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Adenovirus Type 5 Precursor Terminal Protein-Expressing 293 and HeLa Cell Lines

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HeLa and 293 cell lines that express biologically active adenovirus type 5 precursor terminal protein (pTP) have been made. The amount of pTP synthesized in these cell lines ranges from barely detectable to greater than that observed in cells infected with the wild-type virus. The pTP-expressing cell lines permit the growth of a temperature-sensitive terminal protein mutant virus *sub*100r at the nonpermissive temperature. A higher percentage of the stably transfected 293 cell lines expressed terminal protein, and generally at considerably higher levels, than did the HeLa cell lines. While 293 cells appeared to tolerate pTP better than did HeLa cells, high-level pTP expression in 293 cells led to a significantly reduced growth rate. The 293-pTP cell lines produce infectious virus after transfection with purified viral DNA and form plaques when overlaid with Noble agar after infection at low multiplicity. These cell lines offer promise for the production of adenoviruses lacking pTP expression and therefore completely defective for replication.

The adenovirus chromosome is linear, with inverted terminal repeats that serve as origins of replication. Replication proceeds by leading-strand synthesis only. Replication is primed by precursor terminal protein (pTP), which forms a tight complex with the virally encoded DNA polymerase and is then covalently attached to dCMP, the first base in the genome. Terminal protein (TP) is synthesized as a 77-kDa precursor and processed to the mature 55-kDa form covalently attached to the viral DNA, within the virion (reviewed in references 15 and 32). TP serves as the site of primary attachment of the viral chromosome to the nuclear matrix (1, 26). Mutations within the pTP gene have been shown to reduce the efficiency of replication in vivo (6) and in vitro (21) as well as the affinity of the viral chromosome for the nuclear matrix and the efficiency with which viral early-region genes are expressed (28, 29). In other studies, all of the viable viral pTP mutations recovered were silent (23, 24). Given the importance of pTP in binding to the nuclear matrix (5) and controlling transcription and replication, the recovery of viruses mutated within this gene offers promise for understanding the regulation of transcription and replication. The difficulties encountered in obtaining such mutants suggest that their growth may require a complementing system.

We have made cell lines that express pTP under the direction of the tetracycline repressor-VP16 fusion protein. While this promoter system should lead to inducible expression of the test gene, the great majority of the cell lines examined expressed TP at very similar levels before and after induction. The pTP synthesized in these cell lines is biologically active, as demonstrated by the fact that a mutant with a temperature-sensitive mutation in the TP gene grows efficiently in these cell lines but not in the parental cell lines lacking pTP expression at the nonpermissive temperature. pTP expression appears to be poorly tolerated by mammalian cells, but high levels of pTP expression were generally obtained in 293 cells expressing E1A and E1B proteins.

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MATERIALS AND METHODS

Cell lines. We used 293 cells, human embryonic kidney cells that are transformed by and express the E1A and E1B genes of adenovirus serotype 5 (Ad5) (10), and HeLa cells in these studies. Cells attached to a solid substratum were grown in Dulbecco's modified Eagle's medium containing 10% calf serum, and HeLa suspension cells were grown in Joklik's modified minimal essential medium containing 7% horse serum.

Cloning of a pTP cDNA. The coding region of pTP is split into two exons, with three amino acids encoded by a small exon from near 39 map units and the remainder of the protein encoded by the main exon encoded between 29.4 and 23.8 map units. DNA encoding the main exon of pTP was cloned from the wild-type adenovirus chromosome by digestion with *Xba*I and *Kpn*I and ligation with *Xba*I- and *Kpn*I-digested pGem2 (Promega Biotec) to generate plasmid pGem-pTP. To generate a complete cDNA, pGem-pTP was digested with *Xba*I followed by mung bean nuclease to remove the 5′ overhang. The blunt-ended fragment was then digested with *Hin*dIII and ligated with an adaptor encoding the first three amino acids of pTP (31), 5′AGCTTCGGTGCCATGGCCTTG3′ plus 5′CAAGGCCATGGCACCGA3′. The resultant pseudo-cDNA clone, called ppTP cDNA (Fig. 1A), was demonstrated by sequence analysis to have a correct 5′ end (data not shown). The cDNA clone was transcribed in vitro with T7 RNA polymerase, and the mRNA was translated in the presence of [^{35}S]methionine. The main translation product was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to migrate with the mobility expected for a protein of approximately 80 kDa (data not shown).

Preparation of polyclonal antiserum recognizing TP. The C-terminal 65% of the pTP gene was cloned in frame behind the β-galactosidase gene in the inducible expression vector pUR292 (25) by digesting pTP partially with *Sal*I, filling in with T4 DNA polymerase in the presence of deoxynucleoside triphosphates (dNTPs), digesting with *Eco*RI, isolating the DNA fragment from positions 9841 to 8533, and ligating with pUR292 digested with *Bam*HI, filled in with T4 DNA polymerase in the presence of dNTPs, and digested with *Eco*RI. The resultant clone (Fig. 1B) was used to transform *Escherichia coli* 71-18. The bacterial clone was grown overnight in a small volume to saturation, diluted 100-fold, grown for 2 h at 37°C with vigorous aeration, and then induced to synthesize the fusion protein by addition of 1 mM IPTG (isopropylthiogalactopyranoside). After an additional 2 h of vigorous aeration, the cells were pelleted, washed in phosphate-buffered saline (PBS), lysed by incubation with lysozyme, and pelleted, and the supernatant was collected. Ammonium sulfate was added slowly to 12.5% saturation. The precipitate was collected by centrifugation. The

