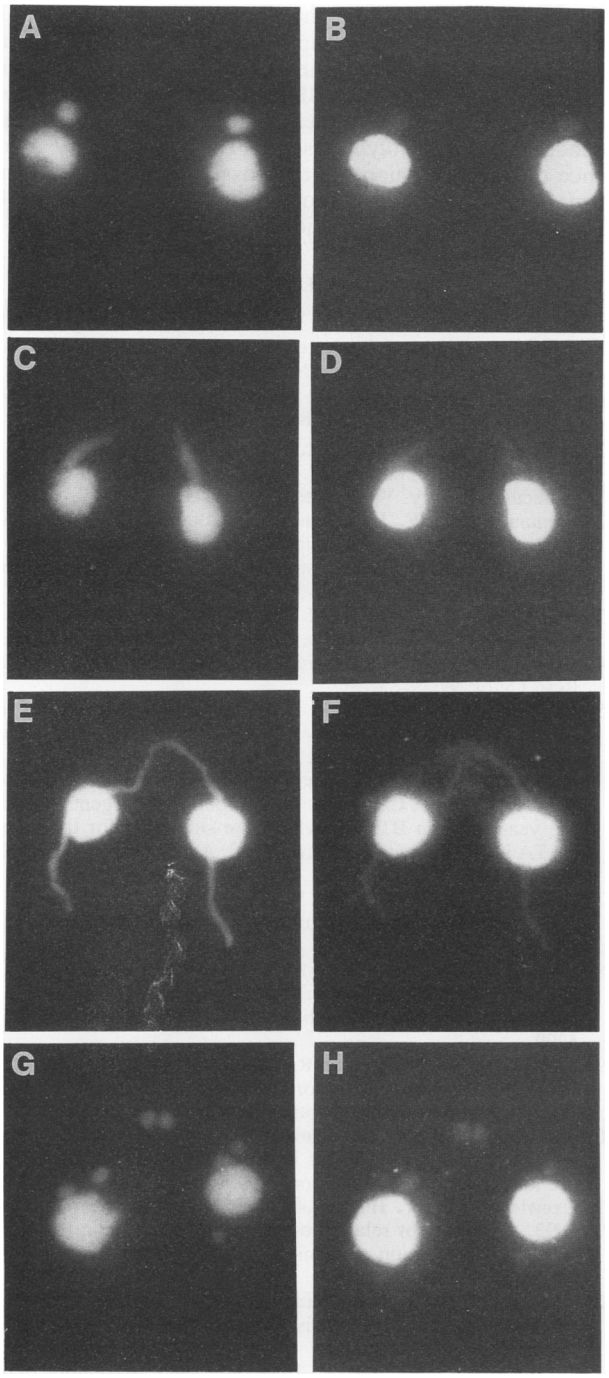

FIG. 8. TBP is localized to both the macronucleus and the micronucleus of mating cells. Immunofluorescent images of mating pairs at various stages of conjugation stained with DAPI (A, C, E, and G) or anti-TBP (B, D, F, and H). Stages represented are premeiotic micronuclei at 2 h postmixing (A and B), early crescent stage of meiotic prophase at 3 h postmixing (C and D), late crescent stage at around 3.5 h (E and F), and just after the second prezygotic division at around 5 h (G and H).

organisms, *T. thermophila* produces two morphologically and functionally distinct types of nuclei. Anti-TBP serum strongly stained the parental macronucleus throughout conjugation, until its breakdown and subsequent elimination.

