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Growth and Developmental Functions of a Human Immunodeficiency Virus Tat-Binding Protein/26S Protease Subunit Homolog from *Dictyostelium discoideum*

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We have characterized a newly identified gene from *Dictyostelium discoideum*, *DdTBPα*, that encodes a member of the family of eukaryotic proteins. These proteins contain a conserved ATPase domain, include subunits of the 26S protease subunit, and are homologous to the mammalian human immunodeficiency virus Tat-binding protein TBP1. While information indicates that some family members are involved in the regulation of transcription in mammalian and yeast cells during growth, these proteins are also involved in other cellular functions, and nothing is known about their possible function in multicellular development. The *Dictyostelium DdTBPα* gene is developmentally regulated, with its expression at the highest levels occurring during growth and early development. The gene is present in two copies in the genome. Disruption of one copy by homologous recombination leads to aberrant morphogenesis, which lasts from the formation of the first finger until the onset of culmination. The gene appears to be essential for growth since we were unable to obtain a complete null phenotype and since expression of an inducible antisense construct in the partial null background resulted in cell death. Expression of the antisense construct during development accentuated the partial null phenotype and also resulted in very abnormal fruiting bodies. Overexpression of DdTBPα from its own promoter leads to very large multinucleated vegetative cells when the cells are grown in suspension culture. When the cells are plated onto petri dishes in growth medium, they rapidly split into multiple cells containing one to two nuclei, in a manner similar to that of wild-type cells. Overexpressing cells are significantly delayed in forming a multicellular aggregate, but development proceeds normally once the first finger stage is reached. The results indicate that DdTBPα plays an important role in regulating both growth and morphogenesis in *D. discoideum*.

Dictyostelium discoideum grows as unicellular amoebae. Multicellular development is initiated upon starvation. After several hours, cells within the population emit oscillatory pulses of cyclic AMP (cAMP) that activate intracellular signal transduction pathways that lead to chemotaxis, signal relay, and the activation of gene expression. As the aggregate forms, cAMP and other extracellular signaling molecules regulate morphogenesis and cellular differentiation, and within 24 h, a mature fruiting body is formed. While a significant amount is known about the signaling pathways that control cellular differentiation and gene regulation, little is known about the nuclear factors that mediate this regulation (4, 6, 25, 26, 45). The only cloned transcription factor with a known function is G box binding factor (GBF), which is essential for cAMP-mediated induction of cell differentiation and late gene expression during multicellular development (37).

Recently, a new family of ATPases has been identified. The prominent feature of the members of this family of proteins is that, although their sizes vary from 45 to 120 kDa, they all contain stretches of about 200 amino acids (in some cases, this region is duplicated) that are strongly homologous (28). The conserved region contains a putative ATP binding site with the consensus G-PG-GKT and a putative helicase motif DEI/LD. Members of the family are found in the cytosol and the nucleus (33). The biological functions of the family members are very divergent. A yeast protein, Cdc48 (15), is involved in spindle body duplication and nuclear division. Sec18 (10), a yeast protein, and the corresponding mammalian protein, NSF (3), are required in vesicle-mediated protein secretion. Another gene product, Pas1 (13), is essential for peroxisome biogenesis in yeast cells. Deletion of another yeast family member, Yme1, results in the elevated escape of mitochondrial DNA to the nucleus (42). Other family members are components of the 26S protease involved in ubiquitin-dependent proteolysis pathways (7, 16, 17). Interestingly, an *Escherichia coli* protein, FtsH (43), which is involved in septum formation during cell division, shares a 30% identity with Yme1 at the protein level in a region of about 550 amino acids and also contains the putative ATP binding site and helicase motif, suggesting that the family may have derived from procaryotic progenitors.

A subfamily of these proteins, which are located in the nucleus, has been suggested to be involved in transcriptional regulation. The gene encoding the prototype of this subfamily, Tat-binding protein 1 (TBP1), was cloned by using biotinylated human immunodeficiency virus (HIV) Tat protein to screen a human λgt11 cDNA expression library for interacting proteins (31). The gene is found in all mammals examined, and its product is localized in the nucleus. Overexpression of the TBP1 protein in many mammal cell lines inhibits Tat-dependent *trans* activation of a reporter construct driven by the HIV long terminal repeat. Further work by Ohana et al. (33) demonstrated that when fused to the GAL4 DNA binding domain, TBP1 can activate GAL_{UAS}-thymidine kinase (UAS, upstream activation site) or GAL_{UAS}-HIV promoter-driven reporter gene expression. In addition, the transcription activity depends

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on both the putative ATP binding site and the helicase motif; however, GAL4-TBP1 cannot stimulate GAL_{UAS}-mouse mammary tumor virus promoter, suggesting that the transcriptional activation is promoter specific. TBP1 is able to form a heterodimer with a related human protein, TBP7, through the putative leucine zippers in their respective N-terminal regions; however, a GAL4-TBP7 fusion cannot activate transcription of the promoters examined (33). A third related human gene, *MSS1* (39), which was cloned by complementing a yeast cold-sensitive mutant, positively modulates Tat-dependent transactivation when overexpressed. Three *TBP1* homologs have been identified in yeast cells (18, 28, 41). One of these, the *SUG1* gene, was isolated by its ability to relieve the requirement of the C-terminal 28 amino acids of GAL4 for GAL4-mediated transcriptional activation (41). During the process of our work, genes encoding two TBP1 homologs (*TBP2* and *TBP10*) from *D. discoideum* were cloned by PCR (38). Transcripts complementary to both genes are present at maximal levels during the vegetative stage and then decrease during multicellular development. The function of these genes during *D. discoideum* growth and development is unknown.

In this paper, we report the use of a genetic screen to clone a new *TBP1* homolog from *D. discoideum* (*DdTBP*α) and describe its function during vegetative growth and multicellular development. *DdTBP*α is developmentally regulated and maximally expressed during growth and early development. Overexpression of DdTBPα leads to growth defects, including large, multinucleated cells in suspension culture and developmental abnormalities. The gene encoding DdTBPα is duplicated. A partial knockout strain created by disruption of one copy of the gene by homologous recombination shows developmental abnormalities, which are intensified by expression of a *DdTBP*α antisense construct. Our results suggest that DdTBPα has specific functions in controlling growth and development in *D. discoideum.*

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains XL1-Blue (Stratagene) and B/r were used. *Klebsiella aerogenes* (40) was used to isolate clones of *D. discoideum* (14). The plasmids pNN388 and pNN396 (11, 12) were kindly provided by R. Davis at Stanford University. The plasmids Exp4$^+$ (8), pVEII (2, 44), pBluescript SK(−) (Stratagene), pATSP (BioLab), and pGEX-KG (19) were also used.

Cloning of the *DdTBP*α gene. pNN396 was digested with *Kpn*I and blunt ended with T4 DNA polymerase in the presence of deoxynucleoside triphosphates (dNTPs) according to the procedure of Sambrook et al. (36). The resulting plasmid was then digested with *Hin*dIII. The CAE3 oligonucleotide (20) containing *Hin*dIII cohesive ends was ligated to pNN396 that had been treated as described above. The cohesive ends of the resulting product were filled in with the *E. coli* DNA polymerase I large fragment (Klenow fragment) as previously described (36) and ligated. The pNN396 plasmid containing CAE3 in the opposite direction was screened by restriction enzyme digestion with *Hin*dIII. The *Not*I-*Hin*dIII fragment was isolated and cloned into *Not*I- and *Hin*dIII-treated pNN388 (pNN388-CAE3). The *Not*I-*Hin*dIII fragment, which does not contain CAE3, was also cloned into pNN388 as a negative control. A λZapII (Stratagene) cDNA library constructed with the mRNAs isolated from developing *D. discoideum* at the slug stage (12 to 16 h) (37) was converted to a Bluescript SK(−) plasmid-borne library according to the procedure of the manufacturer. The plasmid library was then transformed into JM107 cells containing pNN388-CAE3. The colonies which appeared on Luria-Bertani plates containing 50 μg of ampicillin per ml, 40 μg of chloramphenicol per ml, and 30 μg of streptomycin per ml were collected and further characterized. One of the cDNA clones (p6-1) contains a 1.5-kb cDNA encompassing a full-length gene termed *DdTBP*α.