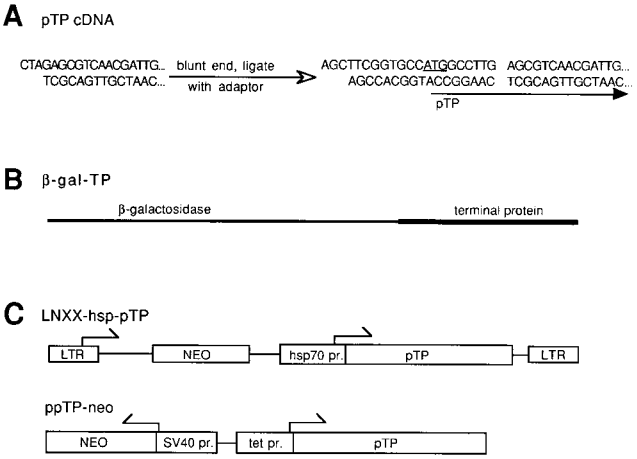

FIG. 1. TP plasmids. Schematic representations of the DNA constructs used are presented. (A) Construction of pTP cDNA. The coding sequence near the site of translation initiation is presented. The fragment encoding the main exon of pTP was digested with *Xba*I, and the ends were repaired with T4 DNA polymerase in the presence of dNTPs and ligated with the adaptor encoding the first three amino acids of pTP. A gap is left between the adaptor sequence and the main exon sequence. The pTP coding region is indicated below by the solid arrow, with the initiator ATG underlined. (B) TP fusion protein plasmid. The C-terminal 65% of pTP was cloned in frame behind β-galactosidase for synthesis of the fusion protein. (C) pTP expression vectors. The retrovirus construct in which pTP expression was driven by the *Drosophila* hsp70 promoter (pr.) and the construct in which pTP expression was driven by the artificial tetracycline (tet)-repressible promoter are shown. The direction of transcription is indicated by the arrows.

pellet was resuspended in PBS. The fusion protein was resolved on 7.5% polyacrylamide gels containing SDS. The bands were visualized by incubation with 0.3 M KCl at 4°C. The fusion protein was electroeluted. After dialysis against H_2O, the protein was concentrated by vacuum drying. Antibodies were raised in rabbits injected with the fusion protein in accordance with National Institutes of Health guidelines (Cocalico Biologicals, Reamstown, Penn.). Serum obtained from the rabbit prior to inoculation with the TP fusion protein antigen did not recognize TP prepared from virions, in contrast to the serum obtained after inoculation (data not shown).

Preparation of cell lines expressing pTP. The pTP cDNA was cloned as a *Hin*dIII-*Eco*RI fragment into the *Hin*dIII and *Eco*RI sites of pSK$^+$ (Stratagene). pSK-pTP was digested with *Hin*dIII, blunt-ended with T4 DNA polymerase in the presence of dNTPs, digested with *Bam*HI, and ligated with the large fragment from plasmid 10-3 (kindly provided by H. Bujard) (9) that had been digested with *Sac*II, blunt-ended with T4 DNA polymerase in the presence of dNTPs, and digested with *Bam*HI. This placed the pTP gene under the control of a promoter containing the tetracycline operator (9). This construct was then digested with *Xmn*I and *Bam*HI and ligated with the large fragment from an *Xmn*I-*Bam*HI digest of plasmid pβ-HAPr-1-neo (11) to place the pTP gene and the selectable neomycin phosphotransferase gene in the same plasmid. The plasmid, called ppTP-neo (Fig. 1C), was then cotransfected by either $Ca_3(PO_4)_2$ precipitation or Lipofectamine (Life Sciences) with plasmid 15-1 (kindly provided by H. Bujard) (9), which encodes a fusion protein consisting of the tetracycline repressor with the activating domain of VP16 of herpes simplex virus. The cells were grown for 3 days in the presence of 400 μg of G418 per ml (to select transfectants) and 10 μg of tetracycline per ml (to repress expression of pTP) and then passed to new plates. The cells were grown in the presence of G418 (400 μg/ml) and tetracycline (10 μg/ml) until colonies were apparent. Colonies were cloned and grown in the presence of G418 (100 μg/ml) and tetracycline (10 μg/ml).

The pTP cDNA was cloned into the retrovirus vector LNXX (a kind gift of Ian Maxwell; derived from the vector LNSX [20], a kind gift of A. Dusty Miller, by deletion of the simian virus 40 [SV40] promoter), which expresses resistance to G418, under the direction of the *Drosophila* hsp70 promoter (33). The pTP cDNA was cloned behind the hsp70 promoter in plasmid pSP65 (a kind gift of Robert Kingston) by digesting the hsp70 promoter plasmid with *Pst*I (which digests at the 3′ end of the promoter-containing fragment), blunt-ending with T4 DNA polymerase, digesting with *Eco*RI, and ligating with the pTP cDNA fragment digested with *Hin*dIII, blunt-ended with T4 DNA polymerase, and digested with *Eco*RI. The hsp70-pTP cDNA plasmid was digested with *Eco*RI, blunt-ended with T4 DNA polymerase, digested with *Hin*dIII, and ligated with retroviral LNXX plasmid DNA digested with *Bam*HI, blunt-ended with T4 DNA polymerase, and digested with *Hin*dIII. The resultant plasmid (Fig. 1C) was used to transform ψ2 and HeLa cells. Stably transformed cells were selected in the presence of G418.

