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Simian Virus 40 Large T Antigen Affects the *Saccharomyces cerevisiae* Cell Cycle and Interacts with p34^{CDC28}

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Simian virus 40 tumor (T) antigen, an established viral oncoprotein, causes alterations in cell growth control through interacting with, and altering the function of, cellular proteins. To examine the effects of T antigen on cell growth control, and to identify the cellular proteins with which it may functionally interact, T antigen was expressed in the budding yeast *Saccharomyces cerevisiae*. The yeast cells expressing T antigen showed morphological alterations as well as growth inhibition attributable, at least in part, to a lag in progression from G_1 to S. This point in the cell cycle is also known to be affected by T antigen in mammalian cells. Both p34^{CDC28} and p34^{CDC2Hs} were shown to bind to a chimeric T antigen–glutathione S-transferase fusion protein, indicating that T antigen interacts directly with cell cycle proteins which control the G_1 to S transition. This interaction was confirmed by in vivo cross-linking experiments, in which T antigen and p34^{CDC28} were coimmunoprecipitated from extracts of T-antigen-expressing yeast cells. These immunoprecipitated complexes could phosphorylate histone H1, indicating that kinase activity was retained. In addition, in autophosphorylation reactions, the complexes phosphorylated a novel 60-kDa protein which appeared to be underphosphorylated (or underrepresented) in p34^{CDC28}-containing complexes from cells which did not express T antigen. These results suggest that T antigen interacts with p34^{CDC28} and alters the kinase function of p34^{CDC28}-containing complexes. These events correlate with alterations in the yeast cell cycle at the G_1 to S transition.

The large tumor (T) antigen of simian virus 40 (SV40) is a potent oncoprotein whose effects are mediated through direct interactions with cellular proteins affecting transcription, DNA replication, and the cell cycle, particularly the entry of cells into the mitotic cycle (5, 29, 30). T antigen interacts with at least two cellular proteins which are involved in negative regulation of cell proliferation, p53 and the retinoblastoma (Rb) protein (4, 13, 16, 32). These interactions with p53 and Rb suggest a mechanism by which T antigen can abrogate cell cycle control as a part of the transformed phenotype.

Definitive identification of the mechanisms by which T antigen mediates its pleiotropic effects, especially its effects on the cell cycle, requires the use of genetic approaches. Such approaches have been quite fruitful in determining the mechanisms of cell cycle control in the yeast *Saccharomyces cerevisiae* (reviewed in reference 22). The remarkable conservation of key cell cycle regulators between *S. cerevisiae* and higher eukaryotes has facilitated the discovery of homologous mammalian cell cycle components (12, 14, 16, 34).

In the budding yeast *S. cerevisiae*, the G_1 to S phase transition is controlled by the product of the *CDC28* gene, p34^{CDC28} (24, 33), a 34-kDa protein kinase which is homologous to p34^{CDC2}, the catalytic subunit of the mammalian mitosis-promoting factor (reviewed in reference 23). The activity of the p34^{CDC28} kinase in G_1 is believed to be regulated by the G_1 cyclins CLN1, CLN2, and CLN3 (3, 10). The in vivo substrates of this kinase remain unknown (23).

Interestingly, the human p34^{CDC2} protein kinase phosphorylates SV40 large T antigen in vitro, at a site which regulates the ability of T antigen to support in vitro replication of SV40 DNA (18). An in vivo interaction between the two proteins has been suggested by the data of Adamczewski et al. (1). That there may be a functional significance to the interaction is indicated by data showing that terminally differentiated myotubes (which have withdrawn from the cell cycle) expressing a temperature-sensitive mutant of T antigen are able to reinduce p34^{CDC2} kinase activity when the cells are grown at the permissive temperature for T-antigen activity (9).

In this report, we have demonstrated that SV40 large T antigen affects the G_1 to S phase transition in *S. cerevisiae* and that T antigen interacts, in vivo, with p34^{CDC28}. Complexes immunoprecipitated with anti p34^{CDC28} from cells containing T antigen have altered kinase function compared with similar complexes immunoprecipitated from normal cells. That T antigen may alter the kinase function of a complex containing a known cell cycle regulator suggests a possible mechanism for the observed T-antigen-induced phenotypes.

MATERIALS AND METHODS

Strains, media, and growth conditions. All yeast strains in this study were derivatives of BF264-15DU (15DU; *matα ade1 his2 leu2-3 trp1-1a*) (25) except that strain X100 was used for the experiment shown in Fig. 2. The relevant genotype for X100 is *mat***a** *leu2 ura3*; no other markers were determined. DU13-cdc28ts is a congenic derivative of 15DU which contains the temperature-sensitive allele *cdc28-13* (7).

All cultures were grown in 2% raffinose minimal medium supplemented with amino acids but lacking uracil or leucine as necessary. For induction of expression from the *GAL10* promoter, the medium was supplemented with 2% galactose unless otherwise indicated.

Plasmids. YEcT#2 expresses large T antigen from the *GAL10* promoter of YEp51, and both plasmids were a generous gift of D. McVey and Y. Gluzman. YEpG2[CDC28] and YEpG2[CDC2Hs] express Cdc28 and human Cdc2, respectively, from the *GAL1* promoter (28). For the in vitro transcription-translation experiments, CDC28 and CDC2Hs were excised from the YEpG2 vectors by *Bam*HI, and each gene was cloned into the unique *Bam*HI site of pGEM3Zf-(Promega) to generate pGEM3Zf-[CDC28] and pGEM3Zf-[CDC2Hs]. The genes were inserted in both orientations so that they could be transcribed by either the T7 or SP6 polymerase. pAB23BXN-T expresses T antigen constitu-

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tively from the glyceraldehyde phosphate dehydrogenase (GAPDH) promoter. It was constructed by inserting a cDNA copy of the gene for T antigen into the *Bgl*II site of pAB23BXN (27). The glutathione *S*-transferase (GST)–T antigen fusion recombinants have been previously described (8).