DNA sequencing. The cDNA fragments were subcloned into the pBluescript SK(−) plasmid and sequenced by the dideoxy termination method with the T3 and T7 primers as described elsewhere (36). Nucleotide and amino acid sequence analyses were conducted with the BLAST program and the GenBank and Swissprotein databases (1).

Southern and RNA blots and screening of the genomic library. DNA and RNA were isolated according to the procedures of Nellen et al. (32). Southern and RNA blots were performed as previously described (30, 35). Antisense expressors were raised in the presence of 1 mM folate (pH 6.5) for total RNA isolation. Standard protocols (36) were followed in the screening of the genomic library (9) for the *DdTBP*α promoter.

Growth of *D. discoideum* cells. The wild-type strain KAx-3 and its thymidine auxotroph JH10 (21, 30) were cultured at room temperature in axenic medium HL5 (40) supplemented with 0.5 μM (each) vitamin B_{12} and folic acid (Sigma) either on shakers at 230 rpm, on Sm^+/3 plates (21) with a *K. aerogenes* lawn (KA plates), or on DM plates (24) with a lawn of *E. coli* B/r. G418 (Gibco/BRL), as applicable, was added to the HL5 medium to a concentration of 15 μg/ml and to the DM plates to a concentration of 80 μg/ml.

Gene disruption constructs. The p6-1 cDNA clone was cut at the unique *Acc*I site, which was flush ended with Klenow, in the presence of dNTPs according to the procedure of Sambrook et al. (36). A *Hin*dIII linker was then added, resulting in p6-1(*Hin*dIII). A 3.2-kb *Hin*dIII fragment containing the *THY1* gene (9) was isolated from the JH60 plasmid (21) and ligated to p6-1(*Hin*dIII). The resulting plasmid was digested with *Eco*RI and used to electroporate JH10 (thy1 null) cells (growing to 1×10^6 to 2×10^6 cells per ml of culture) as previously described (21, 29). After settling for 1 day on HL5 medium containing 100 μg of thymidine per ml, the cells were cultured in unmodified HL5 until they were confluent, at which time a mixture of about 100 cells was transferred onto each KA plate so that the cells could be cloned.

Antisense expression of *DdTBP*α. The *DdTBP*α cDNA clone, p6-1, was digested with *Sac*I and *Bgl*II. The DNA fragments were isolated and ligated to the pVEII vector (2), which was digested with *Bam*HI and *Sac*I. The electroporations of a JH10-derived strain in which one copy of the *DdTBP*α gene was disrupted were carried out as previously described (22). The cells were grown for 12 h on petri dishes containing the HL5 medium. G418 (10 μg/ml) was then added, and the culturing was continued for 24 h before the cells were collected and spun down and the pellets were mixed with G418-resistant *E. coli* B/r. After 3 to 4 days, the plaques appearing on the plates were transferred to HL5 medium in the presence of 1 mM folate or 10^8 cells of heat-killed *E. coli* B/r per ml until the cells were confluent, and they were then used for the Northern (RNA) blot analysis and developmental biological studies.

Overexpression experiments. To overexpress the *DdTBP*α gene under its own promoter, an ~3.0-kb *Xba*I-*Acc*I genomic fragment containing 490 bp of the coding region and located 2.5 kb upstream of the translational start site of the gene was isolated from a genomic clone (Fig. 2B) and ligated to p6-1 treated with the same enzymes, resulting in p6-1-PC. The p6-1-PC plasmid was restricted with *Xba*I and *Xho*I, and the fragment containing the promoter and whole coding region was isolated and ligated to Exp4$^+$ (8), which was digested with the same enzymes, removing the actin 15 promoter. The electroporation, clonal selection, and cell culturing were described in the previous sections.

DAPI staining of nuclei. Staining of nuclei with 4′,6-diamidino-2-phenylindole (DAPI) was performed as described previously by De Lozanne and Spudich (5), except that the fixing of the cells was done at room temperature for 10 min, the staining with the DAPI solution was done twice for 5 min each time, and *p*-phenylenediamine was used to prevent fading of the fluorescence.

RESULTS

Cloning and sequence analysis of the *DdTBP*α gene. We adopted the transcription interference system developed by Elledge et al. (12) (see Materials and Methods) to try to identify transcription factors other than GBF that are capable of binding to the *cis*-acting DNA regulatory sequences designated G boxes and CAEs. These sequences have been shown to be essential for the developmental and cAMP-mediated expression of late genes in *D. discoideum* (37). The CAE3 *cis*-acting element from the prespore-specific gene *SP60* was chosen to be the operator because it exhibits the weakest interaction with GBF by comparison with the other *cis*-acting elements and because of the possibility it might bind an additional factor in vivo (20). In the selection method developed by Elledge et al. (12), a weak promoter drives the expression of spectinomycin while a strong promoter in the opposite direction interferes with the weak promoter. The binding site for a DNA-binding protein is inserted in the strong promoter. Protein binding will occlude the strong promoter and will thus allow the weak promoter to express spectinomycin resistance (Spc^r). Eight Spc^r colonies from 4×10^6 bacterial cells containing the *D. discoideum* 12- to 16-h developmental cDNA library were obtained on plates containing 35 μg of spectinomycin per ml. When these colonies were streaked onto plates containing 50 μg of spectinomycin per ml, six of the eight colonies grew. At a spectinomycin concentration of 80 μg/ml, none of colonies grew. The six plasmids that initially conferred resistance to 50

μg of spectinomycin were isolated from the original positive clones and retransformed into *E. coli* JM107 carrying the spectinomycin expression plasmid pNN388 with the CAE3 element (pNN388-CAE3). All plasmids retransformed the cells at an estimated frequency of 10^6 transformants per μg of the plasmid DNA, indicating that these plasmids conferred Spc^r. However, when the same plasmids were assayed with JM107 harboring pNN388 lacking CAE3, the same frequency of transformation was obtained. A control plasmid from the λZapII library did not confer resistance. The results indicated that the selection depended on the insert in the six plasmids, but it did not depend on the CAE3 binding site.

Sequence analysis of one of the cDNA clones (p6-1) identified a single, long open reading frame encoding a 439-amino-acid protein with a calculated molecular mass of 57.6 kDa (Fig. 1). The cDNA contains a 151-bp, 5′-untranslated region and a 45-bp, 3′-untranslated region (Fig. 2). Comparison of the deduced amino acid sequence with the known sequences in the GenBank by means of the BLAST program (1) revealed that the protein has strong homology to the proteins of the HIV Tat-binding protein (TBP1) family (Fig. 1) (31, 33, 39, 41), which is in turn a subfamily of a family of ATPases (3, 10, 13, 15, 28, 42, 43). Members of this family have recently been shown to be components of the 26S protease (see the introduction). The sequence identity of this gene product with the other members of the TBP1 family is 28.5% over the most conserved stretch of 250 amino acids. When conservative substitutions are taken into account, the degree of similarity is over 75%. This gene was, therefore, named *DdTBP*α for *D. discoideum* Tat-binding protein α. In addition to the homology of DdTBPα to the TBP1 family of proteins, the N terminus of DdTBPα contains two short basic amino acid regions that might represent a nuclear localization sequence or possibly a DNA binding sequence. Such regions are absent in other Tat-binding proteins. Like the other TBP1 family members, DdTBPα also has a putative leucine zipper structure, although the number of possible turns is smaller for DdTBPα. DdTBPα shows no more sequence homology to the other two *Dictyostelium* members of this gene family (TBP2 and TBP10) than to related proteins from other organisms.