Analysis of pTP expression. Expression of pTP in cloned and pooled cell lines was examined by Western immunoblot analysis with polyclonal antiserum raised against the fusion protein, consisting of *E. coli* β-galactosidase and the C-terminal region of pTP. The presence of antibody-recognizing proteins on the Western filter was demonstrated by using an anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase conjugate in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (12).

Analysis of mRNA and protein. Cytoplasmic RNA from cell lines and infected cells was isolated by lysing cells in 10 mM Tris (pH 8)–10 mM NaCl–3 mM $MgCl_2$–0.5% Nonidet P-40. The cytosolic fractions were digested with proteinase K, phenol extracted, and ethanol precipitated three times. The pellets were resuspended in RNase-free water, and the concentration was determined spectrophotometrically. RNase protections (18) were performed with an antisense strand synthesized in the presence of [α-^{32}P]UTP from a pTP cDNA probe in which the *Eco*RV fragment between positions 10433 and 9195 was replaced by an *Xba*I linker.

Viruses. Viruses (Ad5) were grown and titers were determined on 293 cells. The viruses used were *dl*309, which is phenotypically wild type (13), and *sub*100, which has an in-frame insertion in the pTP gene leading to temperature sensitivity and mutations in the E1A and E1B genes (6). To create a virus containing only the pTP mutation that would grow in HeLa cells, *sub*100 was rescued for E1A and E1B functions by preparing *sub*100 DNA, digesting with *Cla*I, which cleaves uniquely within the E1A gene, and cotransfecting 293 cells with plasmid pXC15, which encodes wild-type E1A and E1B gene products. The resultant plaques were grown and assayed, using spermine to precipitate unencapsidated DNA (11a) from minilysis samples, for the presence of the *Xba*I and *Hin*dIII sites (indicative of the wild-type genes) within the E1A and E1B coding regions. The resultant virus, which grows at the permissive temperature in HeLa cells that do not supply E1A and E1B gene products, is called *sub*100r.

RESULTS

Tolerance of pTP expression by mammalian cells. Initial attempts to stably express pTP in HeLa cells from constructs in which the pTP cDNA clone was driven by either the Moloney murine leukemia virus (MMTV) long terminal repeat (LTR) or the *Drosophila* hsp70 promoter were unsuccessful. In the case of the MMTV LTR-pTP construct, a plasmid encoding neomycin phosphotransferase was cotransfected at a ratio of 1:10, while the hsp70-pTP construct contained the neomycin phosphotransferase gene as part of a retrovirus cassette (see Materials and Methods). The presence of pTP mRNA before and after induction with 10^{-6} M dexamethasone (for the MMTV LTR-pTP construct) or heat shock (for the hsp70-pTP construct) was examined by RNase protection in both pooled and cloned G418-resistant cell lines. No specific signal was detected (Fig. 2A). All 293 cells into which the constructs were transfected were killed in the presence of G418.

The pTP cDNA in the retrovirus construct under the control of the hsp70 promoter was introduced by transfection into mouse ψ2 cells, a packaging cell line for the retrovirus. Cells initially grew quite slowly in the presence of G418. Eventually, the rate of growth increased. Pooled and cloned cell lines were examined in comparison with HeLa cells infected with wild-type adenovirus for the presence of pTP mRNA. pTP-specific mRNA was detected in the transfected ψ2 cells, but it was less than full length (Fig. 2A). The presence of a protected band comigrating with that from the DNA polymerase mRNA from infected cells and the loss of the large protected band from the pTP mRNA suggested that a cryptic splice donor was used with the DNA polymerase splice acceptor (Fig. 2B) to generate a retrovirus that no longer encoded pTP. Given that pTP mRNA was not detectable in the HeLa cells and that only a partially deleted form of pTP mRNA was apparent in the ψ2 cells, it appears that pTP expression is selected against in mammalian cells. The lack of pTP mRNA expression in the HeLa cells suggested that cloning of the hsp70 promoter-pTP gene in the opposite orientation in the retroviral plasmid (to avoid cryptic splicing within the genomic RNA) would not necessarily aid in