Transformations. Transformations were performed essentially as described by Schiestl and Geitz (26). Briefly, overnight cultures grown in YPD (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% dextrose) were diluted to approximately 5×10^6 cells per ml and grown for 4 h at 30°C (two doublings). The cells were collected by centrifugation and washed once with sterile water. The cells were resuspended in 1.5 ml of sterile 1× TE plus 1× lithium acetate (LiAc) (made fresh from 10× sterile stocks; 10× TE is 0.1 M Tris-HCl [pH 7.5] plus 0.01 M EDTA, and 10× LiAc is 1 M LiAc [pH 7.5]) and incubated for 1 h at 30°C on a roller. Two hundred microliters of cells was incubated with 5 to 10 μg of plasmid DNA and 200 μg of denatured sonicated salmon sperm carrier DNA for 1 h at 30°C on a roller; 1.2 ml of sterile 40% polyethylene glycol 3350 in 1× TE plus 1× LiAc (made fresh from 50% polyethylene glycol stock, 10× TE, and 10× LiAc) was added, and the suspension was incubated for 30 min at 30°C on a roller. The cells were heat shocked at 42°C for 15 min, collected by centrifugation, washed twice with sterile 1× TE and once with YPD, and finally resuspended in 200 μl of YPD. The cells were spread on selective agar made from synthetic medium with glucose as the carbon source and the appropriate amino acid supplements.

Growth kinetics. Where indicated, overnight cultures, grown in raffinose, were diluted to an A_{600} of 0.05 to 0.1 with medium containing 2% raffinose and 2% galactose. Cells were grown at 30°C unless otherwise noted. At various intervals, 1 ml of culture was collected and the A_{600} was measured, or the cell number was determined by counting a 1:100 dilution of cells on a Hy-Lite hemacytometer. For the DU13-cdc28ts growth kinetics experiments, cells were grown at 23°C (permissive) and 37°C (nonpermissive) as specified. Duplicates were done for each time point, and the standard errors were determined.

Cross-linking. Cultures were first grown overnight in glucose, then diluted into raffinose at 1:10 (A_{600} of ≈0.2), and grown overnight again. Cultures were diluted to an A_{600} of ≈0.1 in 7 ml of raffinose minimal medium with 2% galactose and grown for 16 h at 30°C on a roller. The cells were pelleted, washed with 1× phosphate-buffered saline (PBS), and resuspended in 100 μl of PBS; 20 mM dithiobis(succinimidylpropionate) (DSP; Pierce), dissolved in dimethyl sulfoxide, was added to a final concentration of 0.4 mM, and the mixture was incubated at room temperature for up to 30 min. Because the cross-linking reagent must first penetrate the cell wall before acting in the cytoplasm, the optimal cross-linking time varied in each experiment depending on the density of the cell pellet. Therefore, for each experiment, a time course of cross-linking was done to ensure a point of optimal cross-linking. Caution was taken not to cross-link the cells excessively, which results in attachment of proteins to the cell membrane and loss by centrifugation during preparation of cell lysates. The cross-linking reaction was stopped by adding Tris-HCl to a final concentration of 50 mM (pH 8). Cells were pelleted, washed with PBS, and resuspended in 1 ml of lysis buffer to make protein extracts as described below.

Preparation of extracts. Total soluble protein was prepared from cells as described previously (19), with minor modifications. All procedures were performed on ice or at 4°C. Cells were harvested by centrifugation and washed once in ice-cold water. Cells were resuspended with 2 pellet volumes of cold lysis buffer (1% sodium deoxycholate, 1% Triton X-100, 50 mM Tris-HCl [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], 1 mM sodium pyrophosphate) plus protease inhibitors (1 mM each phenylmethylsulfonyl fluoride and *N*-α-*p*-tosyl-L-lysine chloromethylketone and 8 μg each of pepstatin A and leupeptin [both from Boehringer Mannheim] per ml); 1.5 g of acid-washed glass beads (425 to 600 μm) was added. The mixture was subjected to four cycles of vortexing for 30 s and cooling on ice for 30 s. The lysates were clarified by centrifugation at 17,000 × *g* for 15 min. The protein content of the supernatant was determined using the Bio-Rad protein assay as instructed by the manufacturer.

Immunoprecipitations. One hundred micrograms of total soluble protein was incubated with 15 μl of PAb419, a monoclonal antibody against T antigen provided by M. Bradley, or with 2 μl of anti-p34^{CDC28} serum 9406 (19) for 1 h on ice; 30 μl of a suspension of protein A-Sepharose (Pharmacia) at 100 mg/ml in lysis buffer was added, and the samples were incubated on a rocking platform for 1 h at 4°C. The beads were sedimented and washed three times in lysis buffer containing 300 mM NaCl and then washed once with 50 mM Tris-HCl (pH 7.2). The samples were prepared for electrophoresis by adding an equal volume of 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiling the mixture for 5 min before loading it on an SDS–12% (30:0.8 acrylamide/bisacrylamide) polyacrylamide gel with a 4% stacking gel.

Gel electrophoresis and immunoblotting. Proteins were separated on SDS-polyacrylamide gels as described above and transferred to nitrocellulose (Amersham) as previously described (2). Proteins were detected by incubation with a monoclonal antibody which recognizes the amino-terminal end of T antigen (PAb419) and/or a monoclonal antibody which recognizes the PSTAIRE motif of the Cdk family of proteins (generous gift of M. Yamashita and Y. Nagahama). The proteins were visualized by using an enhanced chemiluminescence system (Amersham) as described by the manufacturer.

Protein kinase assays. Cultures were induced and cross-linked as described above. Protein extracts were prepared, immunoprecipitated with 2 μl of anti-p34^{CDC28} serum, and washed as described above. The protein kinase assays were performed as described previously (33).

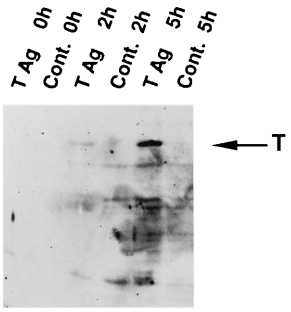

FIG. 1. Immunoblot of extracts from yeast cells containing plasmid YEcT#2, which expresses T antigen (T Ag), or the control plasmid, YEp51 (Cont.). Extracts were made at 0, 2, and 5 h after induction with 2% galactose. The proteins were separated on an SDS–12% polyacrylamide gel. The blot was probed with monoclonal antibody PAb419.