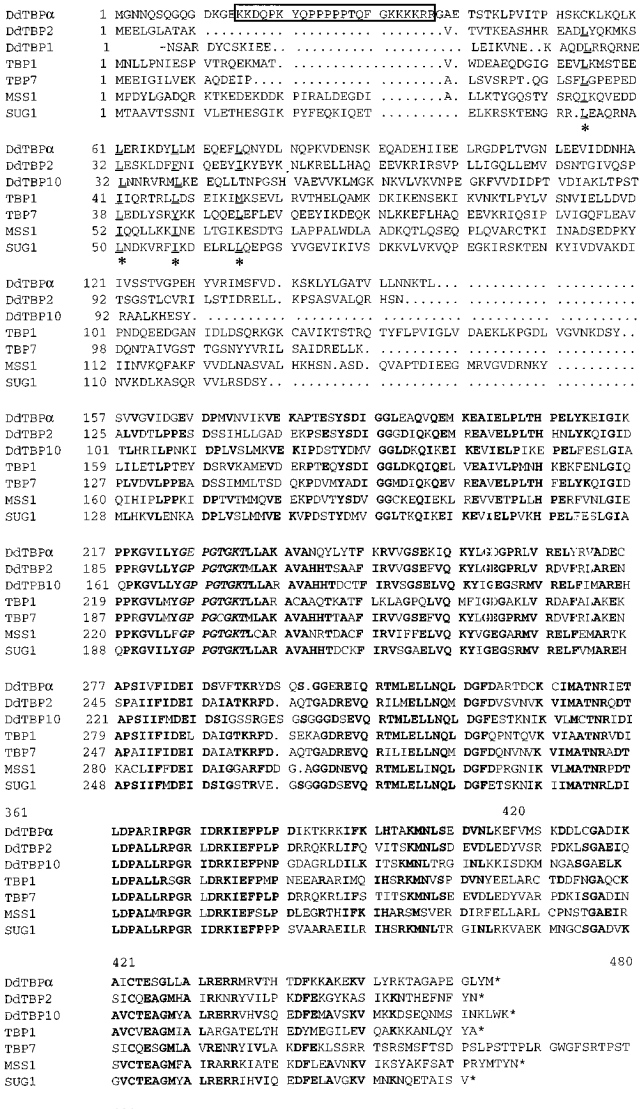


FIG. 1. Comparison of the amino acid sequence of DdTBPα with those of the Tat-binding protein subfamily of proteins. Amino acids conserved between DdTBPα and two or more other family members or between at least four family members not including DdTBPα are in bold. The amino acids at the beginning of each turn of the putative leucine zipper sequences are underlined. The positions of the start of each turn are marked with an asterisk. The basic, proline-rich domain in the N terminus of DdTBPα is boxed. The sequences shown are from the following references: DdTBP2 and DdTBP10, reference 38; TBP1, reference 31; TBP7, reference 33; MSS1, reference 39; and SUG1, references 18 and 41.

Expressed DdTBPα does not bind DNA. To directly test if the DdTBPα gene product has the ability to bind to CAE3, we expressed and purified a glutathione *S*-transferase–DdTBPα fusion and tested the ability of the fusion protein or the fusion protein cleaved with thrombin (to release DdTBPα) to shift the CAE3 probe in a native gel assay (see Materials and Methods). Neither protein caused a gel shift, suggesting that DdTBPα does not bind to the CAE3 probe (data not shown). This result was consistent with the finding that DdTBPα confers Spc^r to cells carrying pNN388 lacking CAE3. When tested, the expressed DdTBPα did not bind labeled restriction fragments of the pNN388 plasmid. We note that Ohana et al. (33) suggested that purified TBP1 does not have DNA-binding activity.

Developmental expression of *DdTBP*α. Figure 3 shows an RNA blot analysis of the temporal expression of the *DdTBP*α gene during growth and development. It is maximally expressed during growth and early development, after which the level of the *DdTBP*α mRNA decreased severalfold throughout the remainder of development.

Disruption of the *DdTBP*α gene: growth and developmental phenotypes. The *D. discoideum* gene *THY1* (9) was used to create a vector for homologous recombination into *DdTBP*α (Fig. 2B; see Materials and Methods for details). *THY1* was inserted immediately before the most-conserved region of the TBP1 family of proteins. The DdTBPα-THY1 fragment was transformed into the *thy1*$^-$ null thymidine auxotrophic strain JH10 (21, 29). Independent thymidine prototrophs were isolated from several experiments and analyzed by Southern blot analysis for disruption of the *DdTBP*α gene (Fig. 2C). A diagram of the constructs and expected integration results is shown in Fig. 2B. The analysis showed that there were two copies of the *DdTBP*α gene, one of which was disrupted, and that the flanking restriction sites around the undisrupted gene were identical over at least an ~10-kb range (Fig. 2C; data not shown), suggesting that the chromosomal region carrying *DdTBP*α was duplicated. (See the legend to Fig. 2C for a detailed description of the results.) The frequency for the partial knockout was ~25% of the clones. In multiple attempts