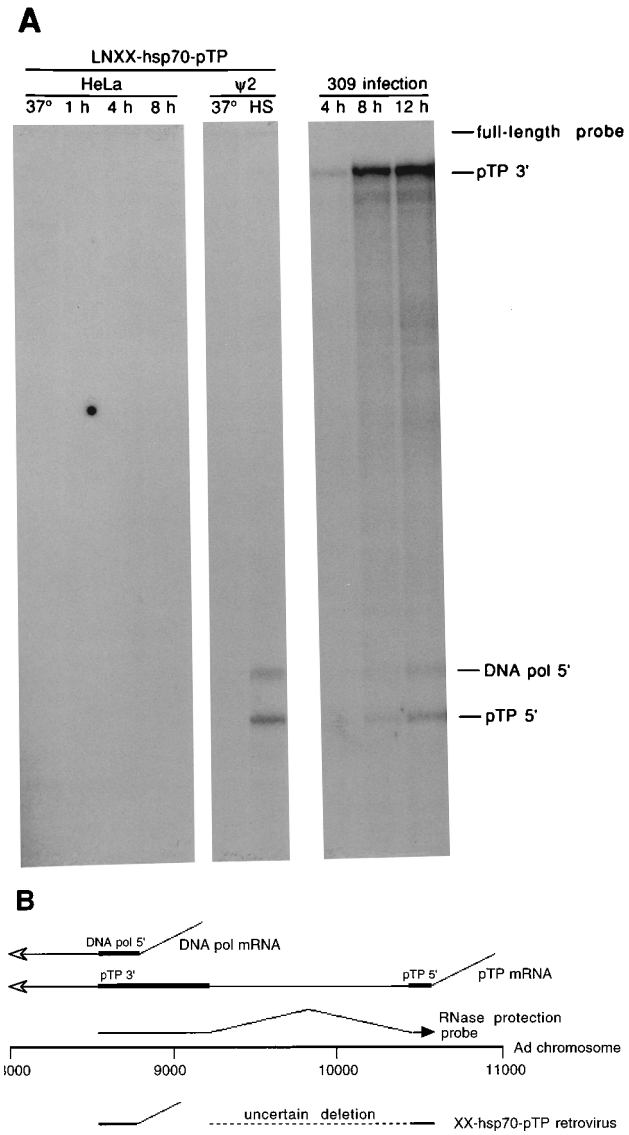

FIG. 2. Expression of pTP mRNA after induction of transduced ψ2 cells. (A) mRNA prepared from heat-shocked and control HeLa cells and ψ2 cells stably transfected with the retrovirus construct LNXX-hsp70-pTP and HeLa cells infected with wild-type virus *dl*309 was subjected to RNase protection with the antisense pTP probe indicated in part B. Transfected HeLa cells were either incubated at 37°C or heat shocked at 44°C for 20 min and then allowed to recover at 37°C for the times indicated above the lanes. Transduced ψ2 cells were induced by incubation at 44°C for 20 min (HS) and then incubated for 2 h at 37°C. HeLa cells infected with *dl*309 were harvested at the indicated times after infection. The presence of protected RNA species was determined by autoradiography after resolution by electrophoresis on a 5% polyacrylamide (19:1 acrylamide-bisacrylamide) gel. The protected species are indicated on the right. pol, polymerase. (B) Schematic transcription map of the region of adenovirus (Ad) DNA encoding the main exon of pTP. Transcripts are indicated above as horizontal arrows, with splice acceptors indicated by angled lines. The regions of each mRNA expected to yield protected fragments during the RNase protection assay are indicated by heavy lines, with identification immediately above. The pTP and DNA polymerase mRNAs use the same splice donor near 39 map units, with the DNA polymerase splice acceptor lying within the C-terminal part of the region encoding pTP (31). The antisense probe used, synthesized from a plasmid in which the *Eco*RV fragment between bp 10433 and 9195 was replaced by an *Xba*I linker (indicated by angled lines), is indicated below the pTP and DNA polymerase transcripts. The region of the adenovirus chromosome encoding the main exon of pTP is presented, with positions in base pairs from the E1A end of the chromosome indicated below. At the bottom of the figure, the putative hsp70-pTP retrovirus product arising from a splicing event with the DNA polymerase splice acceptor (solid angled line) and a cryptic splice donor at uncertain position between bp 10433 and 9195 (dashed horizontal line) is presented.

expressing pTP. Therefore, an alternative strategy was followed.

Preparation of cell lines expressing pTP. A tightly controlled artificial system in which expression of a test gene is driven by binding of a tetracycline repressor-VP16 fusion protein to a minimal promoter containing a tetracycline operator has been described (9). In *E. coli*, tetracycline binds to the tetracycline repressor, weakening its affinity for the tetracycline operator and activating expression of the tetracycline resistance gene. In the artificial mammalian system, tetracycline binding to the repressor part of the fusion protein leads to release of the fusion protein from the operator and reduction in expression of the gene. Thus, the gene should be activated in the absence of tetracycline and relatively inert in its presence.

A plasmid containing the pTP cDNA driven by the tetracycline-regulated promoter and the neomycin phosphotransferase gene driven by the SV40 early promoter (Fig. 1B) was cotransfected with a plasmid encoding the fusion protein activator into both HeLa and 293 cells. The cell lines were cloned, and expression of pTP was examined before and after induction. In addition, the expression of pTP and TP in HeLa cells infected with the phenotypically wild-type virus *dl*309 was examined (Fig. 3 and Table 1). Most of the cloned 293 cell lines expressed a protein that migrated at approximately 80 kDa, was recognized by the antiserum raised against the β-galactosidase/pTP fusion protein (Fig. 1), and was not present in 293 cells. In addition, a protein with a mobility of approximately 100 kDa, which may represent a translation readthrough product, was present in cells expressing pTP (Fig. 3A). Three 293 cell lines, numbers 3, 8, and 13, expressed very high levels of pTP. Of these, clones 3 and 8 expressed constitutively high levels of pTP, grew slowly, rapidly acidified the medium even when significantly subconfluent, and plated at relatively low efficiency. In contrast, clone 13 grown in the presence of tetracycline initially behaved more like the parental 293 cells. After several additional passages, clone 13 began to grow more slowly and was found to express pTP at high constitutive levels similar to those observed in clones 3 and 8.