Expression of GST fusion proteins and in vitro binding assays. GST-T antigen fusion proteins were expressed and purified as described by Gruda et al. (8). Fusion proteins were checked for quantity and quality on silver-stained gels. Equivalent amounts were immobilized on glutathione-agarose beads and incubated with in vitro-transcribed and -translated p34^{CDC28} or p34^{CDC2Hs} according to established protocols (8).

RESULTS

Expression of SV40 large T antigen causes morphological changes in *S. cerevisiae*. SV40 large T antigen was subcloned downstream of the *GAL10*-inducible promoter on YEcT#2, a high-copy-number 2μm plasmid (gift of D. McVey and Y. Gluzman). This plasmid was introduced into *S. cerevisiae* 15DU, and T-antigen expression was induced by adding 2% galactose to the growth medium. As a control, cells were transformed with the parent plasmid lacking the T-antigen cDNA (YEp51). Expression of full-length T antigen was confirmed by Western blot (immunoblot) assays (Fig. 1); T-antigen protein is detectable by 2 h after induction. As T antigen was expressed and accumulated, up to 20% of the cells showed altered morphologies (Fig. 2), whereas control cells grown in 2% galactose were unchanged. Immunofluorescence analysis showed that there was a direct correlation between cells with the greatest nuclear staining for T antigen and morphologically altered cells (data not shown). It is likely that this is due to higher copy numbers of the T-antigen-expressing plasmid in these cells. Interestingly, the morphologically altered cells remained viable. When individual cells were isolated by micromanipulation onto agar dishes containing either glucose or galactose as a carbon source, the cells grew into normal-size colonies. After such growth on galactose, up to 20% of the resultant cells again showed the characteristic T-antigen-induced morphological phenotype (data not shown).

Expression of SV40 large T antigen inhibits growth in *S. cerevisiae*. The effects of T antigen on growth kinetics were examined by first culturing cells for 12 h in selective glucose-containing medium, then diluted into selective raffinose medium, and again grown for 12 h. These overnight cultures were diluted to mid-log phase in raffinose and induced with 2% galactose. At various times after dilution and induction, growth of the cells was measured by counting cells. (Similar growth kinetics were observed with measurement by A_{600}; for example, see Fig. 8.) Figure 3 shows a typical growth curve beginning after induction. We routinely observed that cell growth was inhibited beginning approximately 3 to 4 h after the in-

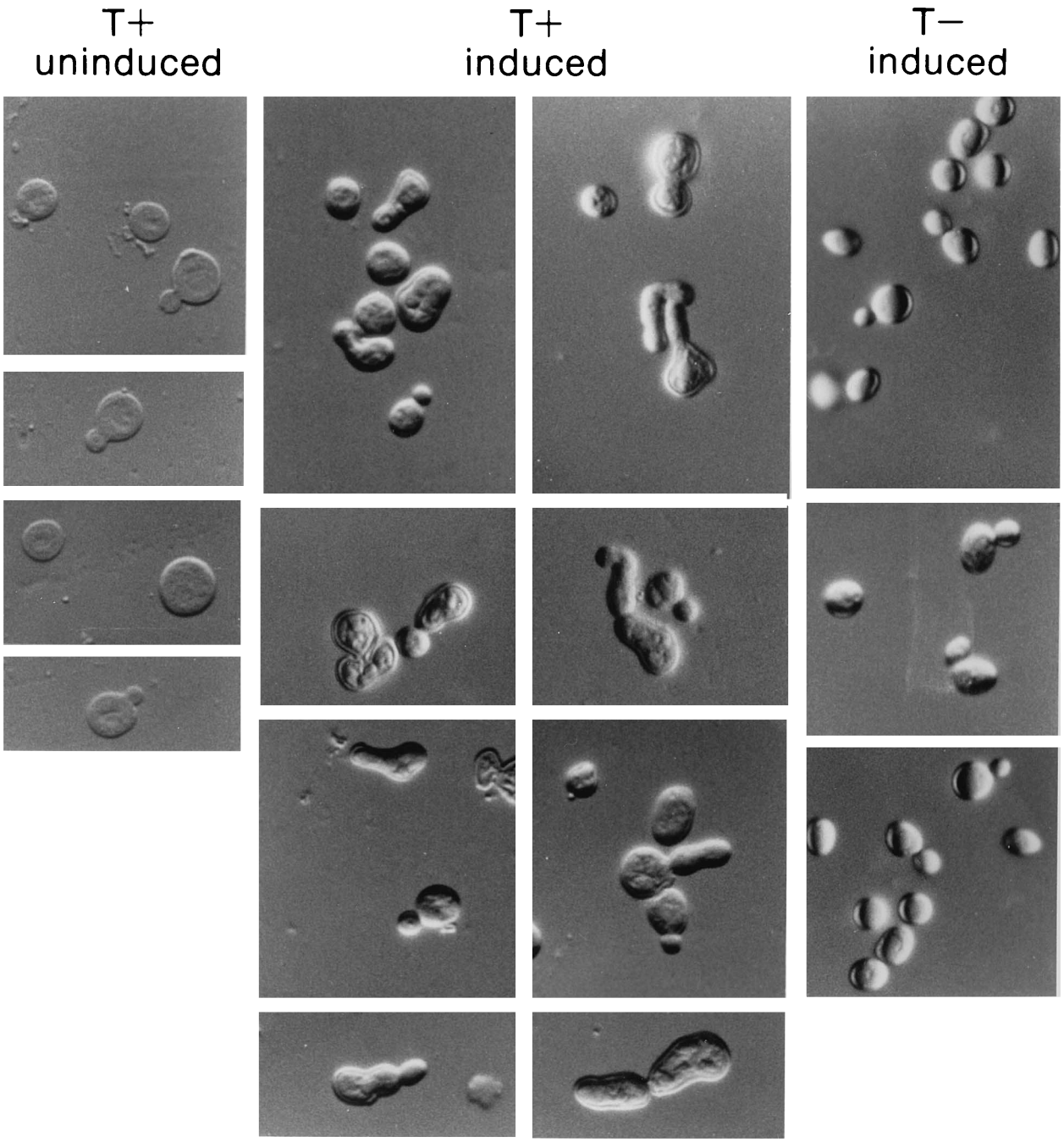

FIG. 2. Morphology of *S. cerevisiae* cells expressing SV40 T antigen. Cells were transformed with a plasmid carrying a galactose-inducible T-antigen gene (YEcT#2; T+) or a control plasmid (YEp51; T−). T-antigen expression was induced with 2% galactose as described in Materials and Methods.

duction of T antigen. This is coincident with the first appearance of the altered morphology described above.