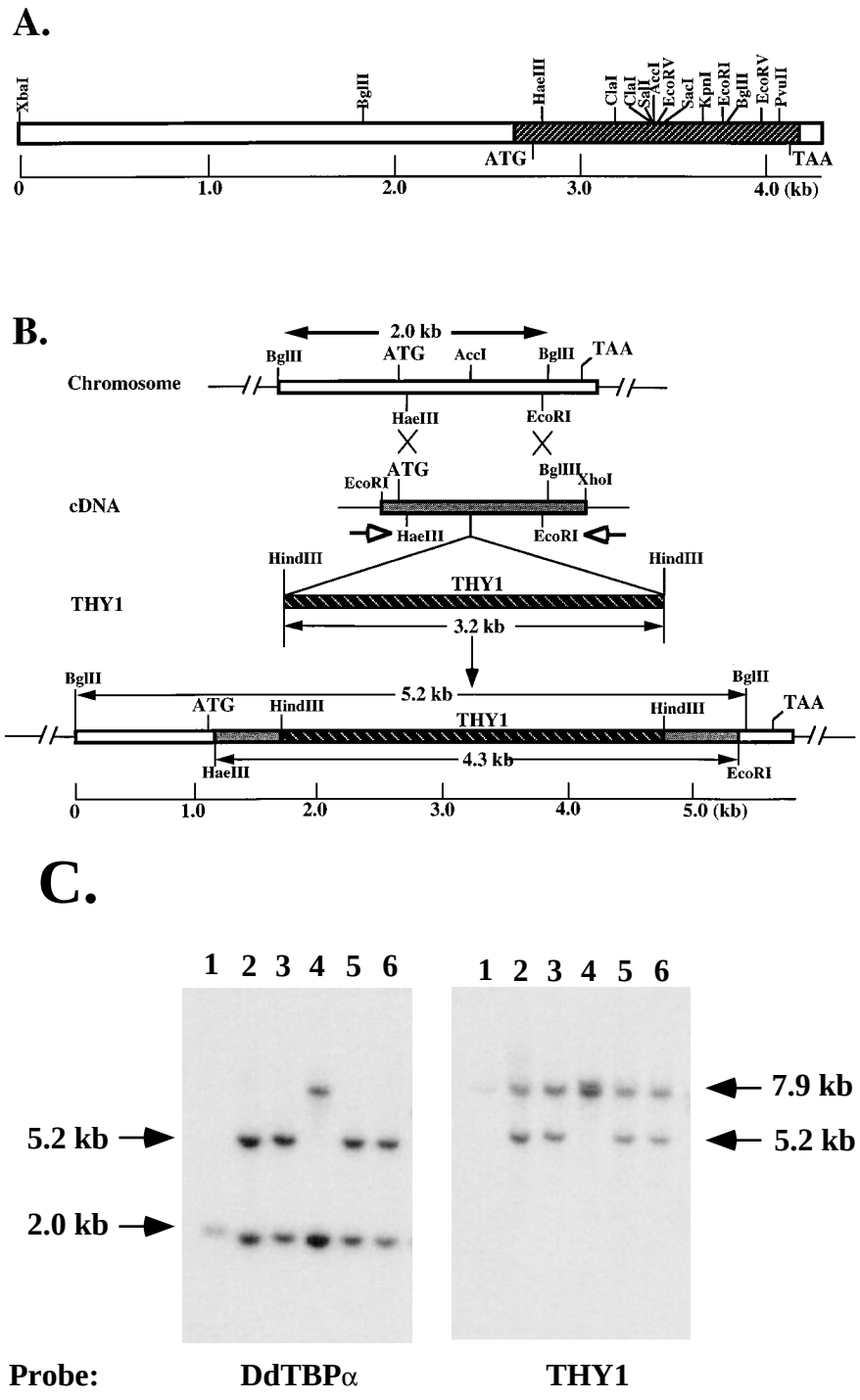


FIG. 2. Restriction maps and gene disruption construct and Southern blot. (A) Partial restriction maps of the *DdTBP*α cDNA (hatched box) and genomic DNA. (B) Maps of cDNA and diagram of the gene disruption strategy. The open arrowheads by the cDNA sequence (p6-1) indicate the enzymes used to digest the plasmid containing the gene replacement construct prior to electroporation. The enzymes used in the Southern analysis and the sizes of the corresponding fragments are also shown. (C) Two identical Southern blots were probed with the *Eco*RI fragment of the p6-1 cDNA and the *THY1* gene as indicated. Genomic DNAs isolated from five independent clones are shown. The DNAs were digested with *Bgl*II. The results are representative of four independent electroporation experiments; for each experiment, 12 clones were randomly chosen and grown, genomic DNAs were isolated, and the Southern analysis was conducted. The isolates A1, A3, B2, and B3 are the partial null mutants in which one of the two identical copies is disrupted. Clone A11 is a clone in which the *THY1* gene is inserted in a different location in the chromosome. DNA from the parental strain (JH10) is also shown. It has a 2.0-kb *Bgl*II fragment of the *DdTBP*α gene, corresponding to the isolated genomic clone, and a 7.9-kb genomic fragment containing the *THY1* gene. The results for strain A11 show that the *DdTBP*α gene is not disrupted. The results show a 5.2-kb fragment that hybridizes to both the *DdTBP*α probe and the *THY1* probe. This size would be expected if a homologous recombination in a *DdTBP*α gene occurred. For strain A11, an 8.1-kb band hybridizes to both probes. This fragment cannot be explained from the known restriction maps.

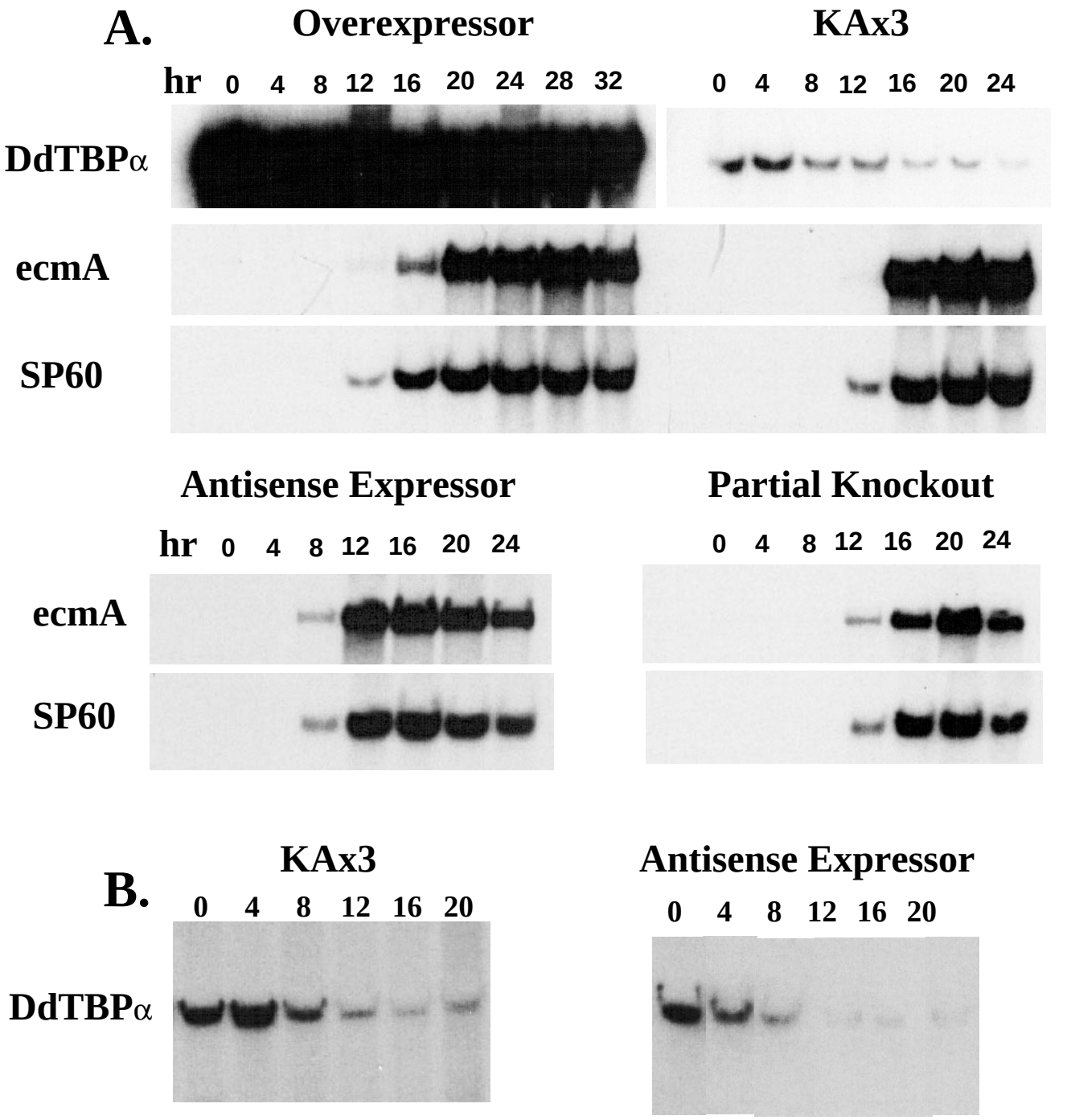


FIG. 3. Developmental expression in wild-type, overexpression, and antisense strains. Total cell RNA was isolated from the strains as indicated in the panels. For synchronous development, cells were plated on filters placed on top of PDF (4a). The probes are indicated on the left sides of the panels. (A) *ecmA* is a prestalk-specific gene. *SP60* is a prespore-specific gene. In the wild-type strain, aggregation was almost complete by 8 h, and a tight aggregate was formed by 12 h. In the overexpressing strain, aggregation occurred between 10 and 18 h, a mound was formed by ~20 h, and mature fruiting bodies were formed by 35 h. (B) Relative levels of *DdTBP*α transcripts in wild-type and hemizygous strains expressing antisense RNA from the discoidin Iγ promoter. Cells were grown in HL5 axenic medium in the presence of 1 mM folate. Cells were washed and plated for development as described above. Equal amounts of the total cell RNA were sized and blotted. The two filters were probed together in the same bag to ensure uniform hybridization conditions.