The levels of pTP expressed by 293-pTP cell lines was compared with the amounts of pTP and TP present within HeLa cells infected with phenotypically wild-type *dl*309 (Fig. 3A). Cells were lysed in the presence of Nonidet P-40 in a low-ionic-strength buffer, a process which disrupts the virions sufficiently that DNase I added to the mixture is able to digest viral DNA which has been encapsidated as well as unencapsidated viral and host DNA. Thus, TP, which is only present after packaging of the viral DNA, is apparent during the latter stages of the infection. The levels of pTP expressed in the 293-pTP cell lines were similar to or greater than the amounts of TP and pTP apparent in cells infected with the wild-type virus, suggesting that the cell lines should be useful for complementation of pTP mutant adenoviruses.

Only 5 of the 19 HeLa cell lines that grew sufficiently to be tested expressed sufficient quantities of pTP for it to be apparent in Western analysis, and only 1 of these expressed relatively high amounts of pTP (Fig. 3B and Table 1). Both HeLa and 293 cell lines expressing pTP were further characterized.

Complementation of a pTP mutant adenovirus by pTP-expressing cell lines. The biological activity of the pTP-expressing cell lines was examined by their ability to complement a temperature-sensitive TP mutant virus for growth at 39.5°C. The pTP mutant virus *sub*100r is temperature sensitive for growth (6) but expresses early-region genes at the nonpermissive temperature (29). Examination of the behavior of this virus during infection at low multiplicity demonstrated that the pTP mutation led to tight temperature sensitivity in 293 cells,

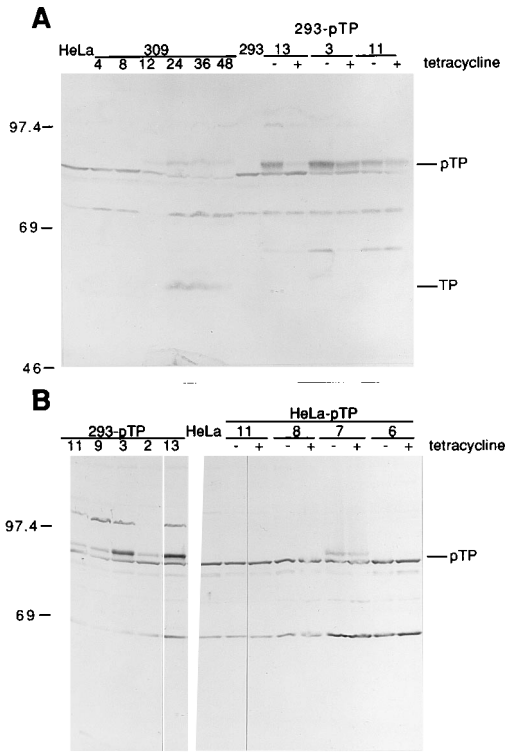

FIG. 3. TP expression in cell lines and in infected HeLa suspension cells. pTP and TP expression was examined by Western analyses. Extracts from approximately 3×10^5 cells were resolved on 8% polyacrylamide gels containing SDS. Proteins were electrophoretically transferred to nitrocellulose. Staining of the filters with Ponceau S confirmed that similar amounts of protein were present in all lanes (data not shown). The presence of pTP- and TP-specific protein bands was determined by using polyclonal antiserum as indicated in Materials and Methods. (A) Expression of pTP in 293-pTP cell lines compared with that in HeLa cells infected with the wild-type virus. The 293-pTP cells were grown in the presence and absence of tetracycline (10 μg/ml) as noted above the lanes. HeLa suspension cells were infected with phenotypically wild-type *dl*309, and aliquots were collected at various times after infection (in hours, as noted above the lanes). Uninfected HeLa and parental 293 cell extracts were used as controls. Size markers are indicated on the left (in kilodaltons) and the positions of pTP and TP are shown on the right side of the panel. (B) Expression of pTP in HeLa-pTP cell lines and in 293-pTP cell lines. HeLa-pTP cell lines were grown in the presence and absence of tetracycline (10 μg/ml) as noted above the lanes. The 293-pTP cell lines were grown in the absence of tetracycline. Parental HeLa cells were used as a control. Size markers are indicated on the left, and the position of pTP is shown on the right side of the figure.

with no cytopathic effect (CPE) apparent after low-multiplicity infection of 293 cells at 39.5°C (Table 2). Use of a lysate of the cells infected at 39.5°C for infection of 293 cells at 32°C did not lead to CPE, indicating that little or no virus escaped the block at the nonpermissive temperature. To test the biological activity of the pTP-expressing cell lines, cells infected with either phenotypically wild-type *dl*309 or *sub*100r were incubated at 32°C continuously, incubated at 32°C for 16 h to permit initial replication to occur in the presence of the wild-type pTP provided in *trans*, and then shifted to 39.5°C, or else they were incubated continuously at 39.5°C. CPE was observed in all cell lines infected with *dl*309 under all conditions. CPE was observed only at the permissive temperature in HeLa or 293 cells infected with *sub*100r. However, CPE was observed under all temperature regimens in 293-pTP and HeLa-pTP cells infected with *sub*100r (Table 2). The 293-pTP cell lines in continuous culture over a 7-month period have been shown both to produce pTP and to complement *sub*100r for growth at the nonpermissive temperature.