SV40 T antigen causes a lag in progression from G_1 to S phase. To determine if T antigen was causing a lag in a particular phase of the cell cycle, the cells were synchronized in G_1 and growth kinetics were analyzed as the cells were released from the G_1 block. A congenic strain carrying a temperature-sensitive mutation in the *CDC28* gene (DU13-cdc28ts) was used to synchronize the cells at START (19). Specifically, DU13-cdc28ts, transformed with YEcT#2 (a galactose-inducible T-antigen-expressing plasmid) or with a matched control plasmid (containing no T-antigen gene), was grown in selective raffinose medium at the permissive temperature for 12 h. The cells were diluted to mid-log phase in raffinose containing medium, induced with 2% galactose, and grown at the permissive temperature (23°C) for 3 h to allow a pool of T-antigen protein to build up in the cells before shifting the cells to the nonpermissive temperature (37°C) for 5 h. The asterisk in Fig. 4 indicates the point of temperature shift. Growth was measured by A_{600}. At the end of the 5-h incubation, approximately 70% of the cells were arrested in G_1, as determined by the characteristic unbudded shmoo morphology (19), and deter-

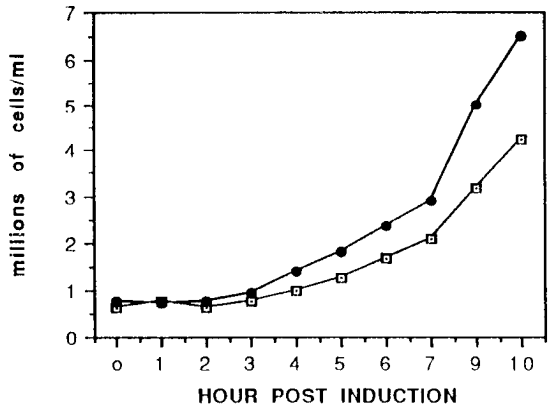

FIG. 3. Growth curve of cells expressing T antigen. Cells were grown for 12 h in glucose medium before being diluted 1:10 in raffinose medium. After 12 h, cells were again diluted 1:10 in raffinose and induced with 2% galactose. For 10 h following induction, samples were taken for cell counts. The graph shows growth curves, based on cell counts, following galactose induction for cultures expressing T antigen (open squares) and control cultures (closed circles).

mination by cell counting indicated that approximately 80% of the cells were arrested (data not shown). After this period, the cultures were shifted back to the permissive temperature; this point is indicated by the delta in Fig. 4. The data in Fig. 4 show that the kinetics of emergence from the G_1 block (starting at the 8-h time point) was altered and slower for the cells expressing T antigen. The T-antigen-expressing cells exhibited little or no change in A_{600} for the first 40 min after release; the A_{600} then began to increase at a slower rate than the control cells. Ideally there should be no apparent increase in the A_{600} reading after the temperature shift initiating the arrest in G_1. However, both the control cells and the T-antigen-producing cells form schmoos in response to the *cdc28ts* arrest; in addition, the T-antigen-containing cells undergo induced morphological changes. Such changes in cell mass and shape increase the A_{600} reading and account for the increase noted between 0 and 8 h in Fig. 4. As mentioned above, examination of cell numbers confirmed that at least 80% of the cells were arrested.

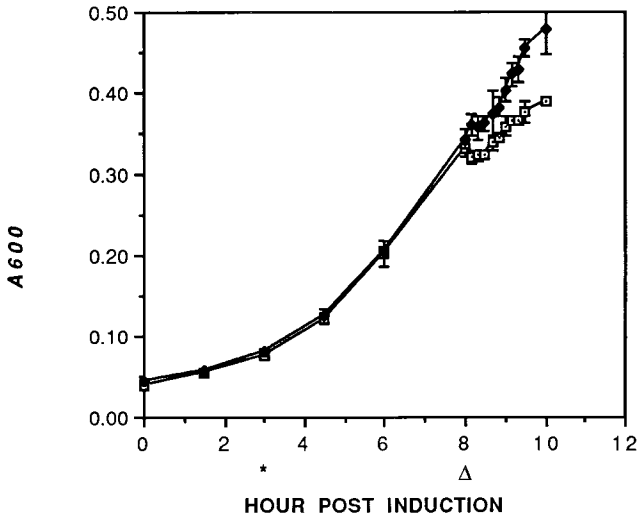

FIG. 4. Growth curves of synchronized cells expressing T antigen. DU13-cdc28ts cells expressing T antigen (open squares) and DU13 control cells lacking T antigen (closed circles) were grown for 12 h in raffinose medium at the permissive temperature (23°C). Cells were diluted 1:10 and induced with 2% galactose. Cells were grown at 23°C for 3 h, shifted to the nonpermissive temperature (37°C) for 5 h to synchronize the cells in G_1, and then released from G_1 by being shifted back to 23°C. Samples were taken at various intervals to measure A_{600}. The asterisk indicates when cells were shifted to 37°C, and the delta indicates when the cells were shifted back to 23°C. Samples were done in duplicate.