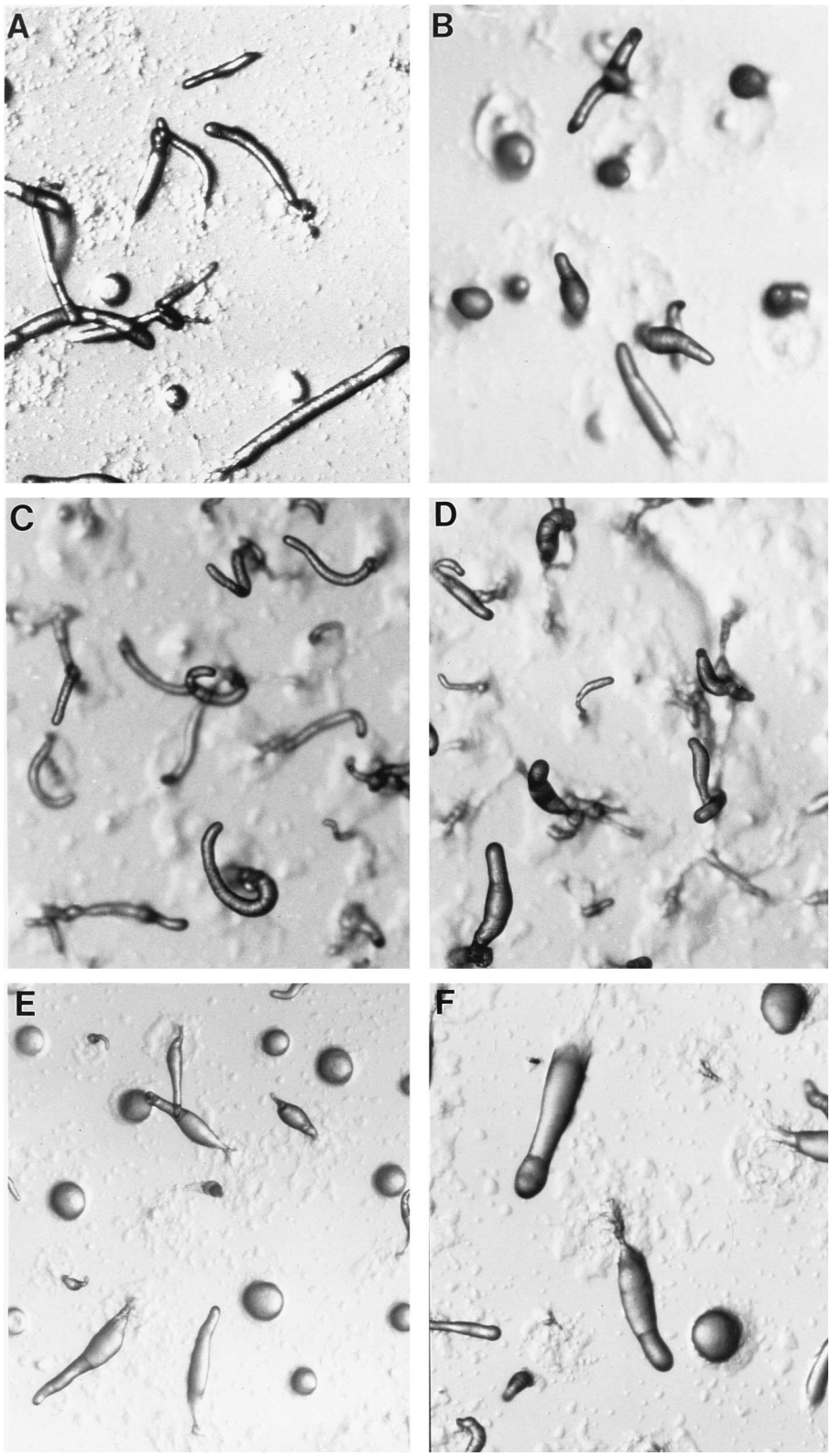
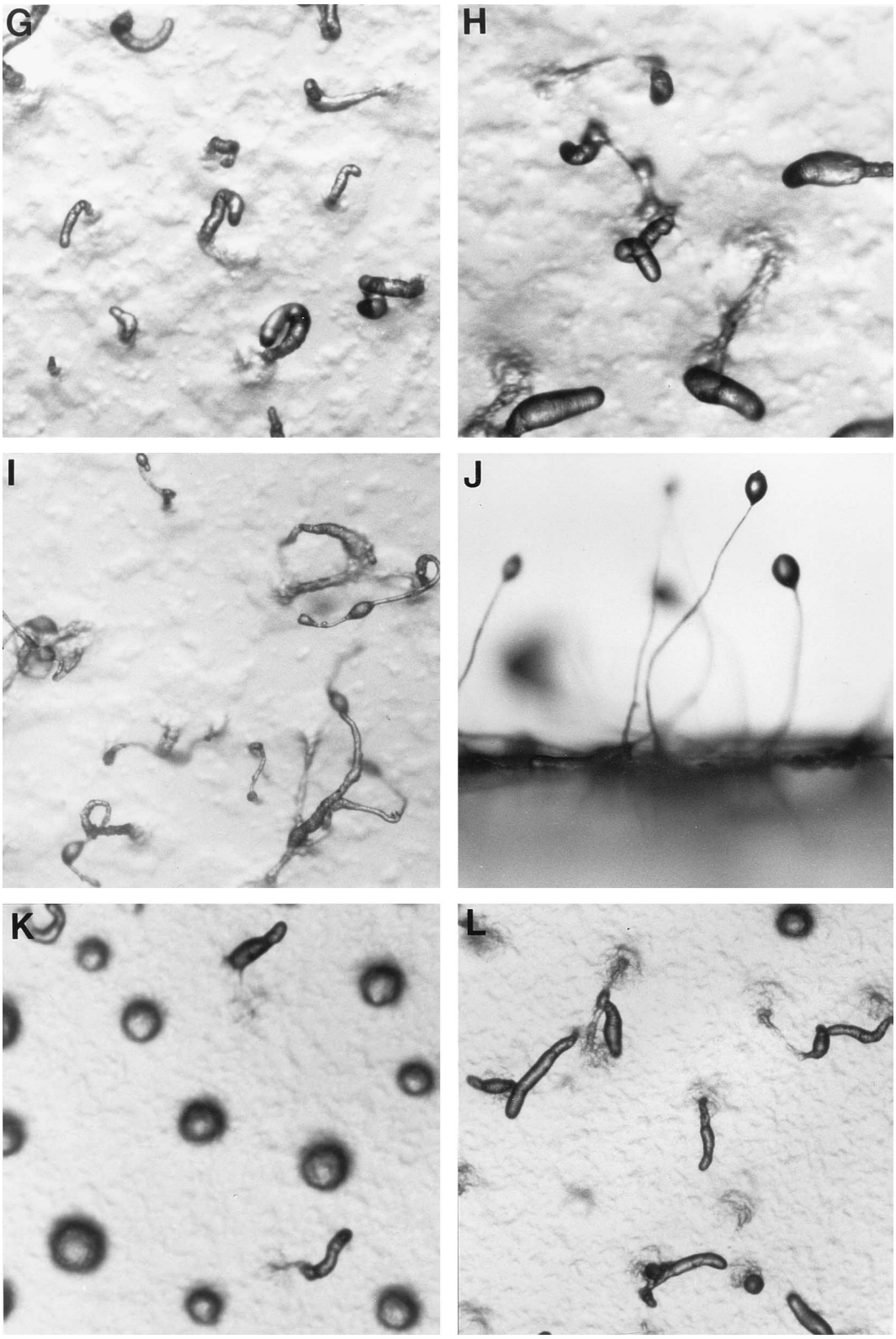


FIG. 4. Morphological phenotypes of the *DdTBP*α mutants. (A, B, and J) (side view) Wild-type cells at 14, 17, and 24 h, respectively; (C and D) partial null mutants at 14 and 17 h, respectively; (E and F) complemented partial null mutants at 14 and 17 h, respectively; (G, H, and I) antisense expressor in the partial null background at 14, 17, and 24 h, respectively; (K and L) overexpressor at 20.5 and 25 h, respectively.