TABLE 1. Expression of pTP in cell lines and infected cells[a]

Clone	pTP expression		*dl*309-infected HeLa cells		
	HeLa-pTP	293-pTP	Time (h)	pTP	TP
1	*	−	4	−	−
2	*	++	8	−	−
3	*	+++	12	+	−
4	*	−	24	++	++
5	−	−	36	+	++
6	−	−	48	+	++
7	++	−			
8	−	++++			
9		++			
10	±	+++			
11	−	++			
12	±	+++			
13	−	++++			
14	*	++			
15	±	−			
16	*				
17	−				
18	−				
19	−				
20	+				
21	−				
22	−				
23	−				
24	−				
25	−				

[a] Relative levels of TP expression, as determined by Western blot analysis after induction in the absence of tetracycline, are indicated for the various HeLa and 293 cell lines as well as for various times of infection of HeLa suspension cells with the phenotypically wild-type virus *dl*309 at 25 PFU/cell. *, cell lines died or ceased growing before reaching sufficient density to permit determination of the level of TP expression; −, not detected; ± to ++++, qualitative level of pTP, ranging from just detectable to high concentration, with ++ being the maximal level observed during infection of HeLa cells with *dl*309.

The CPE observed after infection of pTP-expressing cell lines with *sub*100r at the nonpermissive temperature could result either from complementation in *trans* or from recombination between the resident copy of the pTP gene and the *sub*100r genome. To rule out the possibility that recombination was occurring, lysates of 293-pTP_{13} cells infected at 39.5°C with *sub*100r were used to infect 293 cells at 39.5°C. When the cells were infected at low multiplicity (less than 0.01 PFU/cell), no

TABLE 2. Growth of *sub*100r in pTP-expressing cells[a]

Cell line	Virus	CPE at growth temp (°C):		
		32	32 + 39.5	39.5
HeLa	*sub*100r	+	−	−
293	*sub*100r	+	−	−
	*dl*309	+	+	+
HeLa-pTP_7	*sub*100r	+	+	+
	*dl*309	+	+	+
293-pTP_2	*sub*100r	+	+	+
	*dl*309	+	+	+
293-pTP_{13}	*sub*100r	+	+	+
	*dl*309	+	+	+

[a] Plates (60 mm) of cells were infected with approximately 25 PFU of the temperature-sensitive TP mutant *sub*100r or phenotypically wild-type *dl*309 at either 32°C (permissive temperature), 39.5°C (nonpermissive temperature for *sub*100r), or at 32°C for 16 h following infection and then at 39.5°C. Growth of the viruses was determined by the appearance of CPE. CPE was similar in the presence and absence of tetracycline.

TABLE 3. Plaquing efficiency of *sub*100 on pTP-expressing 293 cells[a]

Cell line	Temp (°C)	*sub*100 plaque titer
293	32	3.9×10^6
	39.5	3.3×10^2
293-pTP$_2$	39.5	1.8×10^7
293-pTP$_{13}$	39.5	2.7×10^7

[a] Plates (60 mm) of cells were infected with dilutions of *sub*100 at the temperature indicated. The cells were overlaid after infection and stained with neutral red 7 days after infection at 39.5°C and 12 days after infection at 32°C. Plaques were counted 14 h after staining.

TABLE 4. Virus yields in 293 and 293-pTP cell lines[a]

Virus	Cell type	Plaque titer	
		32°C	39.5°C
*sub*100r	293	9.6×10^7	1.7×10^5
	293-pTP$_{13}$	8.8×10^6	3.0×10^7
	293-pTP$_2$	2.0×10^8	2.5×10^8
*dl*309	293	8.6×10^7	1.0×10^8
	293-pTP$_{13}$	1.1×10^8	2.0×10^8
	293-pTP$_2$	2.1×10^8	5.0×10^8

[a] Plates (60 mm) of cells were infected with either *dl*309 or *sub*100 at a multiplicity of 20, as determined by using 293 cells at the permissive temperature. After 3 days (for virus grown at 39.5°C) or 5 days (for virus grown at 32°C), 60-mm 293-pTP$_{13}$ plates were infected with dilutions of the viruses at 37°C and overlaid with Noble agar. The cells were stained with neutral red 7 days after infection. Plaques were counted 14 h after staining.

CPE was observed. When 293 cells were infected at relatively high multiplicity (greater than 1 PFU/cell), CPE was observed soon after infection, and the cells died after 5 days. However, the cells did not swell, as expected during a productive adenovirus infection. Further, when lysates of these cells were used to infect 293 or 293-pTP$_{13}$ cells, CPE was not induced (data not shown). This evidence indicates that *sub*100r infection at relatively high multiplicity can lead to cell death in the absence of complementation but that little infectious virus was produced unless wild-type pTP was synthesized. Thus, the pTP-expressing cell lines expressed biologically active pTP, and little or no recombination between the host cell pTP gene and the virus occurred. Finally, it appears that *sub*100 pTP is not dominant in *trans* to wild-type pTP.