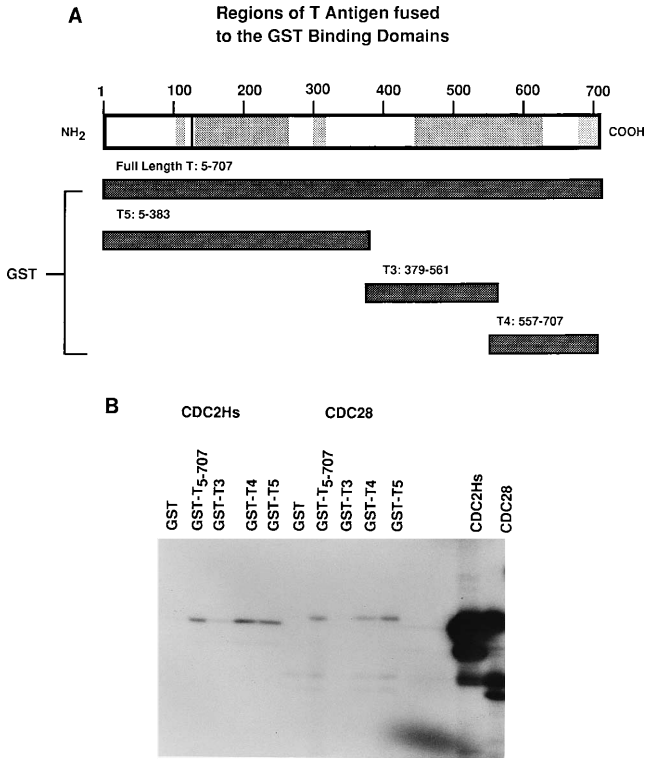

FIG. 5. Binding of in vitro-transcribed and -translated p34^{CDC2Hs} and p34^{CDC28} to GST-T antigen fusion proteins. (A) Regions of T antigen fused to the glutathione binding domain of GST as previously described (8). The numbers next to the name of each region correspond to the included amino acids. The shaded regions on the full-length diagram of T antigen represent, from left to right, the Rb binding domain (amino acids 102 to 115), the DNA binding domain (amino acids 131 to 259), the putative zinc finger (amino acids 302 to 320), the ATPase domain (amino acids 418 to 627), and the host range region (amino acids 682 to 708); the black bar indicates the nuclear localization signal (amino acids 126 to 132) (5). (B) In vitro-transcribed and -translated [^{35}S]methionine-labeled p34^{CDC2Hs} and p34^{CDC28} bound to the GST binding site only (GST), full-length T antigen (GST-T_{5-707}), T3 (GST-T3), T4 (GST-T4), and T5 (GST-T5). The last two lanes show the in vitro-translated products as markers.

The same cells were also examined to determine the percentage which were budding, a measure of progression from G_1 to S phase (reviewed in reference 22). In agreement with the reduced growth kinetics, the cells expressing T antigen showed a lag in the ability to form buds compared with the control cells (data not shown). The lag lasted about 40 min after release from the G_1 block, and then the cells began to bud. These data indicate that T antigen slows the progression of *S. cerevisiae* cells from G_1 to S phase.

SV40 T antigen binds to p34^{CDC28} and p34^{CDC2Hs} in vitro. Given the ability of T antigen to bind to proteins involved in cell growth control, and given the essential role that p34^{CDC28} plays in the G_1 to S transition, we examined the ability of T antigen to interact with p34^{CDC28} in vitro. We used fusion proteins consisting of full-length T antigen, as well as various fragments of T antigen, fused to the glutathione binding domain of GST (6, 8, 20) (Fig. 5A). All of the fusion proteins are expressed well in *Escherichia coli* (8), although the fragments are produced at higher levels than full-length T antigen as seen

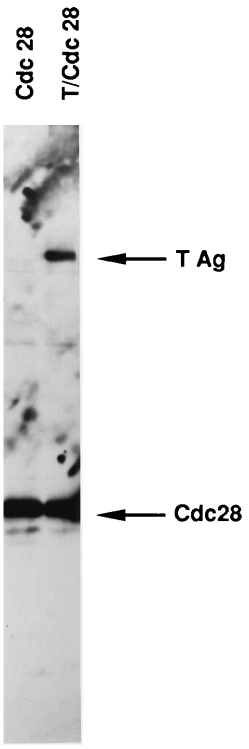

FIG. 6. Immunoblot of lysates from cells which constitutively expressed T antigen (T Ag) and overexpressed p34^{CDC28}. The lane marked Cdc28 represents cells transformed with the control plasmid (pAB23BXN) and YEpG2[CDC28], induced with 2% galactose. These cells overexpress Cdc28 only. The lane marked T/Cdc28 represents cells transformed with the plasmid constitutively expressing T antigen (pAB23BXN-T) and YEpG2[CDC28], induced with 2% galactose. These cells should express T antigen as well as overexpress Cdc28. The blot was probed with PAb419 (anti-T antigen) and with anti-PSTAIRE for p34^{CDC28}.

previously (8). The fusion proteins bound to glutathione-agarose beads were incubated with in vitro-transcribed and -translated [^{35}S]methionine labeled p34^{CDC28} and p34^{CDC2Hs} proteins. After extensive washing, bound proteins were eluted and electrophoretically separated on SDS-polyacrylamide gels. Figure 5B shows that full-length T antigen (GST-T_{5-707}) binds both p34^{CDC28} and p34^{CDC2Hs} under these conditions. Two fragments of T antigen were also found to bind these proteins, the amino-terminal 383 amino acids (GST-T5) and the carboxy-terminal 150 amino acids (GST-T4). Given the complexity of the T-antigen protein, it is not surprising to find more than one domain involved in binding. Amino acids 379 to 561 were not significantly involved in binding to either protein (GST-T3).