(four independent experiments, 48 individual clones tested), no double knockouts were obtained, although such knockouts were obtained for the gene encoding the phosphotyrosine phosphatase PTP1 at a frequency approximately equal to that for a single knockout event (23). This suggests that a double homologous recombination event for DdTBPα is either very rare or possibly that DdTBPα is required for growth. Expression of DdTBPα was examined by RNA blot hybridization in

hemizygous strains and compared with that in the wild type. In three different experiments with hemizygous strains, the level of expression of DdTBPα was not detectably different from that in the wild-type strain on the basis of a visual comparison of the RNA blots. Densitometric scanning showed a level of expression in the partial null strain that was 1.5- to 2.0-fold lower than that in the wild-type strain (data not shown).

The hemizygous partial knockouts manifested a growth phenotype. The cells were larger than wild-type cells (data not shown), and this feature was used in an attempt to isolate cells in which both copies of the *DdTBP*α genes were disrupted. Electroporated JH10 (*thy1*$^-$ null) cells surviving without exogenous thymidine were plated on nutrient agar plates in the presence of *K. aerogenes* so that clones could be isolated. Under these conditions, the bacterial food source forms a confluent lawn on the plate and each *D. discoideum* cell forms a plaque in the lawn as the bacteria are digested and the cell divides vegetatively. A total of 500 independent clones were picked and resuspended into HL5 axenic growth medium in microtiter wells. Clones containing large cells were identified by phase-contrast microscopy. Fifty clones were analyzed further by Southern blot analysis. Of these, 24 had one of the *DdTBP*α genes disrupted; however, none of the strains had both copies disrupted (data not shown).

To examine morphology during multicellular development, cells were plated on nonnutrient PO_4-buffered agar. The partial knockout mutants formed mounds that were more textured than wild-type mounds, and a significant fraction of cells did not participate in aggregation or formed very small, loose associations of cells that did not proceed further in development (an estimated 20 to 25% of the cells). Aggregates at the first finger stage were curled or sometimes spiral and appeared rough (Fig. 4C), in contrast to the smooth and shining extracellular sheaths of the wild-type first fingers and slugs (Fig. 4A and B). No migrating slugs were formed. The second fingers (Fig. 4D) were also different from those of the wild type (Fig. 4B), but the fruiting bodies appeared to be normal, as in the wild type (data not shown for mutant; wild type shown in Fig. 4J).

The location of the *THY1* gene in the gene disruption construct might result in the expression of a truncated protein containing the N-terminal 167 amino acids, including the basic domain and putative leucine zipper. Such a protein might have partial function or act as a dominant negative mutant that could sequester the intact DdTBPα protein from the second copy of the gene in the genome (see below) or heterodimerize with other TBP1 homologs in *D. discoideum* (38). To determine if a truncated or additional RNA was expressed, RNA blots from the partial knockout were examined. Only the normal DdTBPα transcript was detected (see below; data showing the absence of a detectable, truncated transcript is not shown).

Complementation of the partial null phenotypes by the *DdTBP*α gene. To confirm that the partial null phenotypes were caused by the disruption of one copy of the *DdTBP*α gene, we used the *DdTBP*α gene driven by its own promoter to complement one of the partial null strains. To do this, we isolated a genomic clone (p1; Fig. 2A) which contains ~2.5 kb of the 5′ upstream sequence and the entire DdTBPα coding region. The *DdTBP*α gene most likely does not contain an intron within the coding region, since the *Hae*III-*Pvu*II fragments from the genomic and cDNAs (Fig. 2) containing most of the DdTBPα coding region comigrate on agarose gels (data not shown). A genomic fragment containing 2.5 kb of the 5′ flanking sequence was linked to the cDNA (p6-1) at the *Acc*I site located within the coding region. The gene fusion was subcloned into the expression vector EXP4$^+$ from which the *Act15* promoter was deleted (8). The resulting plasmid was used to transform the partial null strain. Transformants were selected for growth at lower G418 concentrations so that the potential effects caused by high copy vector inserts (see below) would be minimized, and clones carrying five copies of the transformation vector, as determined by Southern blots (data not shown), were chosen for further analysis. As shown in Fig. 4E and F, the developmental phenotypes as well as the growth phenotype (determined by the size of the vegetative cells; data not shown) observed in the partial null mutant were no longer detected, indicating that the phenotypes are the result of knocking out one of two genes.

Antisense expression in the partial null background. The partial null strains have both growth and developmental phenotypes, indicating that the function of DdTBPα is at least partially dependent on the gene dosage. To examine the effects of further reducing the level of *DdTBP*α expression, we made an antisense construct by using the growth-repressible discoidin Iγ promoter (2). The discoidin Iγ promoter is expressed in vegetatively growing cells, but its expression can be repressed by 1 mM folate (2, 44). Removal of the folate results in a derepression of the discoidin Iγ promoter. The antisense construct (see Materials and Methods) was transformed into the strain carrying a disruption of one of the *DdTBP*α genes, and G418-resistant cells were selected in the presence of exogenously added 1 mM folate. To examine the effect of the antisense construct on growth, the exogenously added folate was removed from cells growing on plates by changing the growth medium to HL5 lacking exogenous folate. The cells then died within 2 to 3 days, suggesting that DdTBPα is essential for growth. Cells carrying the plasmid lacking the *DdTBP*α antisense insert grew normally.

The discoidin Iγ promoter is also expressed at high levels upon starvation of the cells during the preaggregation stages of development (34), although its expression is not regulated by folate (2). To examine the potential effects of expressing the *DdTBP*α antisense construct during development, we grew the cells in the presence of 1 mM folate, washed the cells, and then plated them on nonnutrient PO_4-buffered agar. When the developmental phenotype of these cells was examined, a very much higher fraction of the cells (as compared with that of the cells of the partial null mutant) did not participate in development, as shown by a large number of cells on the agar that did not form discrete mounds (see the legends to Fig. 4G and H). In terms of morphology, both the first (Fig. 4G) and second finger (Fig. 4H) stages were abnormal and the resulting fruiting bodies were very aberrant (Fig. 4I). We examined the timing of the expression of a prestalk-specific marker (*ecmA*) and a prespore-specific marker (*SP60*) in the partial knockout strain and in the partial knockout strain expressing the antisense construct. No timing difference was seen in either of these strains by comparison with the timing of wild-type cells, although the level of expression was reduced approximately threefold (data not shown). This reduction was probably due to the lower number of cells participating in development. When different clones were isolated, we saw that ~50% of the cells did not participate in aggregation and we also observed the same morphological phenotypes (data not shown), indicating that these observations were not due to heterogeneity in the population.

To determine the effect of the antisense construct on DdTBPα expression levels, we examined the level of *DdTBP*α mRNA during growth and development in the antisense construct-expressing strain. As shown in Fig. 3B, the antisense strain grown in 1 mM folate shows a slightly reduced level of expression of DdTBPα by comparison with that of the wild-

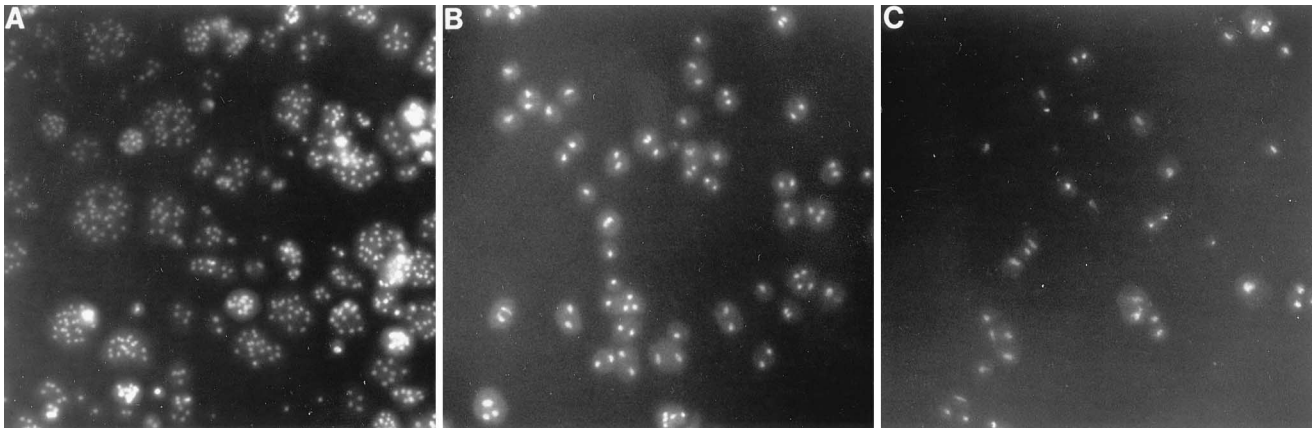


FIG. 5. DAPI staining of nuclei in wild-type and DdTBPα-overexpressing strains. (A) Overexpressor cells growing in suspension shaking culture immediately after being plated on a petri dish. (B) The same population of cells after 20 min. (C) Wild-type cells growing in suspension shaking culture immediately after being plated on a petri dish.

type strain during vegetative growth. However, upon starvation and entry into development, there is a very rapid drop in the level of the transcript, and the level of the transcript in the antisense strain was significantly lower than that in the wild-type control.