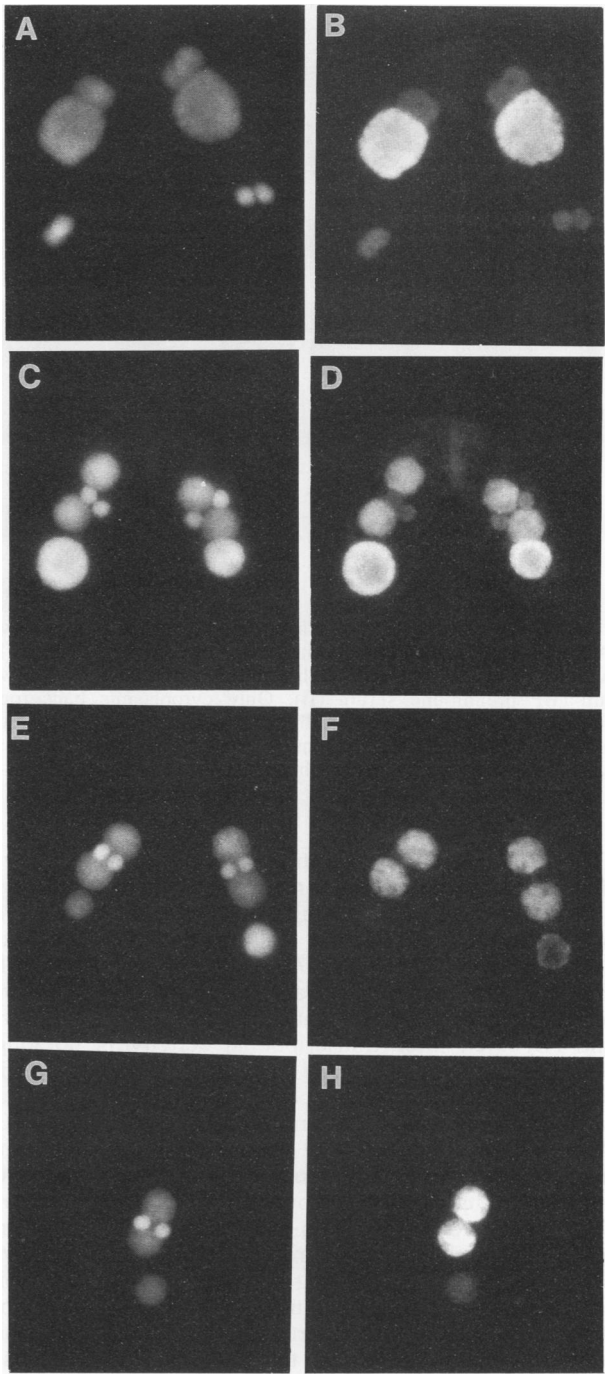

FIG. 9. Elimination of TBP from micronuclei and retention in developing macronuclei accompanies nuclear differentiation in late stages of conjugation. Immunofluorescent images of mating pairs stained with DAPI (A, C, E, and G) or anti-TBP (B, D, F, and H). Anti-TBP staining occurs in all products of the second postzygotic division (A and B), at approximately 8 h postmixing. Anti-TBP staining of all nuclei persists during macronuclear development (C and D). Anti-TBP staining is restricted to developing macronuclei coincident with the degradation of the parental macronucleus (E and F), at around 10 h postmixing, and this pattern remains after cell separation (G and H).

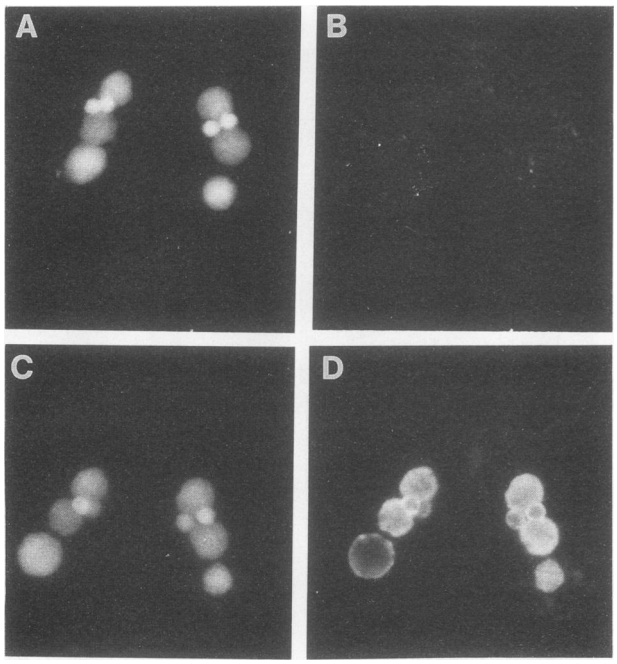

FIG. 10. Immunofluorescent images of mating pairs stained with DAPI (A and C), anti-TBP preimmune serum (B), or anti-H2A (D). A late-stage pair similar to that shown in Fig. 9C and D stained with anti-TBP preimmune serum (B). Only cytoplasmic background fluorescence was observed at all stages with anti-TBP preimmune serum. Anti-H2A staining demonstrates that micronuclei at 10 h are capable of being stained by antibodies (D).

This pattern is in accord with the transcriptional activity of this nucleus. Interestingly, micronuclei also stained with anti-TBP at the onset of conjugation. The appearance of TBP occurs just prior to a burst of transcriptional activity observed in meiotic nuclei (47, 71), consistent with TBP's critical involvement in the transcription process. However, both prezygotic and postzygotic nuclei continue to stain with anti-TBP well after transcriptional activity is no longer detectable. Even those nuclei situated in the posterior region of the cell after the second postzygotic division, which are fated to develop into micronuclei, stained with anti-TBP. Although transcription is not detected in these nuclei, they may still have the potential to transcribe since they possess a critical component of this process (TBP). Consistent with this, these nuclei still retain the capacity to develop into macronuclei when they are relocated to the anterior cytoplasm (50). Anti-TBP staining is finally lost from developing micronuclei very late in conjugation, approximately coincident with the time that differentiating macronuclei become transcriptionally active and the parental macronucleus is degraded. TBP staining is eliminated from differentiating, transcriptionally silenced micronuclei in the absence of nuclear division or swelling that could result in dilution. It is this active elimination of TBP from micronuclei, rather than selective deposition in macronuclei, that initially produces the macronuclear-specific distribution observed in growing and starved cells. Thereafter, there must be either selective deposition of TBP into macronuclei or selective degradation of TBP in micronuclei to maintain the macronuclear-specific pattern of TBP distribution during continued vegetative growth.

The cloning and sequencing of the TBP gene from *T. thermophila* should allow the elucidation of some of the important features of transcriptional regulation in this organism. A primary unsolved question is exactly what, if any, sequence(s) *Tetrahymena* TBP recognizes in the TATA-less, A-T-rich, relatively featureless upstream regions of genes encoded by RNA polymerase II. The evolutionary divergence of this TBP supports the possibility that the mechanism of promoter recognition in *T. thermophila* may be different from that in the majority of organisms.

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