SV40 T antigen binds p34^{CDC28} in vivo. To determine whether T antigen physically interacts with p34^{CDC28} in vivo, we tested whether antisera directed against SV40 T antigen could coimmunoprecipitate the p34^{CDC28} protein from yeast cell extracts. For these experiments, we cotransformed cells with a plasmid that overexpresses p34^{CDC28} when induced with galactose [YEpG2(CDC28)] and a plasmid that expresses T antigen constitutively from the GAPDH promoter (pAB23 BXN-T); as a control, cells were transformed with the parent vector without the T-antigen coding sequences. Expression of both proteins was confirmed by Western blot analysis (Fig. 6). Both extracts have overexpressed p34^{CDC28}, but only one expresses T antigen constitutively (lane T/Cdc28). The cells were treated with the cross-linking reagent DSP for 0 to 30 min before extracts were prepared under nondenaturing conditions (see Materials and Methods) and immunoprecipitated with PAb419, a monoclonal antibody directed against T antigen. Immunoprecipitates were boiled in the presence of SDS and 2-mercaptoethanol to reverse the cross-linking, and the resultant proteins were separated by SDS-PAGE. The proteins were transferred to nitrocellulose and immunoblotted for the presence of p34^{CDC28}, using an antiserum directed against the PSTAIRE motif of p34^{CDC28}. As shown in Fig. 7A, after cross-linking for 30 min, p34^{CDC28} is coprecipitated with the T-antigen polypeptide (Fig. 7A, lane 6). In the absence of T-antigen expression, low background levels of p34^{CDC28} are detected (lanes 1 to 3). This is apparently due to a combination of the very high levels of expression of CDC28 in these cells and a low level of cross-reactivity with the anti-T antibody. The identity of the large band migrating at approximately 60 kDa is not known. However, a 60-kDa protein has been previously detected with use of PSTAIRE antibodies and may be in complex with CDC28 (22a).

The converse experiment was done with an antiserum against the p34^{CDC28} protein to immunoprecipitate the cross-linked complex and immunoblotting with PAb419 to detect T antigen (Fig. 7B). No specific bands are detected with the PAb419 antiserum in extracts from cells which overexpress p34^{CDC28} only (lane 1). However, a 97-kDa band is clearly visible in the extracts in which T antigen is constitutively expressed in the presence of overexpressed p34^{CDC28} (lane 2). Coprecipitation of p34^{CDC28} with T antigen has been detected in the absence of cross-linking; however, cross-linking greatly increases detection (data not shown).

Overexpression of p34^{CDC28} does not rescue the T-antigen phenotypes. One hypothesis from the foregoing results is that the interaction of T antigen with p34^{CDC28} abrogates its normal function, thus giving rise to inhibition of growth and morphological changes. If this is the case, overexpression of p34^{CDC28} should reverse or lessen the T-antigen phenotype. To test this, p34^{CDC28} was overexpressed from a galactose-inducible plasmid (YEpG2[CDC28]) either alone or in the presence of constitutively produced T antigen, and growth kinetics and morphology of the cells were analyzed.

Overexpression of p34^{CDC28} did not rescue the T-antigen-induced growth inhibition. Figure 8 shows the growth kinetics of cells constitutively expressing T antigen in either the presence or absence of overexpressed p34^{CDC28}. The cells carrying the control constitutive plasmid [pAB23BXN] and the plasmid which overexpresses p34^{CDC28} (BXN/28) grew at the same rate as wild-type cells. Thus overexpression of p34^{CDC28} had no effect on growth rate. Cells expressing T antigen with normal levels of p34^{CDC28} showed the expected inhibition of growth, in agreement with the data in Fig. 3. Surprisingly, cells expressing T antigen with overexpressed p34^{CDC28} showed even greater growth inhibition. Thus, overexpressing p34^{CDC28} does not reverse the effects of T antigen on growth; rather, it exacerbates the phenotype. The morphologies of the cells expressing T antigen and overexpressing p34^{CDC28} were also consistent with an inability of overexpressed p34^{CDC28} to rescue the T-antigen effect. Within 5 h of induction, cells expressing both proteins showed the characteristic T-antigen-induced morphologies (data not shown). Overall, the inability of overexpressed p34^{CDC28} protein to overcome the morphological and growth phenotypes induced by T antigen is consistent with a mechanism whereby T antigen does not act simply by titrating out the available p34^{CDC28} protein but alters the function of complexes containing it and p34^{CDC28}.

The SV40 T antigen-p34^{CDC28} complex has altered kinase function. To test whether the T antigen-p34^{CDC28} complex had altered enzymatic function, we examined the kinase potential

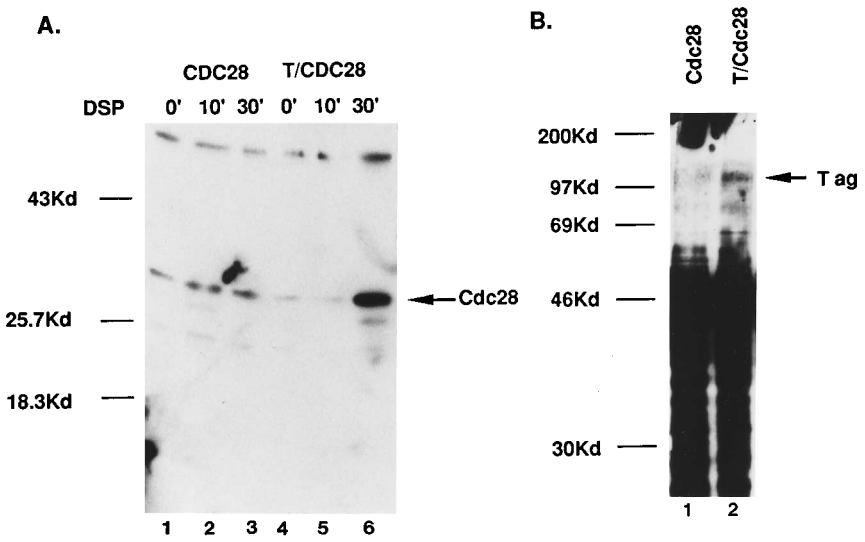

FIG. 7. Immunoblot of immunoprecipitated cross-linked lysates from cells overexpressing p34^{CDC28} either with or without T antigen. (A) Cells were transformed with pAB23BXN (control plasmid) and YEpG2[CDC28] (galactose-inducible p34^{CDC28}) (lanes 1 to 3) or pAB23BXN-T and YEpG2[CDC28] (lanes 4 to 6). Cells were cross-linked with DSP for various times (0, 10, or 30 min [′]) before lysates were prepared (see Materials and Methods). Lysates were immunoprecipitated with PAb419 (anti-T antigen) and separated by electrophoresis on an SDS–12% polyacrylamide gel. The immunoblot was probed for p34^{CDC28} with anti-PSTAIRE. (B) Cells transformed with pAB23BXN (control) and YEpG2[CDC28] (lane 1) or with pAB23BXN-T and YEpG2[CDC28] (lane 2) were cross-linked for 10 min before lysates were prepared. Lysates were immunoprecipitated with anti-p34^{CDC28} antisera and electrophoresed. Immunoblot was probed with PAb419 (anti-T antigen).