Overexpression of DdTBPα results in severe growth and developmental phenotypes. The *DdTBP*α gene was overexpressed in *D. discoideum* from its own promoter, and high-copy-number overexpressors were selected on G418-resistant *E. coli* B/r cells on nutrient plates in the presence of 80 μg of G418 per ml (see Materials and Methods). Figure 3A shows that a high-copy-number clone expressed DdTBPα transcripts at very high levels throughout development. Although they show almost normal growth on tissue culture plates in standard HL5 medium, the DdTBPα overexpression strains showed very abnormal growth in a shaking (suspension) culture. The cells were very large and multinucleated, as determined by DAPI staining (Fig. 5A and 6), suggesting that karyokinesis was normal but cytokinesis was impaired. Interestingly, when the cells were transferred from suspension culture to a tissue culture plate, the cells rapidly attached to the substratum and cytokinesis started immediately (Fig. 7), with the cell number increasing three- to fourfold in 20 min in contrast to the normal doubling time of 8 to 10 h. DAPI staining showed that most of the resulting cells contained two nuclei (Fig. 5B), while most wild-type cells had one to two nuclei (Fig. 5C).

The large-cell, multinucleated phenotype is reminiscent of that of the myosin II heavy chain null or antisense mutant, although not as severe (5, 27). When assayed by RNA blot hybridization, myosin II gene expression in the DdTBPα-overexpressing strains during both growth and development was indistinguishable from that of wild-type cells (data not shown).

The most prominent developmental phenotype resulting from the overexpression of the *DdTBP*α gene was one in which the initial stages of development are significantly delayed. It took ~20 h for the overexpressor to reach the tipped aggregated stage (Fig. 4K) but only about 12 h for the wild-type cells to reach the same stage. Although the overall delay is 8 to 10 h, it appeared that development was only slowed during the early stages, from aggregation through tip formation. After the first finger was formed, the developmental timing was normal, with culmination initiating by 25 h (Fig. 4L) and the formation of mature fruiting bodies containing viable spores by 35 h (data not shown). In terms of morphology, the fingers and slugs had a rough texture, but the fruiting bodies appeared normal.

To gain further insights into the effects of DdTBPα overexpression on development, we examined whether the timing of expression of the prestalk and prespore markers was affected by overexpression of the *DdTBP*α gene. Although morphogenesis was delayed for 8 to 10 h, the expression of the prestalk-specific gene *ecmA* and the prespore-specific gene *SP60* was induced ~4 h after that of wild-type cells (Fig. 3). At that time, the DdTBPα-overexpressing strain was in the late aggregation stage, while expression of these genes is normally first seen at the mound stage in wild-type strains.

DISCUSSION

We have examined the function of DdTBPα, a *D. discoideum* homolog of the human Tat-binding protein TBP1. The human TBP1 is a member of a larger family of eukaryotic proteins that contain a conserved ATPase and are involved in a large number of diverse cellular functions. Recently, members of this family have been shown to include components of the 26S ubiquitin-dependent protease and also to have an effect on transcriptional regulation (see the introduction). Whether this effect on transcription is direct, through the interaction of Tat-binding protein homologs with the transcriptional machinery, or indirect, through effects on protein stability, for example, is unclear. The ability of TBP1, when fused to the GAL4 DNA binding domain, to activate transcription of a GAL_{UAS}-containing promoter suggests that its function could be direct (33). The presence of a possible leucine zipper that is preceded by two short basic domains also suggests that DdTBPα could bind DNA and/or form homo- or heterodimers with other leucine zipper family proteins. Our results did not detect any DNA binding activity; however, we cannot eliminate the possibility that DdTBPα may bind DNA in a sequence-specific manner to a different DNA sequence by itself as a monomer or homodimer or as a heterodimer with another *D. discoideum* factor. If heterodimer formation were essential, we would not have detected DNA binding in our assays. The presence of a putative leucine zipper in DdTBPα, the previous identification of other TBP1 homologs in *D. discoideum*, and the ability of some family members to form heterodimers (see the introduction) suggest that DdTBPα may function in conjunction with other Tat-binding proteins. While genes encoding two other family members have been cloned (38), there is presently no information on their function and on whether their under- and/or overexpression phenotypes are similar to