The level of complementation by the pTP-expressing 293 cells relative to the parental cells was assayed by comparing the plaquing efficiency of *sub*100 at the nonpermissive temperature (Table 3). The plaquing efficiency of the 293-pTP cell lines was 10,000-fold greater than that of the parental 293 cells at the nonpermissive temperature of 39.5°C when measured 8 days after infection (Table 3). The plaques which occurred on 293 plates were small and occurred only at such a high multiplicity of infection that it was likely that some cells were being multiply infected. This latter point suggests that the temperature sensitivity of *sub*100 can be partially overcome in a gene dosage-dependent manner, possibly through increased concentration of active pTP to a critical level or through increased concentration of a factor, such as DNA polymerase, with which pTP interacts and which could act to stabilize the mutant pTP. The number of plaques apparent on 293 cells incubated at the nonpermissive temperature continued to increase with increasing time of incubation (when the plaques were counted at day 12, the relative complementation by the pTP-expressing 293 cells was reduced by approximately 10-fold), suggesting that there is also a kinetic block to growth of *sub*100. The plaquing efficiency on the 293-pTP cell lines at 39.5°C was substantially greater than that on 293 cells at 32°C, suggesting that the mutation in *sub*100 is defective at all temperatures. However, 293 cells grow less efficiently at 32 than at 39.5°C, so the difference may reflect, at least in part, cellular contribution to growth of the viral plaques.

The ability of the 293 and 293-pTP cell lines to support the growth of *dl*309 and *sub*100r was also examined by using one-step growth curves. The 293 and 293-pTP cells were infected at a multiplicity of 20 PFU/cell (as determined by plaque titer on 293 cells at 37°C for *dl*309 and 32°C for *sub*100r) at both 32 and 39.5°C. The resultant cell lysates were titered by plaque assay with 293-pTP$_{13}$ cells (Table 4). The 293-pTP$_{13}$ cells were used because of the greater efficiency of plaquing of *sub*100 (Table 3) and the fact that the cells expressing pTP appear to survive longer after reaching confluence than do 293 cells. The yield of *sub*100r grown in 293 cells at 39.5°C was approximately 1,000-fold less than that at 32°C, while it was slightly greater at 39.5°C than at 32°C in the 293-pTP cells. The yield of *dl*309 was similar at 32 and 39.5°C in all of the cell lines. The yield of viruses was generally greatest from 293-pTP$_2$ cells and least from 293-pTP$_{13}$ cells, suggesting that intermediate levels of pTP expression may increase viral growth but that high-level expression may lead to interference.

The data on the level of complementation of *sub*100r growth by the 293-pTP cell lines must be interpreted with caution. There is evidence that *sub*100 is more infectious on 293-pTP cells than on 293 cells (Table 3), complicating comparison of virus yields from cells infected with the same volumes of infectious *sub*100r. In addition, expression of *sub*100 pTP could lead to inhibition of, or possibly cooperation with, the pTP expressed in the cell lines. Examination of the ability of the pTP-expressing cell lines to complement the growth of a virus lacking expressing of pTP in comparison with the growth of the wild-type virus would provide the best evidence for the level of pTP activity of these cell lines. The abilities of the pTP-expressing cell lines to support the growth of wild-type and mutant viruses will be examined in greater detail.

Transfection of pTP-expressing cell lines. The ability of the 293-pTP cell lines to produce virus after transfection of purified, undigested viral DNA was examined by using DNA from virus 327 $_{Bst}\beta$-gal (this virus is useful for introducing foreign genes into the left end of the viral chromosome [27]). Both 293-pTP$_2$ (59 plaques) and 293-pTP$_{13}$ (27 plaques) plates yielded a similar number of plaques relative to parental 293 cells (43 plaques). The data demonstrate that the pTP-expressing cell lines are likely to be useful for construction of viruses.

Effects of pTP expression on growth rate of cells. The growth rate of 293-pTP$_2$ cells, which express relatively modest levels of pTP, was compared with that of 293-pTP$_{13}$ cells, which express very high levels of pTP, when both cell lines were at early passage. High-level expression of pTP appeared to have deleterious effects on growth rate, as 293-pTP$_2$ cells grew significantly faster than did 293-pTP$_{13}$ cells (Fig. 4), with a doubling time of approximately 2.2 days for 293-pTP$_2$, compared with 3.5 days for 293-pTP$_{13}$. In addition, 293-pTP$_{13}$ cells acidified the medium significantly more rapidly relative to cell density. The growth rate of 293-pTP$_{13}$ cells and the rate of acidification of the medium were not significantly affected by the presence of G418 relative to 293-pTP$_2$ cells (data not shown), indicating that the differences in growth rate between 293-pTP$_2$ and 293-pTP$_{13}$ cells were probably due to pTP expression. With subcloning and prolonged passage of the 293-pTP$_{13}$ cells, the