of p34^{CDC28} immunoprecipitates from cell extracts in which p34^{CDC28} had been overexpressed in the absence or presence of T antigen. In this experiment, T antigen was constitutively expressed from the GAPDH promoter and p34^{CDC28} was overexpressed by galactose induction. The cells were cross-linked with DSP for 0 to 20 min (see Materials and Methods) before extracts were prepared under nondenaturing conditions. Proteins were immunoprecipitated with antisera directed against p34^{CDC28}, and the immunoprecipitated proteins were incubated with [γ-^{32}P]ATP and histone H1 as an exogenous substrate. The kinase reaction mixtures were boiled in SDS loading buffer to reverse the cross-linking, and the proteins were separated by SDS-PAGE. The proteins were then transferred to nitrocellulose, autoradiographed to detect the phosphorylated proteins in the immunoprecipitated complex, and finally immunoblotted for specific proteins. Figure 9 shows the results of the kinase reaction. In all lanes, histone H1 is phosphorylated by the anti-p34^{CDC28} immunoprecipitates, as expected, since all the extracts contained overexpressed p34^{CDC28}. Interestingly, a highly phosphorylated band at approximately 60 kDa appears in autophosphorylation reactions using immunoprecipitated complexes from the cross-linked extracts containing T antigen (lane 4) but appears to be only weakly phosphorylated in the immunoprecipitated complexes from cross-linked extracts without T antigen (lane 3). It is unlikely that the 60-kDa protein is a proteolytic breakdown product of T antigen since (i) it is not detected on Western blots by various monoclonal antibodies to T antigen (not shown) and (ii) a band of similar electrophoretic mobility (but less phosphorylated) is present in the immunoprecipitates from extracts of cells which did not contain T antigen (lane 3). Overall, these data suggests that in the presence of T antigen, the p34^{CDC28} complex has altered kinase potential or altered substrate specificity. In either case, the appearance of a unique, highly phosphorylated band suggests that the T antigen-p34^{CDC28} complexes have altered biochemical properties as suggested above.

In addition, in both the non-cross-linked and cross-linked samples which contain T antigen (Fig. 9, lanes 2 and 4), a modestly phosphorylated band which has the same mobility as T antigen is seen at approximately 97 kDa. This band was also detected by Western analysis when the nitrocellulose was probed with an antiserum against T antigen (PAb419) (data not shown). Therefore, our data suggest that T antigen is modestly phosphorylated by a kinase activity in the anti-p34^{CDC28} immunoprecipitated complex.

CLN1, CLN2, and CLN3 do not appear to be direct targets of SV40 T antigen. In addition to regulation by p34^{CDC28}, the G_1 to S transition in *S. cerevisiae* is also controlled, in part, by the G_1 cyclins CLN1, CLN2, and CLN3 (3, 10). The *CLN* genes are functionally redundant in that a strain with any one functional CLN protein is viable whereas a strain with mutations in all three genes is inviable. We determined whether the

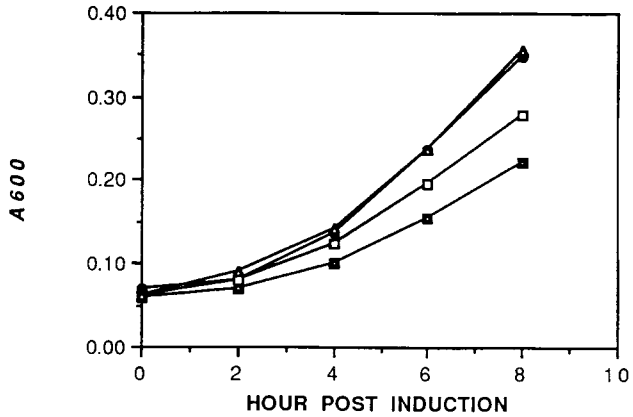

FIG. 8. Growth rates of cells overexpressing p34^{CDC28} in the absence and presence of T antigen. A_{600}s of wild-type (15DU) cells (closed circles), cells with wild-type levels of p34^{CDC28} but constitutively expressing T antigen (open squares), cells overexpressing p34^{CDC28} and constitutively expressing T antigen (closed squares), and cells overexpressing p34^{CDC28} also transformed with the control constitutive plasmid pAB23BXN (open triangles). All cells were grown in 2% galactose.

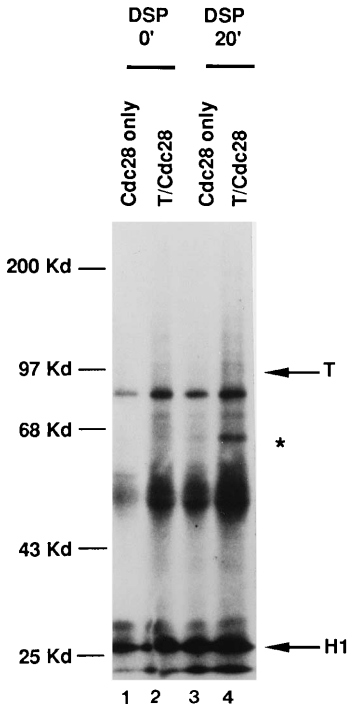

FIG. 9. Autoradiograph of in vitro kinase reactions with immunoprecipitates from cross-linked extracts. Cells overexpressing $p34^{CDC28}$ only (lanes 1 and 3) or overexpressing $p34^{CDC28}$ and constitutively expressing T antigen (lanes 2 and 4) were cross-linked with DSP for 0 or 20 min (′) (see Materials and Methods) before lysates were prepared. Lysates were immunoprecipitated with anti-$p34^{CDC28}$ antisera (see Materials and Methods) and assayed for kinase activity. H1 kinase activity present in all lanes is indicated by the lower arrow. Phosphorylated T antigen in lanes 2 and 4 is indicated by the upper arrow. The approximately 60-kDa band uniquely phosphorylated by the $p34^{cdc28}$-T antigen complex in lane 4 is indicated by the asterisk.