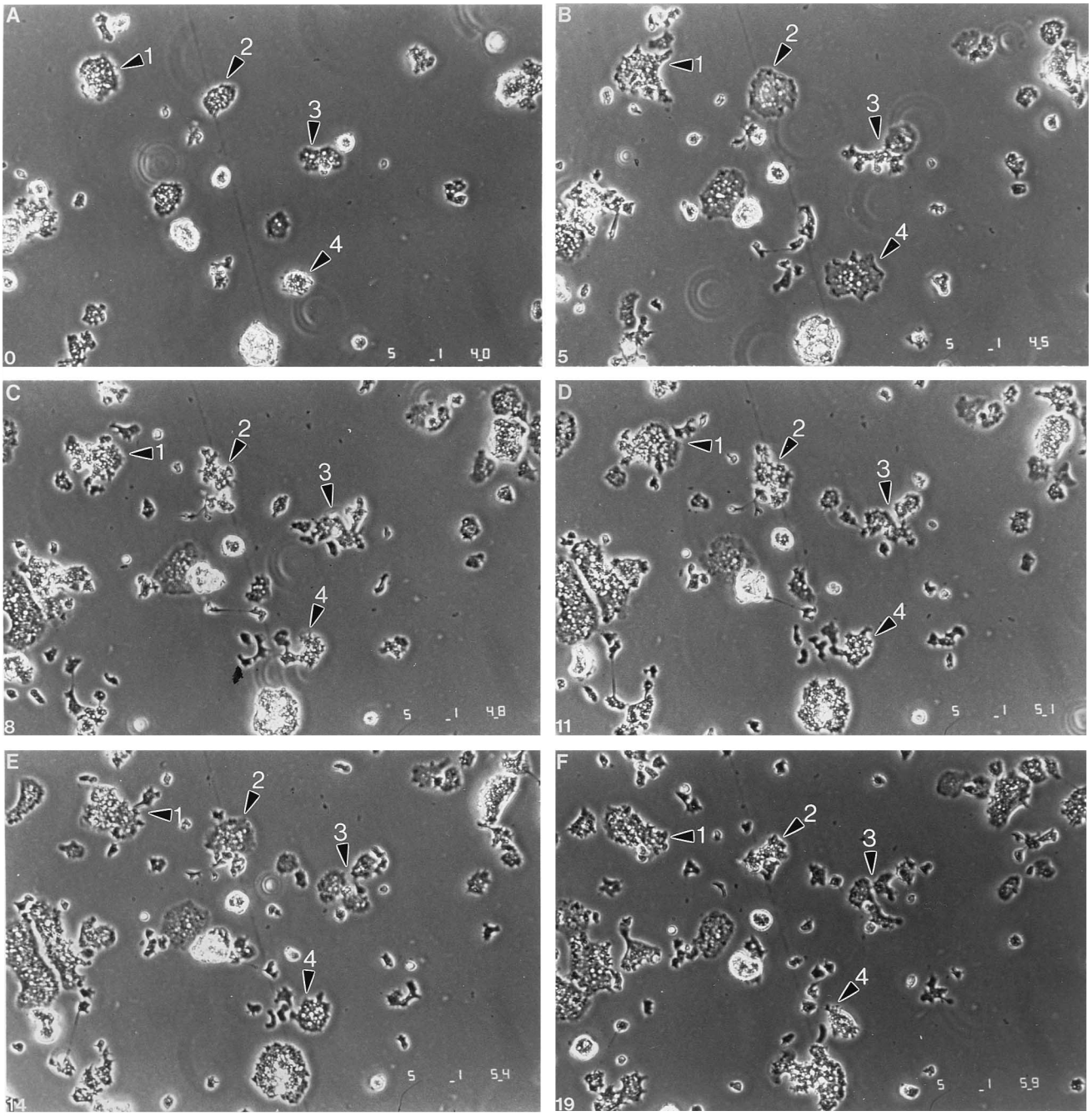


FIG. 6. Kinetics of cell division of wild-type and mutant strains. Photographs of the DdTBPα-overexpressing strain were taken at 0, 5, 8, 11, 14, and 19 min (A through F, respectively). During the first few minutes, additional cells continued to settle. Arrowheads point to large cells that cleaved into multiple small cells over the 19 min recorded. By 19 min, some of the progeny cells had moved away from each other. Times (in minutes) from the start of the experiment (0 min in panel A) are indicated at the lower left corners of the panels. Numbered arrowheads point to individual large cells and their progeny through the course of the experiment. The breaking up of large cells over time can be seen.

those we have described for DdTBPα. If they were similar, it would be an indication that they are involved in regulating the same processes. DdTBPα was isolated by its ability, when expressed in *E. coli*, to increase the level of expression of the spectinomycin gene on a reporter plasmid. It is not clear if this results from the augmentation of the expression of the promoter driving the Spc^r gene or from repression of the expression of the strong promoter that blocks expression of the Spc^r gene.

DdTBPα appears to play an essential role in both growth and multicellular development. DdTBPα transcripts are present maximally during growth and early development, although expression is seen throughout most of multicellular development. Our results suggest that DdTBPα is essential for growth. While we could knock out one of the two *DdTBP*α gene copies, it was not possible to obtain a double knockout. Moreover, when a *DdTBP*α antisense transcript was expressed downstream from the derepressible discoidin Iγ promoter, the

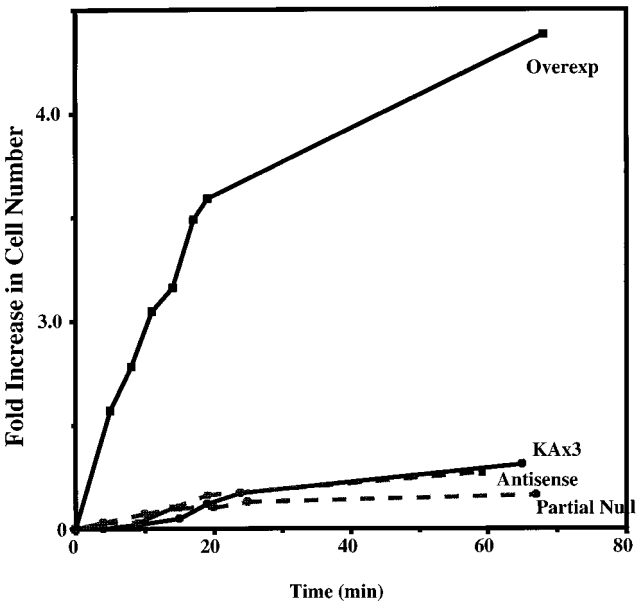


FIG. 7. Cells from the DdTBPα-overexpressing strain, wild-type cells, cells from the partial null strain, and cells from the partial null strain expressing the antisense strain were grown exponentially in shaking cultures to 1×10^6 to 2×10^6 cells per ml, diluted 10-fold, transferred onto tissue culture plates, and allowed 2 min to settle on the bottom (time zero). Pictures were then taken every 2 to 5 min for up to 20 to 25 min, with one last picture taken at 60 to 70 min. The cell numbers were counted and normalized to that at time zero and expressed as the fold increase with time.

cells died after removal of the repressor folate from the medium.

D. discoideum is very sensitive to doses of the *DdTBP*α gene product. The single-gene knockout showed abnormal morphogenesis during multicellular development, starting at the finger stage. The most noticeable aspects of the morphological phenotype were the twisted fingers and the absence of a slug stage. These phenotypes could be complemented by a low-copy-number vector expressing DdTBPα, were strongly accentuated by the antisense construct, and included very abnormal fruiting body formation. The resultant phenotypes suggested that DdTBPα may be important for both aggregation and proper morphogenesis; however, the processes regulated directly by DdTBPα or indirectly through the genes whose expression might be controlled by DdTBPα are not known. In all cases, expression of a prestalk-specific and a prespore-specific gene was normal, suggesting that the abnormal morphogenesis is not due to the inability to induce the individual cell types during multicellular development. The aberrant morphological phenotypes plus the effect of DdTBPα overexpression on cytokinesis suggest that cytoskeletal proteins may be affected in mutant strains.

The most striking phenotype of the overexpressor cells is the one in which there is the almost complete absence of cytokinesis during growth in shaking (suspension) culture and very rapid cytokinesis when the cells are placed on a substratum. The formation of the progeny of the large, multinucleated cells did not appear to be due to amoeboid pulling or stretching of the multinucleated cells, as is observed in the myosin II null and antisense mutants when they are plated on a substratum. In contrast, each large, multinucleated cell appeared to give rise to multiple mono- and dinucleated cells at the same time. The presence of a normal level of myosin II gene expression (data not shown) suggests that the effect is not due to underexpression of myosin II. Since the overexpressing cells divide very poorly in suspension, we assume that a solid substratum is essential for the activation of cytokinesis. Interestingly, conditional mutations of members of this gene family in both *Saccharomyces pombe* and *Saccharomyces cerevisiae* result in mitotic arrest. Our DdTBPα antisense suppressors die, but we did not detect arrest in metaphase. Since cell death in our strains occurred over a period of days, it is possible that the phenotype resulted from a general reduction of ubiquitin-dependent protein degradation in the cell. The formation of large, multinucleated cells is consistent with a role of DdTBPα in mitosis. If DdTBPα is functioning as a component of the 26S protease in causing the observed growth and developmental phenotypes, it is not clear why the overexpression of a single subunit of the protease (which does not have intrinsic proteolytic activity) would result in increased protease activity unless this component is rate limiting. It would be interesting if overexpression of members of this family of proteins would have similar effects on cytokinesis in other cells.

The overexpressor cells also showed a delayed morphological development at the time of aggregation and mound formation. However, when prestalk- and prespore-specific gene expression was assayed, the DdTBPα-overexpressing cells induced cell-type-specific gene expression prior to the mound stage, although this induction was delayed by comparison with that of the wild type. Expression of both the prestalk-specific *ecmA* gene and the prespore-specific *SP60* gene requires the transcription factor GBF that is regulated by high cAMP levels found in the mound (36a, 37). The induction of these genes prior to mound formation might suggest that the overexpressing cells have increased cAMP signaling earlier, relative to the morphological development. The mechanism by which DdTBPα overexpression affects the precocious induction of the cell-type-specific genes will require a more detailed understanding of the mechanisms by which the cAMP signaling pathway mediates GBF function.

The Tat-binding proteins appear to be a diverse family of proteins which affect multiple cellular pathways. *D. discoideum* may represent an experimentally manipulatable system in which to dissect the processes by which these proteins control cellular function.

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