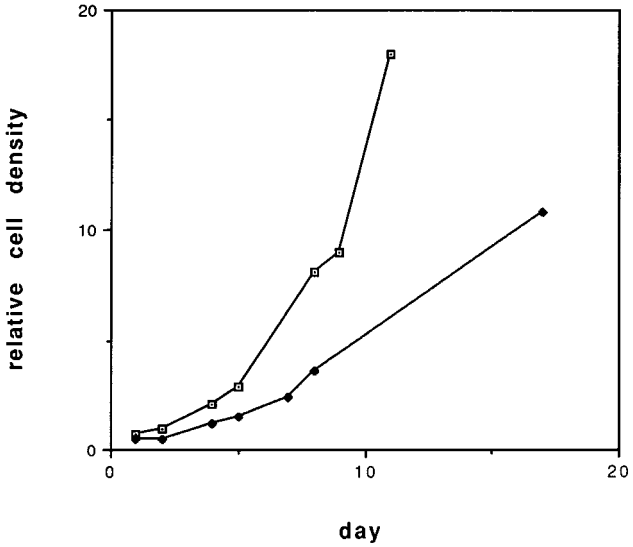

FIG. 4. Growth rates of pTP-expressing 293 cells. The 293-pTP_2 cells (⊡), which express pTP at a moderate level, and 293-pTP_{13} cells (◆), which express a high level of pTP, were examined for relative growth rates in the presence of tetracycline (10 μg/ml) and G418 (100 μg/ml). The data presented are normalized to 10^7 cells.

growth rate has increased, suggesting that adjustment to the presence of pTP is occurring (data not shown).

DISCUSSION

HeLa and 293 cell lines that express relatively large amounts of biologically active Ad5 pTP were made (Fig. 3 and Tables 1 and 2). The level of pTP expression in some of the cell lines was found to be similar to or greater than that of pTP liberated by DNase I digestion of HeLa cells at the height of infection with phenotypically wild-type Ad5. Thus, these cell lines should be useful for complementing adenovirus pTP mutants, including mutants that lack pTP expression altogether and are therefore completely defective for replication. Adenoviruses that do not express E1A are replication competent, albeit at a level considerably lower than that of the wild-type virus (30). Replication leads to an inflammatory response in the absence of E3 gene expression (7, 8). Reduction in replication of transducing viruses has been accomplished through the use of a virus with a temperature-sensitive E2A allele (4). While the approach of using replication-defective viruses offers promise for gene therapy, the use of the temperature-sensitive virus leads to reduced virus yields during growth. The 293-pTP cells described here should permit complementation of viruses that are completely replication defective and in which the E1 region has been replaced by foreign genes for use in gene therapy and transient transduction of tissue culture cells. In addition, the pTP-expressing cell lines offer promise for studies of pTP function, including its interaction with the nuclear matrix, in the absence of the majority of adenovirus-encoded proteins.

High-level expression in mammalian cells of the adenovirus-encoded proteins involved in viral replication has proven difficult to obtain. Considerable effort was required to obtain cell lines expressing high levels of the single-stranded DNA-binding protein (2, 16, 17), and reports of successful stable expression of the adenovirus DNA polymerase have not been found. While all of these gene products are involved in adenovirus replication, the reasons for the difficulties in obtaining cell lines expressing these proteins are likely to differ, as are the mechanisms by which the proteins interfere with cellular growth. For example, expression of single-stranded DNA-binding protein appears to be toxic to cells (16), while expression of pTP does not.

pTP does exert strong effects on cellular proliferation, as indicated by the reduced rate of growth of cells expressing high levels of pTP (Fig. 4), the difficulty in obtaining cell lines that express pTP, and the apparent selection against pTP expression (Fig. 2). pTP was generally expressed at much higher levels in 293 cells than in HeLa cells. While the difference in expression of pTP may reflect a tissue-specific difference in tolerance, it is possible that the presence of the E1A and/or E1B protein in 293 cells protects cells against the growth-limiting effects of pTP. The E1A and E1B genes are transforming, presumably as a result of their role in driving cells toward the S phase. However, infected cells do not generally divide despite the growth-stimulating effects of the E1 proteins. It is possible that pTP, whose expression increases dramatically during the late phase of the infection (Fig. 2 and 3), serves to block further progression through the cell cycle. Such a function of pTP would be consistent both with the difficulties in obtaining cell lines that express high levels of pTP and with the increased frequency with which high-level expression was observed in 293 cells.

The cell lines described here express pTP under the control of a minimal promoter containing a tetracycline operator that binds a fusion protein consisting of the tetracycline repressor and herpes simplex virus VP16 (9). pTP expression should be inducible in the absence of tetracycline. However, only one 293 cell line was found to be inducible, and then only during early passage. It is possible that all of the cell lines expressed pTP in an inducible manner early after transfection and that low-level expression of pTP was essential for the establishment but not the maintenance of the cell lines.

High levels of pTP were obtained in cells in which expression was directed by the artificial tetracycline operator promoter. In contrast, no full-length pTP mRNA expression was observed under the direction of the weaker *Drosophila* hsp70 promoter. The fact that greater success was obtained with the stronger promoter raises the possibilities that pTP expression is particularly deleterious during specific phases of the cell cycle, such as during formation of the nuclear matrix, and that the tetracycline operator and hsp70 promoters differ in activity with respect to the cell cycle. The human hsp70 promoter shows a low level of activity in the absence of cell stress (28), and it is likely, based on comparison with the activity of the hsx70 promoter (3, 14, 19) and other heat shock promoters (22), that a low level of activity of the hsp70 promoter occurs in a cell cycle-dependent manner. Expression of pTP during times when the hsp70 promoter is active may be particularly deleterious.

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