cell cycle phenotypes caused by expressing T antigen involved functional interference with one or more of the CLN proteins. For this experiment, we used strains that were congenic to the aforementioned strains, and to each other, except for double mutations in two of the three *CLN* genes. Therefore, each strain expressed only one of the three CLN proteins. The double-mutant *cln* strains were transformed with the galactose-inducible T-antigen-expressing plasmid (YEcT#2) or its corresponding control plasmid. In all three cases, T antigen's effect on growth was comparable to the phenotype observed in the *CLN* wild-type strain. We found that strains expressing each CLN protein individually were inhibited no more by T antigen than a strain expressing all three CLN proteins (data not shown). Therefore, we conclude that the CLN proteins are not direct targets for the effects of T antigen on the cell cycle.

DISCUSSION

We have presented evidence that SV40 large T antigen affects the *S. cerevisiae* cell cycle at the G_1 to S phase transition, a point at which T antigen is also known to affect the mammalian cell cycle (29, 30). In the budding yeast *S. cerevisiae*, we find that T antigen causes morphological changes and slows the progression from G_1 to S. We have also shown that T antigen binds in vitro to both $p34^{CDC28}$ and its mammalian homolog, $p34^{CDC2}$, and that T antigen complexes with $p34^{CDC28}$ in vivo. Previous data have suggested in vivo interactions between T antigen and cyclin-dependent kinases in SV40-infected and -transformed mammalian cells (1).

Using peptides representing various domains of T antigen fused to the amino terminus of GST, we have mapped two regions of T antigen which are involved in binding both $p34^{CDC28}$ and $p34^{CDC2}$ in vitro. The first region is composed of the amino-terminal 383 amino acids of T antigen, which also contain the binding site for at least one other cell cycle-regulatory protein, the product of the retinoblastoma gene (Rb) (4), and contains the binding regions for the TATA-binding protein and the transcription factor Tef-1 (8). This domain has been shown to have transforming potential in some cells (reference 17 and references therein) and contains several phosphorylation sites which regulate some of T antigen's properties. One of these sites, threonine 124, has previously been shown to be a substrate for the $p34^{CDC2}$ kinase in vitro (18). The second region of T antigen which binds both $p34^{CDC28}$ and $p34^{CDC2}$ is within the carboxy-terminal 150 amino acids. This domain of T antigen includes several serine and threonine residues which are phosphorylated in mammalian cells.

Given the central cell cycle role of $p34^{CDC28}$, it is likely that the interaction between T antigen and $p34^{CDC28}$ is at least partially responsible for growth and morphology phenotypes produced by T antigen in yeast cells. One hypothesis is that by binding to $p34^{CDC28}$, T antigen prevents it from performing its normal functions. Our results suggest that T antigen does not simply regulate the availability of $p34^{CDC28}$ by binding to it but that $p34^{CDC28}$ complexes containing T antigen have an altered biochemical function. In this regard, the data of Adamczewski and coworkers (1), who used SV40-infected or -transformed mammalian cells, suggest that only a fraction of the cell's cyclin-Cdk complexes and T antigen are associated with each other. If only a fraction of these complexes contain T antigen, then it can again be argued that any effects caused by T antigen would not arise from regulating the availability of the complexes but from altering their biochemical function. Using the yeast system, we have presented evidence for such alteration of function. Specifically, a protein of approximately 60 kDa is highly phosphorylated in $p34^{CDC28}$ complexes from T-antigen-containing cells, whereas it is much less phosphorylated (or unphosphorylated) in the same complexes from cells which do not contain T antigen. However, at this time neither the identity of the protein nor the mechanism by which T antigen alters its phosphorylation state is known.

The correlation between T antigen's binding to $p34^{CDC28}$ and its phenotypic effect seen at the G_1-S transition in the yeast cell cycle suggests a causal relationship. Interestingly, the phenotype that T antigen produces in *S. cerevisiae* is the opposite of its mitogenic potential in mammals, in which it accelerates the cell cycle. Therefore, while T antigen interacts with homologous yeast and mammalian cell cycle-regulating proteins (such as $p34^{CDC28}$ and $p34^{CDC2}$, respectively), some aspect of these interactions apparently differs between the lower and higher eukaryotes. Studies of adenovirus E1a suggest that it can interact with both $p33^{CDK2}$ and $p34^{CDC2}$ in vivo (11, 20, 31). However, complexes between cyclin A and E1a contain only $p33^{CDK2}$ (11, 31). Hence, cyclin A interaction may influence which specific cyclin-dependent kinase interacts with the viral proteins in mammalian cells. In this regard, Adamczewski and coworkers (1) have shown that $p33^{CDK2}$ and cyclin A interact with T antigen in SV40-infected and -transformed cells. Our genetic studies suggest that T antigen does not directly interfere with the functions of the G_1 cyclins CLN1, CLN2, and CLN3; however, other cyclins may be involved. Therefore, the difference in growth effects by T antigen in yeast and mammalian cells may arise from the level of involvement of specific types of cyclins.

We initiated these studies in order to use *S. cerevisiae* ge-

netics to examine interactions between T antigen and cellular proteins. The studies described here provide biochemical and genetic evidence that T antigen interacts with at least one major cell cycle regulating protein, p34^{CDC28}, and may affect its function. Our data, taken with the observation that T antigen can interact with cyclin A and p33^{CDK2} in SV40-infected and -transformed mammalian cells (1), suggest that biochemical changes in the complexes containing p34^{CDC2} or p33^{CDK2} could contribute to some of the mammalian cell cycle aberrations known to be induced by T antigen.

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